

**Hepatic and Enteric Viral Infections:
Molecular epidemiology, immunity
and antiviral therapy**

Mohamad Saifudin Hakim

The studies presented in this thesis were performed at the Laboratory of Gastroenterology and Hepatology, Erasmus MC-University Medical Center Rotterdam, the Netherlands.

The research was funded by:

- Netherlands Organization for Scientific Research (NWO)
- Dutch Digestive Foundation (MLDS)
- Daniel den Hoed Foundation
- The Indonesia Endowment Fund for Education (LPDP)

© Copyright by Mohamad S. Hakim. All rights reserved.

No part of the thesis may be reproduced or transmitted, in any form, by any means, without express written permission of the author.

Cover design: MD. Anasanti and MF. Rizal. Layout design: MS. Hakim and A. Wira.

Printed by: Ridderprint BV, Ridderkerk, the Netherlands

ISBN: 978-94-6375-098-1

**Hepatic and Enteric Viral Infections:
Molecular epidemiology, immunity and
antiviral therapy**

Hepatitis en enterale virus infecties:
Moleculaire epidemiologie, immuniteit en antivirale therapie

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof. dr. R.C.M.E. Engels

and in accordance with the decision of the Doctorate Board

The public defense shall be held on

Wednesday, 12nd September 2018 at 11.30

by

Mohamad Saifudin Hakim

born in Rembang, Central Java, Indonesia

Erasmus University Rotterdam



Doctoral Committee

Promotor:

Prof. dr. M.P. Peppelenbosch

Inner Committee:

Prof. dr. M.J. Bruno

Prof. dr. W.J. de Jonge

Prof. dr. B. van Hoek

Copromotor:

Dr. Q. Pan

Nina, Sarah and Shafiya

I'm not good with words, nor I'm good in expressing my feelings for you three

But for whatever its worth, I took the liberty to dedicate this to you

as a showcase of my compassion, love and gratitude

Let this cocktail of affection and cognition dances

to kindle the flame of passion, hope and illumination

as we build together a better world ahead in His Grace.

Dedicated in the memory of my mother and father

You will always continue to live in my heart

CONTENTS

Chapter 1 General introduction and aim of the thesis -- **1**

Part I. Hepatitis E Virus

Chapter 2 The global burden of hepatitis E outbreaks: A systematic review (*Liver International, 2017*) -- **19**

Chapter 3 Immunity against hepatitis E virus infection: Implications for therapy and vaccine development (*Reviews in Medical Virology, 2018*) -- **47**

Chapter 4 Distinct antiviral potency of sofosbuvir against hepatitis C and E viruses (*Gastroenterology, 2016*) -- **69**

Chapter 5 Genotype-specific acquisition, evolution and adaptation of characteristic mutations in hepatitis E virus (*Virulence, 2018*) -- **79**

Part II. Rotavirus and Norovirus

Chapter 6 Basal interferon signaling and therapeutic use of interferons in controlling rotavirus infection in human intestinal cells and organoids (*Scientific Reports, 2018*) -- **111**

Chapter 7 TNF- α exerts potent anti-rotavirus effects via the activation of classical NF- κ B pathway (*Virus Research, 2018*) -- **145**

Chapter 8 6-thioguanine potently inhibits rotavirus infection through suppression of Rac1 GDP/GTP cycling (*Antiviral Research, 2018*) -- **175**

Chapter 9 Significance of continuous rotavirus and norovirus surveillance in Indonesia (*World Journal of Pediatrics, 2018*) -- **201**

Chapter 10 Norovirus and rotavirus infections in children less than five years of age hospitalized with acute gastroenteritis in Indonesia (*Archives of Virology, under revision*) -- **221**

Chapter 11 Identification of rotavirus strains causing diarrhea in children under five years of age in Yogyakarta, Indonesia (*Malaysian J. Medical Sciences, 2017*) -- **239**

Chapter 12 Summary and discussion -- **259**

Chapter 13 Dutch summary -- **267**

Appendix -- **273**

Acknowledgement

Publications

PhD Portofolio

Curriculum Vitae

Chapter 1

General Introduction and Aim of The Thesis

Hepatitis E virus as an important cause of viral hepatitis

Infectious diseases are a significant global health problem. Viral hepatitis, including the diseases caused by hepatitis B (HBV) and C (HCV) viruses, affected millions of people, and especially so the substantial proportion of infected patients in which pathology progressed into chronic infection. The latter patients have an increased risk of developing severe complications, and major associated pathology includes liver fibrosis and cirrhosis which finally culminates into liver failure and hepatocellular carcinoma.¹ Among viral hepatitis, however, it is the hepatitis E virus (HEV) that is the most dominant cause of acute hepatitis worldwide.²

HEV is a member of the picornavirus family and represents a non-enveloped and positive-strand RNA virus of 32-34 nm in diameter. Its complete genome is 7.2 kb in length, and includes three or four open reading frames (ORFs).² ORF1-derived proteins are non-structural viral proteins and are essential for viral replication. Various domains have been identified in this region, including a methyltransferase (MeT), a Y domain (Y), a papain-like cysteine protease (PCP), a proline-rich hinge domain, an X domain, an RNA helicase domain (Hel), and an RNA-dependent RNA polymerase (RdRp) domain. ORF2 encodes a 72 kDa protein that is incorporated into the viral capsid and represents the predominant antigen targeted by the human host immune system.³⁻⁵ ORF3 encodes a 13 kDa protein that mediates virus release from infected cells.⁶ It also interacts with several host proteins and thus plays an essential role in pathogenesis caused by this virus.^{7,8} A novel ORF4 (nt 2835-3308) has been recently identified from HEV genotype 1 and has been shown to drive HEV replication.⁹

Several different genotypes of HEV exist which all cause infection in humans, yet only one single serotype has been identified.¹⁰ HEV genotype 1 and 2 are mainly transmitted via the faecal-oral route and are responsible for many large water-borne outbreaks of Hepatitis E in developing countries.¹¹ In contrast, HEV genotype 3 and 4 are zoonotic, mainly causing chronic infection in immunocompromised organ transplant recipients in the Western world.¹² In addition, more distantly HEV-related viruses have been identified in several animals, including ferrets, rats and bats.¹³

Generally, HEV infection results in mild disease and thus no specific anti-viral treatments are required, irrespective of the genotype involved.² However, high mortality in pregnant women following HEV genotype 1 infection has been observed during many large HEV outbreaks in developing countries.¹¹ With respect to developed countries, the burden provoked by HEV-related diseases is born by immunocompromised populations, especially in orthotopic liver transplant recipients in which HEV genotype 3 infection rapidly causes liver cirrhosis and ultimately, loss of the transplanted liver.¹² Therefore, HEV represents an emerging issue in global health.

Immunity and antiviral therapy against hepatitis E

Similar to other viral infections, adequate immune responses to infection are critical with respect to the outcome of HEV infection. Innate immunity constitutes the first line of defense against viral infections. Recognition of relatively invariant viral components activates innate immune signaling pathways that culminate in the production of type I interferons (IFNs), tumor necrosis factor α (TNF α) and other antiviral cytokines. Subsequently, more specific adaptive immunity develops, including HEV-specific B and T cells, and these cell subsets attempt to achieve complete elimination of the virus. Improved understanding of HEV-provoked immunity may further the development of better HEV-targeting vaccines, although the use of anti-HEV vaccines is currently limited to mainland China.¹⁴

In the clinic, immunocompromised patients represent the main population requiring antiviral therapy for hepatitis E.¹² In these patients, i.e. orthotopic organ transplantation recipients, dose reduction of immunosuppressive agents is initially considered as the preferred intervention and results in viral clearance in a subset of patients.¹⁵ For patients failing this strategy, treatment with pegylated IFN α may be appropriate as it has been successful in chronic HEV patients in a number of case series and case reports. However, its associated adverse events, including graft rejection, limiting its use in the clinic.¹⁶

Antiviral ribavirin (RBV) monotherapy is considered the first line of antivirals suitable to treat chronic HEV.¹⁶ RBV leads in about 70-80% of patients to a sustained virological response. However, treatment failure and recurrences of HEV viremia have been reported in subsets of

patients.^{16,17} Thus, novel anti-HEV therapy is urgently required to overcome these limitations of PEG-IFN α and RBV therapy.¹⁴ Currently, the search for new anti-HEV drugs very much depends on the alternative use of clinically available antiviral medicine. This approach is highly relevant in a clinical setting as there is currently no registered medication for hepatitis E. Sofosbuvir (SOF), the direct-acting anti-hepatitis C virus (HCV) drug (targeting HCV RdRp; RNA-dependent RNA polymerase), has recently been reported to be a potential anti-HEV drug.¹⁸ However, subsequent studies showed discrepancies in different models of HEV, hampering its further development as novel anti-HEV therapy.¹⁹⁻²²

Importantly, the reaction of the human immune system against HEV (production of HEV-specific B and T cells) as well as the use antiviral medications in infected patients, provoke emergence of novel HEV mutations, leading to evasion of antiviral activity and further pathogenesis. HEV evolution may result in escaping variants that evade the host immunity and are resistant to antiviral treatment.²³ Therefore, development of novel antiviral medications and HEV vaccines are necessary for better control of HEV infection-associated diseases in the future.¹⁴

Rotavirus and norovirus: The most important viral agents in acute gastroenteritis

Diarrhea poses a high burden to global diseases and significantly contributes to overall morbidity and mortality, especially in developing countries. Acute diarrhea is a serious global health problem, with 3-5 billion cases and nearly 2 million deaths annually in children under five years of age.²⁴ In children aged five years and older, adolescents and adults, there are approximately three billion episodes of diarrhea annually, emphasizing the point that diarrhea is not only a significant disease in young children.²⁵ The main causes of diarrhea are infectious agents, including various bacteria, parasites and viruses. Noteworthy, rotavirus and caliciviruses (norovirus and sapovirus) have been identified as the main viral agents of acute diarrhea in children under five years of age when the entire world is taken into account.²⁶

Rotavirus mainly infects enterocytes in the gastrointestinal tract and clinically manifests as fever, vomiting and watery diarrhea.²⁷ Severe complications, including bloody diarrhea and

necrotizing enterocolitis, have been described in rotavirus-infected patients.²⁸ Rotavirus diarrhea is highly contagious and most children are infected before the age of five years.²⁹ Similar to rotavirus, norovirus is highly contagious and norovirus-infected patients shed the virus with a high viral load.³⁰ In fact, norovirus is the most frequent cause of acute diarrhea outbreaks when assessed globally.^{31,32} Generally, norovirus infection is self-limiting and no specific antiviral treatments are required. However, in a subset of patients, including immunocompromised individuals, the elderly and in young children, norovirus is associated with severe complications, such as diarrhea recurrence, villous atrophy and malabsorption.^{33,34} Recently, norovirus has emerged as an important cause of chronic infections in organ transplant patients.³⁵

Molecular virology and classification of rotavirus and norovirus

Rotavirus is a 70-nm icosahedral virus and belongs to the *Reoviridae* family. The viral particle seems like a wheel (Latin word: “rota”), hence its name.^{36,37} Rotavirus is a double stranded RNA virus, containing eleven dsRNA segments which encode six structural proteins (viral proteins, VP1-4, VP6 and VP7) and six non-structural proteins (NSP1-6). The rotavirus particle is composed of three concentric layers of viral structural proteins. The innermost capsid is formed by VP2 proteins. VP6 makes the intermediate shell of the virus and is regarded as the main capsid protein. The outermost layer consists of VP4 and VP7 proteins.³⁸ VP4 forms spikes which protrude from the outer surface. It is a protease-sensitive protein that can be cleaved into VP5* and VP8* to facilitate virus attachment to host cells and also furthers subsequent penetration into these host cells.³⁹ Importantly, upon presentation to the immune system, VP4 and VP7 induce the development of neutralizing antibody responses.⁴⁰ NSPs are synthesized during viral replication cycle and are involved in many aspects of rotavirus biology, pathogenesis and host immune responses.⁴¹ For example, NSP1 is a well-known interferon antagonist. NSP1 induces the proteasome-mediated degradation of IRF3, IRF5 and IRF7, leading to attenuation of the virus infection-combating interferon response.⁴²⁻⁴⁴ NSP4 plays a role as enterotoxin by altering Ca^{2+} release in the infected cells.^{45,46}

Rotavirus is classified into eight different serogroups (Groups A-H) based on the inner capsid protein VP6.⁴⁷ Recently, novel serogroups (I and J) have been suggested to be present as

well.^{48,49} Group A rotavirus is the most common serogroup with respect to human infection.⁴⁰ VP4 (a protease-sensitive protein) and VP7 (a glycoprotein) proteins are employed in a binary classification system of rotavirus into P- and G-genotype, respectively.³⁸ Currently, about 28 G-types, 39 P-types and 70 different G-P combinations have been identified.^{29,50} Among many G-P type combinations, G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] are the most common genotypes when viewed from a mondial perspective.⁵¹

Following serotype based classification, subsequently also a full genome-based classification system for rotaviruses has been introduced. In this system, a specific genotype for each of the eleven segments of particular rotavirus strain was assigned. The full descriptor of each rotavirus strain is described as Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx and represents the genotypes of VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, respectively.⁵² The Rotavirus Classification Working Group (RCWG) works towards further uniformity in rotavirus strain nomenclature and produces guidelines for the naming of newly identified rotavirus strains.⁵³ The RCWG proposed nomenclature for individual strains works as follows:

RV group/species of origin/country of identification/common name/year of identification/G- and P-type.

Norovirus is an icosahedral virus and belongs to the *Caliciviridae* family. It is divided into at least six genogroups (GI to GVI), in which GI, GII and GIV are known to infect humans. It is further subdivided into more than 40 genotypes.³⁰ Human norovirus is a 7.6 kb, non-segmented positive-strand RNA genome consisting of three ORFs. ORF1, ORF2 and ORF3 encode a large non-structural proteins (polyprotein), a major structural protein (VP1) and the minor structural protein (VP2), respectively. The polyprotein encoded by ORF1 is composed of p48 (NS1/2 or N-term); NTPase (NS3 or 2C-like); p22 (NS4 or 3A-like); VPg; Pro (NS6); and RNA-dependent RNA polymerase (RdRp).³⁰ The viral capsid is formed by 90 dimers of VP1, consisting of a shell (S) and a protruding (P) domain. The inner surface of the capsid is also composed of few copies of VP2.⁵⁴ The P domain plays an essential role for binding to histo-blood group antigens (HBGAs) which serve as receptors or co-receptors of the host cells, and thus determine genetic susceptibility to norovirus infection in humans.⁵⁵ Although more than 40 different genotypes infecting humans have been identified, GII.4 is the main genotype and has been responsible for the multiple global pandemics of norovirus-

mediated gastroenteritis in the last two decades. Novel GII.4 variants emerge and replace the previously dominant variants every two to seven years. These variants include US95/96 (1995-2000 pandemic); Farmington Hilss (2002-2004 pandemic); Hunter (2004-2005 pandemic); Den Haag 2006b (2006-2010 pandemic); New Orleans (2010-2012 pandemic) and Sydney (2013 pandemic). GII.4 remained the dominant strain detected in clinical samples in 2016.^{56,57} However, several reports have recently indicated an emergence of GII.17 Kawasaki as a major cause of norovirus pandemics and this strain is predicted to replace the currently dominant GII.4 Sydney norovirus as the major cause of norovirus-associated pathology.⁵⁸

Antiviral therapy and vaccines against rotavirus and norovirus

At present, there are no specific antiviral medicines for the treatment of rotavirus infection. Thus patient management is mainly focused on fluid and electrolyte replacement therapy to prevent dehydration.⁵⁹ For infection prevention, two commercial vaccines are available, i.e. Rotarix (containing G1P[8] strain) and RotaTeq (containing G1, G2, G3, G4 and P[8] strains), and have been universally introduced in more than 50 countries. Both vaccines have reduced the burden of rotavirus diarrhea worldwide.⁶⁰ However, they have not been included in National Immunization Program (NIP) in many countries, including Indonesia. This is probably associated with vaccine costs and policy considerations.⁶¹ In developing countries, sustained availability of affordable and effective vaccines is pivotal to reduce the burden of vaccine-preventable diseases. Therefore, new rotavirus vaccine candidates have been developed and currently are in different phases of clinical development. A monovalent human-bovine 116E strain-based vaccine, developed by an Indian company, has now completed phase III trials.^{62,63} Another vaccine, the RV3-BB rotavirus vaccine which was developed by a consortium that involved collaboration between BioPharma (Indonesia national vaccine company), the Faculty of Medicine Universitas Gadjah Mada (UGM) Indonesia, and the Murdoch Children Research Institute (MCRI) Australia, has completed a phase IIb trial in Indonesia.⁶⁴

For norovirus, no approved specific antiviral medication and vaccines are available to treat or to prevent infection. Development of novel antivirals and vaccines was hampered by the lack of robust cell culture and animal models of norovirus infections.⁵⁴ Currently, there are

several models available, including a Huh7 cell-based replicon system as well as organoid models.⁶⁵⁻⁶⁷ The development of anti-norovirus drugs is currently based on screening existing already approved medications.⁶⁸ Dose adjustments of immunosuppressive medications should be first considered for transplant patients at risk of contracting norovirus.³⁵ Should those clinical management techniques not successfully clear norovirus, effective antivirals are highly needed. Ribavirin, a guanosine analogue, exerts antiviral effects against a wide range of RNA and DNA virus, including rotavirus and norovirus.^{69,70} Mycophenolic acid (MPA), an uncompetitive inosine monophosphate dehydrogenase (IMPDH) inhibitor, potently inhibits rotavirus and norovirus replication.^{69,71} These results, although preliminary, should provide guidance for the management of transplantation patients at risk for contracting rotavirus and norovirus infections.

AIMS AND OUTLINE OF THE THESIS

Hepatitis E, rotavirus and norovirus provoke a substantial disease burden worldwide, both in developed and in developing countries. Hepatitis E virus is the predominant cause of acute hepatitis, while rotavirus and norovirus are the main agents for causing acute gastroenteritis in children. Improved understanding of the disease burden involved is essential to raise awareness of the importance of the diseases. To achieve better control of these diseases, development of vaccines and novel antiviral therapies will prove exceedingly useful. Viral infections are tightly regulated by many cellular signaling pathways. Dynamic virus-host interactions, including host responses and viral mutations, will determine the outcome of viral infections.

Aims of The Thesis

The aims of this thesis are to describe the burden and epidemiology of hepatitis E, rotavirus and norovirus infections, and to improve our understanding of virus-drug-host interactions, by exploring antiviral drugs and cellular signaling pathway, including interferon and NF- κ B pathway.

Thesis Outline

In **chapter 2**, I first aim to generate a comprehensive description of the global burden associated with HEV outbreaks. By performing a systematic review of published studies, I show that HEV is responsible for repeated water-borne outbreaks of acute hepatitis over the past century, and thus clearly represents an emerging public health issue warranting further research effort. Currently, control measures mainly depend upon the improvement of sanitation and hygiene. Although important, this will not prove sufficient to control the problem. Therefore, in **chapter 3**, I discuss the recent progress on understanding innate and adaptive immunity in HEV infection. Since immune responses are critical for determining the clinical outcome of HEV infection, understanding of HEV immunopathogenesis should provide the basis for the development of effective vaccines and therapies to achieve a better control of HEV-associated diseases. In this context, it is important to note that the discovery of new anti-viral therapies for HEV has hitherto mainly been based on the screening of currently clinically available antiviral medicines. In **chapter 4**, I thus explored the potency of sofosbuvir (SOF), a direct-acting antiviral (DAA) agent against hepatitis C virus, to inhibit HEV infection. Contrary to a previously published study, I demonstrated that SOF is likely not of value for the treatment of hepatitis E. In search for alternative strategies, in **chapter 5**, I explore the molecular evolution of HEV in the human population, documenting characteristic mutations and their associations with susceptibility, pathogenesis and therapeutic responses. My results indicated that HEV is under substantial evolutionary pressure to develop mutations which enable evasion of the host immune response and resistance to antiviral treatment. Thus, HEV for now remains versatile in developing novel strategies to evade its eradication, and I discuss the implications of these findings in **chapter 12**.

Not discouraged, I then focused on rotavirus and norovirus infections. In **chapter 6**, I explored the role of different types of interferons (IFNs) in regulating the course of rotavirus infections. I found that rotavirus predominantly induces type III IFNs (IFN- λ 1), and to a lesser extent, type I IFNs (IFN- α and IFN- β) in human intestinal cells. In addition, I established the essential role of constitutive IFN signaling in constraining rotavirus replication in an experimental approach involving the silencing of *STAT1*, *STAT2* and *IRF9* genes. Next,

chapter 7 describes potent antiviral effects of TNF- α against rotavirus infections, independent of type I interferon productions. I then established that the anti-rotavirus effect of TNF- α depended on the induction of transcription of NF κ B-target genes via the activation of classical nuclear factor κ B (NF- κ B) signaling. My study thus uncovered a somewhat unexpected antiviral action of TNF- α which may act against a diverse types of viruses and exploiting this novel avenue may prove useful in the fight of humankind against these diseases. In **chapter 8**, I describe the potency of 6-TG, a commonly used drug as an immunosuppressive agent for organ transplantation and inflammataory bowel disease (IBD), as anti-rotavirus drug associated with a high barrier to drug resistance emergence. In conjunction this work opens the way to improve anti-rotavirus therapy as I also discuss in **chapter 12**.

Following these mechanistic studies, I subsequently turned my attention to a more molecular epidemiological characterization of viral infection. In this context diarrhea is especially relevant as it significantly contributes to the overall global burden of disease, especially so in developing countries. It is well known that rotavirus and norovirus are the most dominant viral agents responsible for diarrheal disease globally. Therefore in **chapter 9**, I first performed a comprehensive review of rotavirus and norovirus study in Indonesia. I identified, however, very limited data regarding the incidence and circulating norovirus genotypes in Indonesia, but with respect to rotavirus the situation was much better. Subsequently in **chapter 10**, I describe the prevalence of norovirus and rotavirus infections in children less than five years of age hospitalized with acute gastroenteritis in Indonesia. This study reveals a considerably high burden of norovirus and rotavirus gastroenteritis in Indonesian children under five years of age. Finally in **chapter 11**, I describe rotavirus surveillance data conducted in Yogyakarta and demonstrated a high burden of rotavirus-associated diarrhea. Again I integrate these data with the other data in this thesis in **chapter 12**, and in conjunction my thesis thus provides insight into the global burden and the epidemiological dynamics of important viral infections, the potential of medication and immune-based strategies for combating viral disease and the molecular evolution of the virus in reaction to such strategies. My conclusion will be that although humanity is currently gaining the upperhand, its victory over viral disease is still quite far away.

References

1. Sarpel D, Baichoo E, Dieterich DT. Chronic hepatitis B and C infection in the United States: a review of current guidelines, disease burden and cost effectiveness of screening. *Expert Rev Anti Infect Ther* 2016;14(5):511-21.
2. Hoofnagle JH, Nelson KE, Purcell RH. Hepatitis E. *N Engl J Med* 2012;367(13):1237-44.
3. Holla RP, Ahmad I, Ahmad Z, Jameel S. Molecular virology of hepatitis E virus. *Semin Liver Dis* 2013;33(1):3-14.
4. Cao D, Meng XJ. Molecular biology and replication of hepatitis E virus. *Emerg Microbes Infect* 2012;1(8):e17.
5. Nan Y, Zhang YJ. Molecular biology and infection of hepatitis E virus. *Front Microbiol* 2016;7:1419.
6. Yamada K, Takahashi M, Hoshino Y, et al. ORF3 protein of hepatitis E virus is essential for virion release from infected cells. *J Gen Virol* 2009;90(Pt 8):1880-91.
7. Emerson SU, Nguyen HT, Torian U, Burke D, Engle R, Purcell RH. Release of genotype 1 hepatitis E virus from cultured hepatoma and polarized intestinal cells depends on open reading frame 3 protein and requires an intact PXXP motif. *J Virol* 2010;84(18):9059-69.
8. Huang YW, Opriessnig T, Halbur PG, Meng XJ. Initiation at the third in-frame AUG codon of open reading frame 3 of the hepatitis E virus is essential for viral infectivity in vivo. *J Virol* 2007;81(6):3018-26.
9. Nair VP, Anang S, Subramani C, et al. Endoplasmic reticulum stress induced synthesis of a novel viral factor mediates efficient replication of genotype 1 hepatitis E Virus. *PLoS Pathog* 2016;12(4):e1005521.
10. Smith DB, Simmonds P, Izopet J, et al. Proposed reference sequences for hepatitis E virus subtypes. *J Gen Virol* 2016;97(3):537-42.
11. Hakim MS, Wang W, Bramer WM, et al. The global burden of hepatitis E outbreaks: a systematic review. *Liver Int* 2017;37(1):19-31.
12. Zhou X, de Man RA, de Kneegt RJ, Metselaar HJ, Peppelenbosch MP, Pan Q. Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* 2013;23(5):295-304.
13. Debing Y, Moradpour D, Neyts J, Gouttenoire J. Update on hepatitis E virology: Implications for clinical practice. *J Hepatol* 2016;65(1):200-12.
14. Hakim MS, Ikram A, Zhou J, Wang W, Peppelenbosch MP, Pan Q. Immunity against hepatitis E virus infection: Implications for therapy and vaccine development. *Rev Med Virol* 2018; 28(2): e1964.
15. Wang Y, Metselaar HJ, Peppelenbosch MP, Pan Q. Chronic hepatitis E in solid-organ transplantation: the key implications of immunosuppressants. *Curr Opin Infect Dis* 2014;27(4):303-8.
16. Peters van Ton AM, Gevers TJ, Drenth JP. Antiviral therapy in chronic hepatitis E: a systematic review. *J Viral Hepat* 2015;22(12):965-73.
17. Debing Y, Gisa A, Dallmeier K, et al. A mutation in the hepatitis E virus RNA polymerase promotes its replication and associates with ribavirin treatment failure in organ transplant recipients. *Gastroenterology* 2014;147(5):1008-11 e7; quiz e15-6.
18. Dao Thi VL, Debing Y, Wu X, et al. Sofosbuvir inhibits hepatitis E virus replication in vitro and results in an additive effect when combined with ribavirin. *Gastroenterology* 2016;150(1):82-5 e4.
19. Todesco E, Demeret S, Calin R, et al. Chronic hepatitis E in HIV/HBV coinfecting patient: lack of power of sofosbuvir-ribavirin. *AIDS* 2017;31(9):1346-8.
20. Wang W, Hakim MS, Nair VP, et al. Distinct antiviral potency of sofosbuvir against hepatitis C and E viruses. *Gastroenterology* 2016;151(6):1251-3.

21. Wang W, Peppelenbosch MP, Pan Q. Targeting viral polymerase for treating hepatitis E infection: How far are we? *Gastroenterology* 2016;150(7):1690.
22. Kamar N, Wang W, Dalton HR, Pan Q. Direct-acting antiviral therapy for hepatitis E virus? *Lancet Gastroenterol Hepatol* 2017;2(3):154-5.
23. Ikram A, Hakim MS, Zhou JH, Wang W, Peppelenbosch MP, Pan Q. Genotype-specific acquisition, evolution and adaptation of characteristic mutations in hepatitis E virus. *Virulence* 2018;9(1):121-32.
24. Elliott EJ. Acute gastroenteritis in children. *BMJ* 2007;334(7583):35-40.
25. Walker CL, Black RE. Diarrhoea morbidity and mortality in older children, adolescents, and adults. *Epidemiol Infect* 2010;138(9):1215-26.
26. Lanata CF, Fischer-Walker CL, Olascoaga AC, et al. Global causes of diarrheal disease mortality in children <5 years of age: a systematic review. *PLoS One* 2013;8(9):e72788.
27. Ramig RF. Pathogenesis of intestinal and systemic rotavirus infection. *J Virol* 2004;78(19):10213-20.
28. Sharma R, Hudak ML, Premachandra BR, et al. Clinical manifestations of rotavirus infection in the neonatal intensive care unit. *Pediatr Infect Dis J* 2002;21(12):1099-105.
29. Durmaz R, Kalaycioglu AT, Acar S, et al. Prevalence of rotavirus genotypes in children younger than 5 years of age before the introduction of a universal rotavirus vaccination program: report of rotavirus surveillance in Turkey. *PLoS One* 2014;9(12):e113674.
30. de Graaf M, van Beek J, Koopmans MP. Human norovirus transmission and evolution in a changing world. *Nat Rev Microbiol* 2016;14(7):421-33.
31. Fankhauser RL, Noel JS, Monroe SS, Ando T, Glass RI. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. *J Infect Dis* 1998;178(6):1571-8.
32. Fankhauser RL, Monroe SS, Noel JS, et al. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. *J Infect Dis* 2002;186(1):1-7.
33. Green KY. Norovirus infection in immunocompromised hosts. *Clin Microbiol Infect* 2014;20(8):717-23.
34. Munir N, Liu P, Gastanaduy P, Montes J, Shane A, Moe C. Norovirus infection in immunocompromised children and children with hospital-acquired acute gastroenteritis. *J Med Virol* 2014;86(7):1203-9.
35. Angarone MP, Sheahan A, Kamboj M. Norovirus in transplantation. *Curr Infect Dis Rep* 2016;18(6):17.
36. Bishop R. Discovery of rotavirus: Implications for child health. *J Gastroenterol Hepatol* 2009;24 Suppl 3:S81-5.
37. Flewett TH, Bryden AS, Davies H, Woode GN, Bridger JC, Derrick JM. Relation between viruses from acute gastroenteritis of children and newborn calves. *Lancet* 1974;2(7872):61-3.
38. Greenberg HB, Estes MK. Rotaviruses: from pathogenesis to vaccination. *Gastroenterology* 2009;136(6):1939-51.
39. Ludert JE, Krishnaney AA, Burns JW, Vo PT, Greenberg HB. Cleavage of rotavirus VP4 in vivo. *J Gen Virol* 1996;77 (Pt 3):391-5.
40. Angel J, Franco MA, Greenberg HB. Rotavirus vaccines: recent developments and future considerations. *Nat Rev Microbiol* 2007;5(7):529-39.
41. Hu L, Crawford SE, Hyser JM, Estes MK, Prasad BV. Rotavirus non-structural proteins: structure and function. *Curr Opin Virol* 2012;2(4):380-8.
42. Arnold MM, Barro M, Patton JT. Rotavirus NSP1 mediates degradation of interferon regulatory factors through targeting of the dimerization domain. *J Virol* 2013;87(17):9813-21.
43. Sen A, Rott L, Phan N, Mukherjee G, Greenberg HB. Rotavirus NSP1 protein inhibits interferon-mediated STAT1 activation. *J Virol* 2014;88(1):41-53.
44. Bagchi P, Bhowmick R, Nandi S, Kant Nayak M, Chawla-Sarkar M. Rotavirus NSP1 inhibits interferon induced non-canonical NFkappaB activation by interacting with TNF receptor associated factor 2. *Virology* 2013;444(1-2):41-4.

45. Einerhand AW. Rotavirus NSP4 acts as a viral enterotoxin to induce diarrhea and is a potential target for rotavirus vaccines. *J Pediatr Gastroenterol Nutr* 1998;27(1):123-4.
46. Berkova Z, Crawford SE, Trugnan G, Yoshimori T, Morris AP, Estes MK. Rotavirus NSP4 induces a novel vesicular compartment regulated by calcium and associated with viroplasms. *J Virol* 2006;80(12):6061-71.
47. Desselberger U. Rotaviruses. *Virus Res* 2014;190:75-96.
48. Mihalov-Kovacs E, Gellert A, Marton S, et al. Candidate new rotavirus species in sheltered dogs, Hungary. *Emerg Infect Dis* 2015;21(4):660-3.
49. Banyai K, Kemenesi G, Budinski I, et al. Candidate new rotavirus species in Schreiber's bats, Serbia. *Infect Genet Evol* 2017;48:19-26.
50. Durmaz R, Bakkaloglu Z, Unaldi O, et al. Prevalence and diversity of rotavirus A genotypes circulating in Turkey during a 2-year sentinel surveillance period, 2014-2016. *J Med Virol* 2018;90(2):229-38.
51. Kawai K, O'Brien MA, Goveia MG, Mast TC, El Khoury AC. Burden of rotavirus gastroenteritis and distribution of rotavirus strains in Asia: a systematic review. *Vaccine* 2012;30(7):1244-54.
52. Matthijnssens J, Ciarlet M, Rahman M, et al. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. *Arch Virol* 2008;153(8):1621-9.
53. Matthijnssens J, Ciarlet M, McDonald SM, et al. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). *Arch Virol* 2011;156(8):1397-413.
54. Karst SM, Wobus CE, Goodfellow IG, Green KY, Virgin HW. Advances in norovirus biology. *Cell Host Microbe* 2014;15(6):668-80.
55. Ruvoen-Clouet N, Belliot G, Le Pendu J. Noroviruses and histo-blood groups: the impact of common host genetic polymorphisms on virus transmission and evolution. *Rev Med Virol* 2013;23(6):355-66.
56. Bull RA, White PA. Mechanisms of GII.4 norovirus evolution. *Trends Microbiol* 2011;19(5):233-40.
57. Donaldson EF, Lindesmith LC, Lobue AD, Baric RS. Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations. *Immunol Rev* 2008;225:190-211.
58. de Graaf M, van Beek J, Vennema H, et al. Emergence of a novel GII.17 norovirus - End of the GII.4 era? *Euro Surveill* 2015;20(26).
59. Yin Y, Metselaar HJ, Sprengers D, Peppelenbosch MP, Pan Q. Rotavirus in organ transplantation: drug-virus-host interactions. *Am J Transplant* 2015;15(3):585-93.
60. Tate JE, Parashar UD. Rotavirus vaccines in routine use. *Clin Infect Dis* 2014;59(9):1291-301.
61. Hakim MS, Nirwati H, Aman AT, Soenarto Y, Pan Q. Significance of continuous rotavirus and norovirus surveillance in Indonesia. *World J Pediatr* 2018;14(1):4-12.
62. Bhandari N, Rongsen-Chandola T, Bavdekar A, et al. Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian children in the second year of life. *Vaccine* 2014;32 Suppl 1:A110-6.
63. Bhandari N, Rongsen-Chandola T, Bavdekar A, et al. Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian infants: a randomised, double-blind, placebo-controlled trial. *Lancet* 2014;383(9935):2136-43.
64. Bines JE, At Thobari J, Satria CD, et al. Human neonatal rotavirus vaccine (RV3-BB) to target rotavirus from birth. *N Engl J Med* 2018;378(8):719-30.
65. Papafragkou E, Hewitt J, Park GW, Greening G, Vinje J. Challenges of culturing human norovirus in three-dimensional organoid intestinal cell culture models. *PLoS One* 2014;8(6):e63485.
66. Ettayebi K, Crawford SE, Murakami K, et al. Replication of human noroviruses in stem cell-derived human enteroids. *Science* 2016;353(6306):1387-93.
67. Chang KO, Sosnovtsev SV, Belliot G, King AD, Green KY. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. *Virology* 2006;353(2):463-73.

68. Dang W, Yin Y, Peppelenbosch MP, Pan Q. Opposing effects of nitazoxanide on murine and human norovirus. *J Infect Dis* 2017;216(6):780-2.
69. Dang W, Yin Y, Wang Y, et al. Inhibition of calcineurin or IMP dehydrogenase exerts moderate to potent antiviral activity against norovirus replication. *Antimicrob Agents Chemother* 2017;61(11).
70. Yin Y, Bijvelds M, Dang W, et al. Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. *Antiviral Res* 2015;123:120-31.
71. Yin Y, Wang Y, Dang W, et al. Mycophenolic acid potently inhibits rotavirus infection with a high barrier to resistance development. *Antiviral Res* 2016;133:41-9.

Part I.

Hepatitis E Virus

Chapter 2

The Global Burden of Hepatitis E Outbreaks: A Systematic Review

Mohamad S. Hakim^{1,2}, Wenshi Wang¹, Wichor M. Bramer³, Jiawei Geng⁴, Fen Huang⁵,
Robert A. de Man¹, Maikel P. Peppelenbosch¹, Qiuwei Pan¹

- ¹ Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center Rotterdam, the Netherlands
- ² Department of Microbiology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia
- ³ Medical Library, Erasmus MC-University Medical Center Rotterdam, the Netherlands
- ⁴ Department of Infectious Diseases, The First People's Hospital of Yunnan Province, Kunming, China.
- ⁵ Medical Faculty, Kunming University of Science and Technology, Kunming, China

Liver International 2017; 37(1): 19-31

Abstract

Hepatitis E virus (HEV) is responsible for repeated water-borne outbreaks since the past century, representing an emerging issue in public health. However, the global burden of HEV outbreak has not been comprehensively described. We performed a systematic review of confirmed HEV outbreaks based on published literatures. HEV outbreaks have mainly been reported from Asian and African countries, and only a few from European and American countries. India represents a country with the highest number of reported HEV outbreaks. HEV genotypes 1 and 2 were responsible for most of the large outbreaks in developing countries. During the outbreaks in developing countries, a significantly higher case fatality rate was observed in pregnant women. In fact, outbreaks have occurred both in open and closed populations. The control measures mainly depend upon improvement of sanitation and hygiene. This study highlights that HEV outbreak is not new, yet it is a continuous global health problem.

Keywords: global burden, Hepatitis E, outbreaks

Key Points

- India represents a country with the highest number of reported HEV outbreaks.
- The number of reported HEV outbreaks is most likely underestimation of the actual burden of HEV outbreaks globally.
- In recent years, the burden of HEV outbreaks come from refugee camps in African countries.
- The availability of HEV vaccine should contribute to better control of HEV disease.

Introduction

Hepatitis E virus (HEV) infection is a major cause of outbreaks and acute sporadic hepatitis worldwide. HEV infecting humans consists of four different genotypes (genotype 1-4), with several sub genotypes exist in each. However, only one single HEV serotype was recognized [1, 2]. HEV genotypes 1 and 2 are found mainly in developing countries. They are transmitted via fecal-oral route through a contaminated water source, exclusively infect humans, and are thus responsible for many water-borne outbreaks. In contrast, HEV genotypes 3 and 4 infect humans and animals. They are found mainly in developed countries and are responsible for sporadic cases seen in the western world [3, 4]. In 2005, it was estimated that HEV genotypes 1 and 2 were responsible for about 20.1 million incidents of HEV infections, 3.4 million symptomatic cases, 70,000 fatalities, and 3,000 stillbirths [5]. In general, HEV causes a self-limiting infection and does not need specific treatment. The mortality rate is low. However, fulminant hepatitis may develop and a high mortality rate (as high as 20-30%) is reported in the population of pregnant women after infection with genotype 1 [1].

HEV is a spherical, non-enveloped, single-stranded positive sense ribonucleic acid (RNA) virus that mainly infects the hepatocyte [6]. HEV genome was first entirely cloned in 1991 [7, 8]. Historically, HEV was suggested as a causative agent during jaundice outbreaks with a high attack rate among young adults and resulted in a high mortality rate among pregnant women [9]. Many large, water-borne, jaundice outbreaks in the past were described as non-A, non-B (NANB) hepatitis outbreaks due to failure in identifying hepatitis A (HAV) and hepatitis B virus (HBV) as the responsible agent of the outbreaks [10, 11]. The existence of HEV was already suggested in 1980 during the investigation of the causative agent of a NANB hepatitis outbreak in Kashmir Valley, India [12].

Since the discovery of HEV, many archived samples obtained during NANB hepatitis outbreaks were tested for the presence of HEV [13]. The first retrospectively identified HEV outbreak was a large jaundice outbreaks in New Delhi, India, in 1955-1956 with more than 29,000 suspected cases [13, 14]. Along with the development of serology- and reverse transcription-polymerase chain reaction (RT-PCR)-based diagnostic methods, many HEV outbreaks were then identified (confirmed), both in the past (NANB hepatitis outbreaks) and

in the recent years. Understanding the global distribution of confirmed HEV outbreaks could heighten our awareness of this under-recognized and under-reported human pathogen and improve HEV surveillance.

Therefore, we comprehensively reviewed the confirmed HEV outbreaks in the literature. More specifically, we described the global geographical distribution of (confirmed) HEV outbreaks, the severity (case-fatality rates), outbreak settings and modes of transmission, control measures, and the distribution of HEV genotype responsible for the outbreaks.

Materials and Methods

Literature search

A systematic search of available literature (conducted on 10 March 2015) was performed using the electronic database Embase.com, Medline (Ovid), the Cochrane library, Web of Science, Scopus, and Cinahl (EBSCOhost). Additional references were retrieved from unindexed references from PubMed, Lilacs, Scielo, and Google Scholar. Additional references were sought by reviewing the reference list of selected studies. The search terms were designed by an experienced information specialist (WB). The search was executed without any restrictions of publication date or language. The search terms consisted of two main elements: hepatitis E virus (HEV) and outbreak. For each element, multiple synonyms were searched in title and/or abstract, and when available thesaurus terms (Mesh for medline, Emtree for embase and CINAHL headings for CINAHL). The search strategies for all databases are available in Supplementary Table 1.

Study selection, inclusion and exclusion criteria

After removing the duplicates, we screened the articles based on the title and abstract. The full text copies of included studies based on title and abstract screening were then assessed for eligibility. The inclusion criteria include: 1) Original research articles or reports, informing an outbreak of hepatitis E. An outbreak was identified by: a) reporting an attack rates; b) clearly demonstrated the epidemiological curve; c) reporting large scale, affect several hundred to several thousands of people; d) specify the time course, either short (few weeks) or long period (few months until year[s]); 2) The study used PCR-based and/or serology-

based diagnostics (IgM and IgG anti-HEV antibody to confirm the presence of HEV as a responsible agent for the outbreak; 3) Studies showing NANB hepatitis outbreak that was confirmed later by another study showing that the outbreak was due to HEV; 4) Any studies that confirmed previous NANB hepatitis outbreak as an HEV outbreak; 5) Any studies reported sequencing analysis of HEV strains derived from the outbreak. The following exclusion criteria were used for full-text screening: 1) full-text not available; 2) language other than English; 3) not primary study during the outbreak; 4) not sufficient information. The selection procedure was performed by two independent investigators (M.S.H. and W.W.). Disagreements were resolved by discussion.

Data extraction

M.S.H. extracted the data with help of W.W. Data were extracted from the full-text papers of the included studies. The following items were extracted: author, year of publication, country, specific region (if available), the time of the outbreak (month and year), number of suspected cases, attack rate in general population, diagnosis used (serology, RT-PCR, sequencing), number of sample tested, number of confirmed cases, case fatality rates (CFR) both in general population and pregnant women, outbreak settings, risk factors (modes of transmission), control measures, and HEV genotype. Attack rate was defined as the number of suspected cases divided by the number of exposed population times 100. CFR was defined as the number of deaths divided by the number of suspected cases times 100. Our procedures accorded with the PRISMA guidelines for reporting systematic review and/or meta-analysis (Supplementary Table 5).

Results

Description of the included studies

Using our search strategy, we identified potentially relevant 3,776 articles. After removal of duplicates, 1,653 articles were recorded for title and abstract screening. Of these, 191 articles met the eligibility criteria based on full-text and abstract screening and 10 articles identified from manual search. After assessing 201 full-text articles, we ultimately included 98 articles in this systematic review (Figure 1).

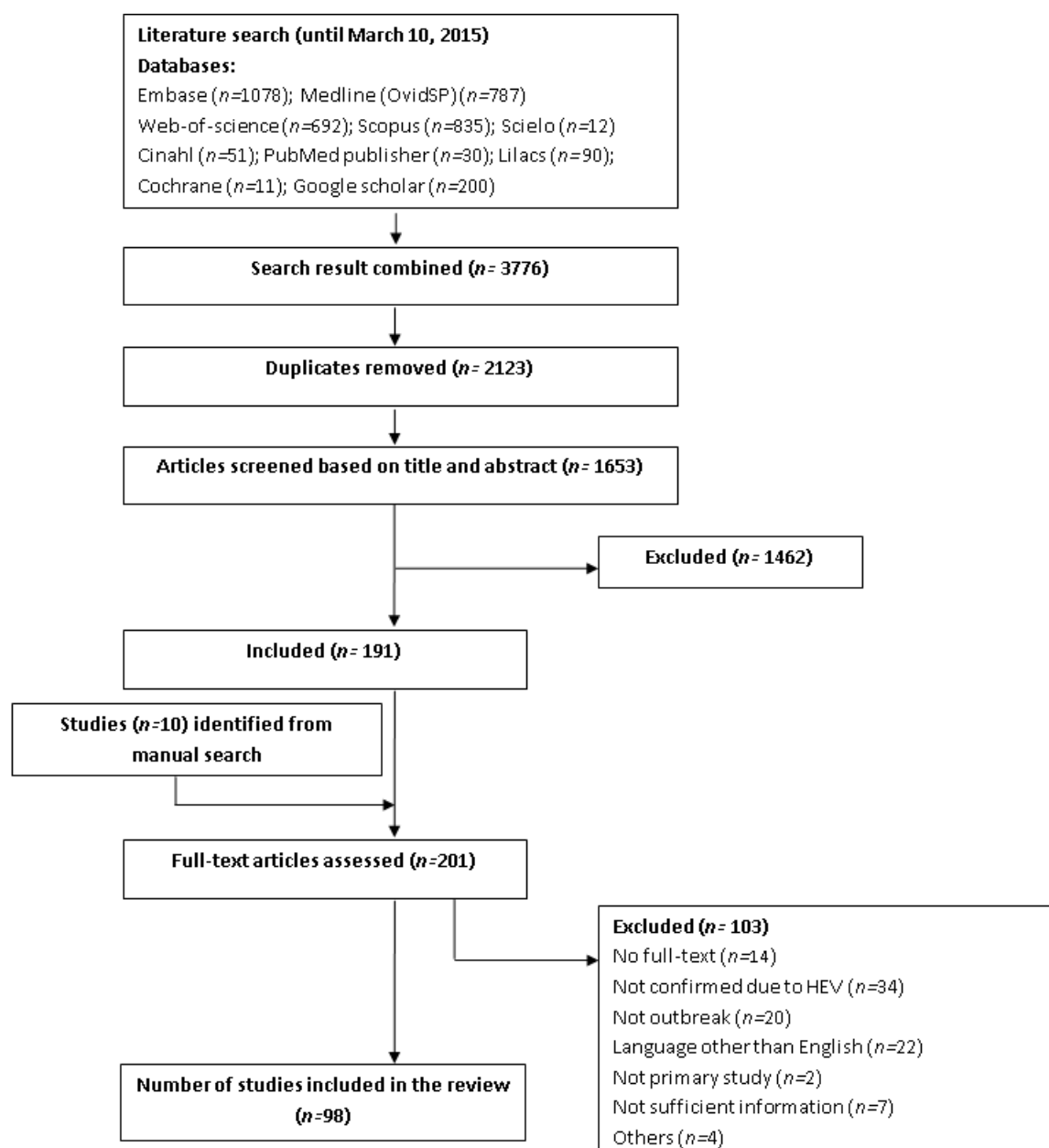


Figure 1. Flow diagram showing literature search and selection results.

Since we did not restrict the publication date and considering the fact that HEV has caused NANB-hepatitis outbreak far before its identification, the publication dates of the included studies ranged from 1978 to 2015. Most of these studies describe the incident of HEV outbreaks in Asian and African countries, and only 5 studies describe HEV outbreaks in American and European countries. Interestingly, a large number of the included studies describing HEV outbreaks occurred in one country, India.

Confirmed HEV outbreak and overall attack rate**Asia**

HEV outbreaks have been reported from 12 countries: Indonesia [15-17], Myanmar [18], Vietnam [19], Japan [20], China [21], Bangladesh [22, 23], Pakistan [24-29], Nepal [30], Iraq [31], Uzbekistan [32], Turkmenistan [33], and India [12-14, 34-65] (Figure 2 and Supplementary Table 2 and 3). The first confirmed HEV outbreaks occurred in New Delhi, India in 1955 [13]. During this outbreak, about 29,000 suspected cases were reported, with an attack rate 2.05%. Retrospective analysis of archived serum samples from 28 patients successfully detected IgM anti-HEV antibodies in all samples (100%) to confirm that HEV was responsible for this large historical outbreak [13]. After this large outbreak, India has repeatedly reported large HEV epidemics, affecting hundreds to thousands of people (Figure 3). The largest HEV outbreak in India was reported in Kanpur, India during December 1990 - April 1991. About 79,000 suspected cases (jaundice patients) were reported, with an attack rate of 3.76%. Analysis of 41 serum samples showed evidence of NANB hepatitis outbreak [43]. Analysis of stool samples from this epidemic demonstrated the evidence of HEV RNA in 6 out of 10 samples analyzed (60%), confirming that HEV was the etiologic agent of this NANB hepatitis outbreak [42]. Another large HEV outbreak was reported from Nellore (south India) with 23,915 suspected cases [62]. From 1975-1994, India experienced 21 HEV outbreaks, 13 of them (62%) reported more than one thousand of suspected cases. The most recent epidemic in India was reported from Lalkuan (Nainital District, Uttarakhand) with approximately 240 suspected cases [65]. The attack rate ranged from 0.34% [37] to 8.61% [65]. There were only three outbreaks that reported attack rate of more than 10%, i.e. Saharanpur, 1992-1993 (14%) [45]; Nainital district, Uttarakhand, July 2005 (16%) [56]; and Baramulla district, Kashmir, 2007-2008 (21.6%) [60]. These data suggest that India is highly endemic for hepatitis E.

There were four HEV outbreak reported from Pakistan [24-28]. The first reported HEV outbreak was Sargodha outbreak which occurred during March - April 1987 [24, 25]. A large water-borne outbreak was reported from the city of Islamabad, affecting 3,827 people, with 10.4% attack rate [27]. A localized HEV outbreak was occurred in the military unit of Abbottabad (August - September 1988), in which more than 100 suspected cases were

recorded [26]. In all these outbreaks, the reported attack rates were more than 10%, ranging from 10.4% [27] to 20% [24].

Bangladesh reported only two HEV outbreaks [22, 23]. An outbreak with more than 4,000 cases was reported from Arichpur, an urban area near Dhaka, with 4% attack rate [22]. From south-east Asian countries, Indonesia reported two HEV outbreaks, in East Java [17] and Kalimantan island [15, 16]. Other south-east Asian countries, such as Myanmar and Vietnam only reported one outbreak [18, 19].

In east Asia, the largest reported outbreak in the world so far was reported from Xinjiang, China. A huge number of 120,000 suspected cases was reported during prolonged outbreak that lasted from September 1986 - April 1988, with an overall attack rate of 3.0% [21]. In the middle-east region, HEV outbreak was only reported from Baghdad, Iraq at 2005, after the Iraq war. More than 250 suspected cases were reported during this outbreak [31]. From central Asia, a large HEV outbreak occurred in the Dashoguz province of Turkmenistan, with more than 16,000 cases were reported [33].

Africa

HEV outbreaks have been reported from 14 countries: Egypt [66], Kenya [67, 68], Sudan and South Sudan [69-76], Central African Republic (CAR) [77-79], Uganda [80-84], Chad [73, 76, 85-89], Republic of Djibouti [90], Algeria [85, 86, 89, 91], Namibia [92, 93], Morocco [94, 95], Somalia [96, 97], Ethiopia [98], South Africa [99], and Cameroon [100] (Figure 2 and Supplementary Table 2). The first, large, laboratory confirmed HEV outbreak involved more than 140 villages in Somalia on early 1988 - late 1989. There were more than 11,000 suspected cases reported with an overall attack rate of 4.6% [96, 97]. A large HEV outbreak was also reported from Kitgum district, Uganda. More than 10,000 suspected cases from October 2007 - June 2009 were reported with an overall attack rate of 25.1% [80-82]. During the investigation, the outbreak was still ongoing and therefore, the number of suspected cases might be increasing. In the last decade, outbreaks of hepatitis E have been reported from several area with warfare and conflict, causing human displacement. Several large HEV outbreaks, involving hundreds to thousands cases, were reported from refugee camps in

Kenya (1,702 cases) [67]; South Sudan (>5,000 cases) [75]; Darfur, Sudan (2,621 cases) [70, 71]; and Chad (>900 cases) [73, 87].

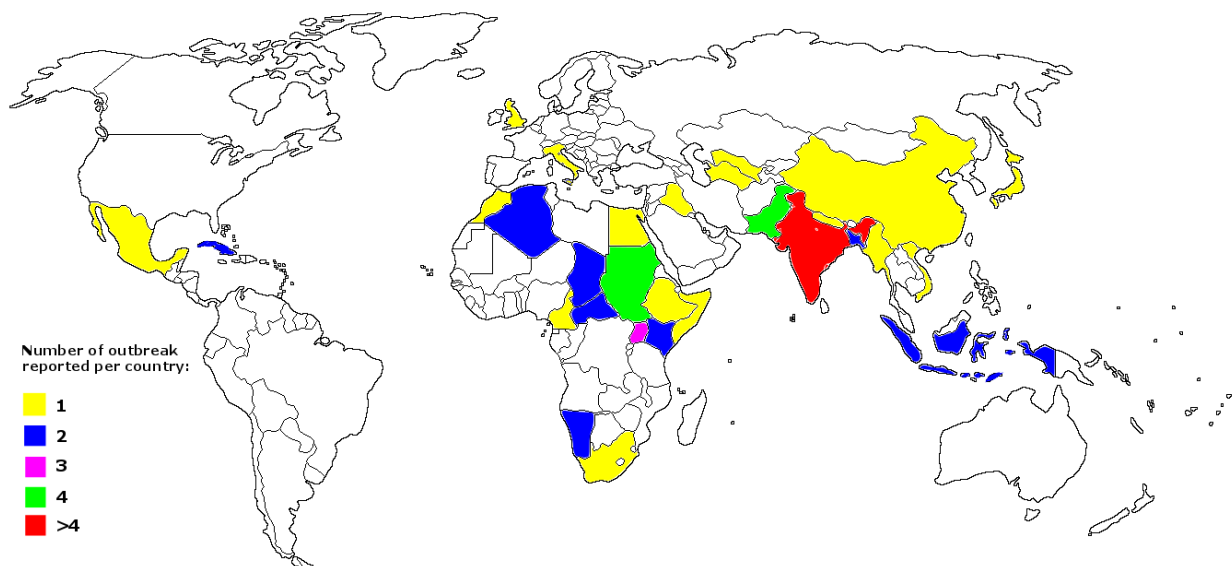


Figure 2. The global HEV outbreak distribution. (Note: Sudan and South Sudan are regarded as one country).

America and Europe

Only few outbreaks were reported from European and American countries. In Europe, a confirmed HEV outbreak probably related to shellfish exposition and involving genotype 3 was reported on cruise ship returning to United Kingdom after a world cruise. 33 of 789 passengers (4%) who provided blood samples were IgM anti-HEV positive, confirming a recent acute HEV infection [101]. A small HEV outbreak was reported from Lazio, Italy. Five suspected cases were reported and all of them were HEV positive (genotype 4) [102]. In America, HEV outbreak was first reported from two villages, Huitzililla and Telixtac, Mexico in 1986, with more than 200 suspected cases. The overall attack rate was 5-6% [103-105]. No HEV outbreak was reported from Mexico thereafter. Another country, Cuba, reported two HEV outbreaks [106].

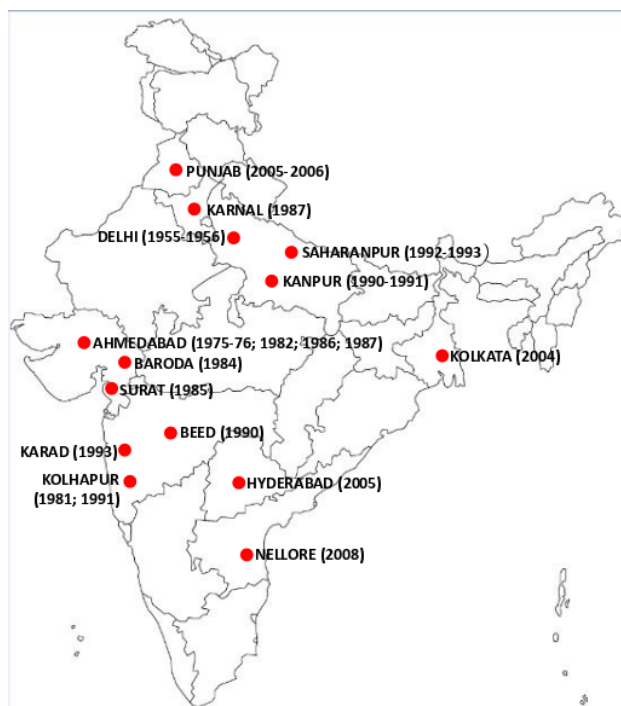


Figure 3. The epidemic history of large HEV outbreak in India with more than 1,000 suspected cases.

Case fatality rate (CFR)

The CFRs were reported in 38 studies (Supplementary Table 4). In overall population, CFRs were relatively low, between 1 and 3%. The highest reported CFR of overall population was 3.6%, in the Kashmir valley outbreak, India, in 1978 – 1979, involving 275 suspected cases [12]. One study reported an overall CFR of 33% (6 fatalities out of 18 cases) [28]. This outbreak occurred among patients in neurosurgery ward in the hospital. Therefore, the underlying disease and condition might be important factors influencing this high CFR.

Compared with overall population, fatalities are higher in pregnant woman. The CFR among pregnant woman ranging from 5.1% in Rajasthan, India during February 2006 [58] to 31.1% in refugee camp, Darfur, Sudan during July - December 2004 [70, 71]. From 15 studies which reported CFR of both overall and pregnant women population, we found a significantly higher CFR in pregnant women compared to overall population (Figure 4). One study specifically compared the CFR among non-pregnant and pregnant females population. It was shown that the CFR of pregnant females was significantly higher than non-pregnant females (11% vs. 1.5%, $p < 0.01$) [96].

In addition to a high CFR among pregnant woman, HEV infection during pregnancy may lead to worse outcome. In HEV outbreak setting, several studies descriptively reported worse pregnancy outcomes such as postpartum hemorrhage, premature delivery, stillbirth, miscarriage, and neonatal death [22, 74, 78]. Since these were descriptive studies, the relative contributions of HEV infection to pregnancy-related outcome could not be determined. Gurley ES *et al.* [22] reported that pregnancies complicated by acute jaundice had an increased risk for miscarriage, stillbirth and neonatal death, as compared to pregnancy without jaundice (OR 2.7; 95% CI 1.2-6.1).

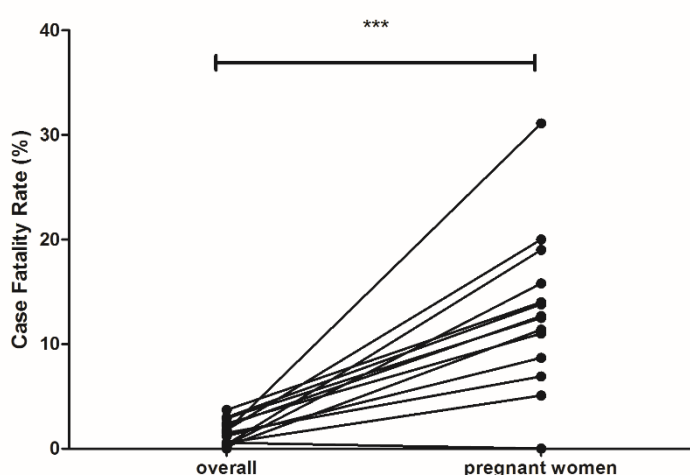


Figure 4. Case Fatality Rates (CFR) of overall population and pregnant women.

Outbreak settings

Most HEV outbreaks occurred in community-based settings, such as village (rural area), city (urban area) or affecting a large area (one province) (Table 1). Several outbreaks occurred in a more-restricted (closed) settings, such as military units [18, 26, 30, 49, 51, 98], college [24], prison [47], and factory [106]. In recent years, several outbreaks were also reported from refugee camps with a big number of suspected cases [67, 68, 70, 75, 92]. Interestingly, one study reported an HEV outbreak that occurred on a cruise ship [101].

Outbreak settings and underlying cause (modes of transmission)	References
Outbreak settings	
City (urban area)	[22]; [23]; [27]; [31]; [37]; [38]; [43]; [45]; [46]; [48]; [52-55]; [57]; [59]; [61]; [62]; [65]; [78]; [79]; [93]; [106] ⁵
Village (rural area)	[12, 36] ³ ; [17] ⁴ ; [19] ⁴ ; [40]; [56]; [58]; [60]; [64]; [66]; [91]; [96] ⁴ ; [100]; [104, 105] ³
Affect large area (district or province)	[33] ² ; [41]; [50] ¹ ; [80-82] ³ ; [84]
Refugee camps	[67]; [68]; [70, 71] ³ ; [75]; [83]; [92]
Military units or military camps	[18]; [26]; [30]; [49]; [51]; [98]
Hospital	[28]; [99]
Cruise ship	[101]
Prison	[47]
Factory	[106] ⁵
College	[24]
Modes of transmission	
Contamination of drinking water	
Leakage of water pipeline (broken, poor construction)	[18]; [22]; [31]; [38]; [41]; [45]; [49]; [51]; [53]; [54]; [57-59]; [61]; [64]; [65]
Failure of water treatment	[24]; [27]; [40]; [43]; [45]; [52]; [60]; [70]
Use of untreated water from river, spring	[12]; [17]; [56]; [91]; [96]
Flooding, heavy rainfall	[19]; [31]; [69]; [75]
Leakage of sewage pipelines	[38]; [55]
Food contamination	[101]

¹ Two district affected; ² One province affected; ³ Refer to one outbreak; ⁴ Situated along the river; ⁵ Two outbreaks reported in one study

Table 1. HEV outbreak settings and underlying cause of HEV outbreaks.

Risk factors and modes of transmission

Several risk factors were reported as the underlying cause of the outbreak (Table 1). The main mode of transmission reported was water-borne transmission. Leakage of water pipeline due to broken or poor construction was the most reported cause underlying the outbreak. The broken water pipelines lead to fecal or sewage contamination of the drinking water supply. Another underlying cause of the outbreak was failure of water treatment (such as filtration or chlorination). This failure led to the supply of grossly contaminated drinking water to the household. The use of untreated water from river and spring was also

reported as the underlying cause of the outbreak. Several HEV outbreaks occurred following flooding or heavy rainfall [19, 31, 69, 75], facilitating contamination of water supplies with feces. One study reported food contamination as the likely cause of the outbreak of HEV aboard a cruise ship [101].

Role of person-to-person transmission

Several studies investigated the occurrence of person-to-person transmission during HEV outbreaks [26, 40, 43-45, 58, 63, 81, 94, 104]. Most of the studies suggest that there was no or minimal evidence of person-to-person transmission during HEV outbreak. However, there were variations between studies to determine the occurrence of person-to-person transmission. Only one study suggested that person-to-person transmission might be responsible for HEV outbreak in a large and prolonged HEV outbreak in Uganda [81]. This conclusion was supported by several observations: 1) prolonged outbreak, which occurred about 2 years; 2) HEV was undetectable from the environment (water sources) and the zoonotic sources (pig); 3) improvement of hygiene (such as chlorination) could not stop the epidemic; and 4) evidence of close contact and time interval between index and secondary cases within household [81]. However, some inquiries have been questioned to argue against the evidence [107, 108]. The relative contribution of person-to-person transmission therefore deserves further investigation, especially in the large and prolonged outbreaks. As HEV transmission occur via fecal-oral route, person-to-person transmission might be possible.

Control measures

To cope with the outbreak, control measures should be taken to prevent more additional cases. However, not all studies described specifically the control measures taken during the outbreaks (Table 2). Chlorination of the water supply was the most reported control measures during HEV outbreaks, followed by repairing of the broken water pipeline. Improving general hygienic precaution (such as hand washing and boiling of drinking water) is a simple and low cost intervention to prevent HEV transmission during outbreak. Provision of an alternatively safe water supply (such as providing containers of safe drinking water) was reported. Lack of proper facilities for disposal of human feces is one of the underlying

factors responsible for outbreaks, especially in refugee camps. Therefore, hastening of latrine construction was reported as a control measure during HEV outbreaks in the refugee camps.

No	Intervention	References
1	Chlorination of the water supply	[26]; [37]; [38]; [40]; [44]; [45]; [54]; [63]; [65]; [77]; [83]
2	Repair of water pipelines	[26]; [45]; [46]; [49]; [53]; [57]; [59]; [61]; [62]; [65]
3	Improving general hygienic precautions (handwashing, boiling water)	[26]; [38]; [65]; [68]; [75]; [83]
4	Provision of alternate water supply	[27]; [30]; [65]
5	Hastening latrine construction.	[68]; [83]
6	Surveillance for additional cases (active case finding)	[26]; [75]
7	Simultaneous closure of of the water supply	[24]; [27]
8	Improving safe drinking water availability	[75]
9	Training of health care workers	[68]
10	Increasing community awareness	[68]

Table 2. Control measures of HEV outbreak.

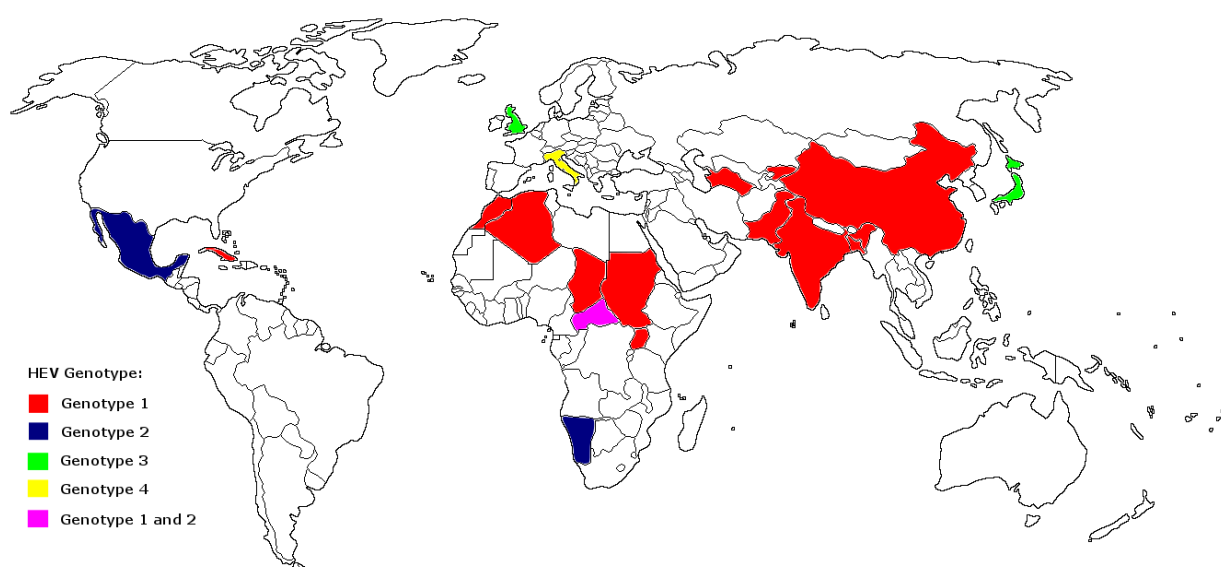


Figure 5. HEV genotype distribution responsible for the outbreaks. (Note: Sudan and South Sudan are regarded as one country.)

Country	Year	HEV Region sequenced	HEV Genotype	Reference
Asia				
India	2008	RNA-dependent RNA polymerase (RdRp gene)	Genotype 1, subtype A	[62]
India	2010	ORF1	Genotype 1, subtype A	[63]
India	1981	RNA polymerase	Genotype 1, subtype A	[109]
India	1975 - 1976	RNA polymerase	Genotype 1, subtype B	[109]
India	1984	RNA polymerase	Genotype 1, subtype A	[109]
India	1990	RNA polymerase	Genotype 1, subtype A	[109]
India	1991	RNA polymerase	Genotype 1, subtype D	[109]
Kyrgyzstan	1987 - 1989	ORF 2 (nt 5972-6319) and ORF 1 (nt 71-353)	Genotype 1	[114]
Bangladesh	2010	ORF2	Genotype 1, subtype A	[23]
Turkmenistan	1985	ORF2	Genotype 1	[33]
Pakistan	1987	Full genome (7195 nt)	Genotype 1, subtype B	[29]; [109]
China	1986 - 1988	Full genome	Genotype 1, subtype B	[21]
Japan	2005	ORF1	Genotype 3	[20]
Africa				
Morocco	1994	nt 5,014 - 7,186 (the 3'-terminal region of ORF1, full length ORF2 and ORF3, and a portion of the 3'-noncoding region)	Genotype 1	[95]
Central African Republic	2002	NS	Genotype 1 and 2	[77]
Sudan and Chad	2004	ORF 2 nucleotides 6,653-7,100	Genotype 1	[76]
Chad	1983 - 1984	ORF2 and ORF3	Genotype 1, subtype C	[89]; [109]
Uganda	2007 - 2009	ORF2	Genotype 1	[80]
Algeria	1986 - 1987	ORF2	Genotype 1	[91]
Algeria	1979 - 1980	ORF2 and ORF3	Genotype 1, subtype C	[89]; [109]
Namibia	1995 - 1996	451 bp region of a subgenomic fragment from the 3' end of the genome in ORF2	Genotype 2	[93]
Europe				
United Kingdom	2008	NS	Genotype 3	[101]
Italy	2011	ORF1 and ORF2	Genotype 4	[102]
America				
Cuba	1999 and 2005	ORF1	Genotype 1	[106]
Mexico	1986	Nearly complete genome (7185 nt)	Genotype 2	[109]; [110]

Table 3. HEV genotype responsible for the outbreak.

HEV genotypes responsible for the outbreak

Data on the genotype responsible for HEV outbreak were available only from limited number of studies (as summarized in Table 3 and shown in Figure 5). The open reading fragment 2 (ORF2) region was the most frequently region sequenced to determine the HEV genotype, followed by ORF 1 region (including RNA polymerase region). In accordance with the global

distribution of the HEV genotypes, genotype 1 and 2 were mainly responsible for the outbreaks occurred in developing countries (Asia and Africa), while genotype 3 and 4 were responsible for small outbreaks in the western world (Europe), i.e. United Kingdom [101] and Italy [102]. Genotype 2 was responsible for outbreaks in CAR [77], Namibia [93], and Mexico [109, 110]. In Asia, all but one outbreak were due to genotype 1. In Asia and Africa, it seems that genotype 1 was more responsible than genotype 2 as the causative agent of HEV outbreaks. Moreover, genotype 1 was also responsible for several large HEV outbreaks, such as in China (1986-1988, with 120,000 suspected cases) [21]; India (2008, with 23,915 suspected cases) [62]; Turkmenistan (1985, with 16,175 suspected cases) [33]; and Uganda (2007 - 2009, with >10,000 suspected cases) [80]. No large HEV outbreaks so far were reported due to genotype 3 and 4.

Discussion

Historically, epidemic of jaundice and hepatitis with high attack rates in young adults and predominant or exclusive deaths among pregnant women was believed to be due to HEV [9]. The first laboratory-confirmed HEV outbreak is Delhi outbreak (1955 - 1956) [111]. Since then, many HEV outbreaks were reported in the literature, especially after the availability of HEV diagnostic assay (HEV serology and RT-PCR). Our data suggest that HEV outbreak occurred repeatedly up to the recent years in many different countries, especially in Asian and African countries. It indicates that HEV outbreak is not new, yet it is a continuous health problem in developing countries. It is highly possible that our data only represent a tip of the iceberg. A higher percentage of HEV outbreaks that have occurred in many (other) countries might be not reported and not well-documented, mainly due to the absence of a surveillance system of HEV infection or lack of serology and PCR confirmation. For example, about 33 outbreaks of acute viral hepatitis in Cuba were not well-reported and therefore excluded from our analysis [112]. Similarly, reports from 10 different Asian and African countries were not well-documented [113]. We also found a report of an HEV sequence derived from a Kyrgyzstan outbreak, but we could not find the outbreak description [114]. Consequently, the actual number of HEV outbreaks should be much higher than what we present in this study. Therefore, the problem of HEV infection should not be underestimated by national and international health agencies.

HEV represents a significant health problem, especially in the developing countries. Acute sporadic form of HEV disease is the most frequent cause of acute viral hepatitis globally [2]. Epidemics of HEV, either in a small or large scale, occur periodically up to this moment, as reported from India [115, 116]. Many large outbreaks of hepatitis E have been reported especially from west and north part of India and thus represent a major health problem in the country (Figure 3). Several outbreaks have also been reported from neighboring countries such as Bangladesh, Pakistan, and Nepal (Figure 2). The Indian subcontinent, therefore, could be the best representation of areas with high endemicity of HEV infection.

In recent years, several large HEV outbreaks reported from refugee settlements. Because of warfare and conflict in some African countries, displaced populations occupy refugee settlements and this has led to a new epidemic setting for HEV [67, 68, 70, 75, 92]. As the disease is mainly transmitted by fecal contamination of drinking water, the density of the resident population, a limited access to a good quality of drinking water, lack of adequate sanitation and personal hygiene, may predispose to the occurrence of HEV outbreaks in refugee camps [117]. Currently, increasing number of refugee population, resulted from wars, persecution, conflict and human rights violations, imposes one of the most pressing global challenges. This led to a complex humanitarian crisis, partly due to lack access of health service [118]. The most common causes of death in this population are communicable diseases, such as diarrheal diseases, measles, and malaria [119]. These refugee camps are potential risk settings for water-borne outbreaks including HEV, cholera, HAV, and rotavirus [120-123], and they deserve the access of more timely, appropriate, and quality health-care services.

Although our data showed a limited number of reported HEV outbreaks in European and American countries, we cannot fully exclude the possibility that HEV could be the future threat in the region. HEV was considered as one of the emerging zoonotic swine pathogens [124]. Autochthonous HEV infection was reported from several countries in Europe, with evidence of zoonotic transmission from pigs [125]. A recent study has reported a small outbreak in China, which is caused by the zoonotic genotype 4 HEV and is related to the food in the company's cafeteria [126]. Therefore, it is highly possible that HEV genotypes 3 and 4 could be the potential cause of small-scale outbreaks in the developed countries in the near

future, especially with the lack of transmission route identification and the lack of effective intervention strategies.

During HEV infection, the risk of progression towards fulminant hepatitis is higher among pregnant women as compared to men and non-pregnant women [127, 128]. Several studies during HEV outbreaks demonstrated that HEV infection could result in worse maternal and fetal outcome [22, 74, 78]. Similarly, studies of pregnant women presenting with jaundice due to acute viral hepatitis in hospital-based setting showed that FHF and mortality rate was greater in HEV-infected women than in non-HEV-infected women [128]. HEV-infected pregnant women have also a significantly higher risk of developing obstetric complications, intrauterine fetal death, preterm delivery, and stillbirth as compared to non-HEV-infected pregnant women [128]. It is estimated that HEV is responsible for ~9.8% of pregnancy-associated deaths in Bangladesh and about 10,500 of annual maternal death in southern Asia [129]. Some immunological and hormonal factors have been associated with high mortality rate in HEV-infected pregnant women [130-132]. Interventions to prevent the occurrence of HEV infections in this high-risk population are therefore urgently required [129].

Most studies reported fecal contamination of drinking water as the major route of transmission during HEV outbreak. The most commonly reported underlying cause of this contamination is leakage of water pipeline distribution system, either due to damaged or poor construction. As the water pipelines located close to drain or sewerage system, the damaged facilitate mixing of sewage materials and drinking water supplied to the household, causing water-borne outbreaks such as HEV, hepatitis A virus (HAV), shigellosis and cholera [133-135]. A water-borne outbreak of pesticide poisoning was also reported due to damage of water pipeline distribution system [136]. Therefore, this kind of outbreak could be prevented by proper construction of water pipelines, keeping them away from the drain system, and also by monitoring of pipelines for damage.

Since HEV outbreak is mainly due to contaminated-drinking water, its control would depend upon improved hygiene and sanitation, such as increased access to safe water, provision of soap and chlorine tablets to improve personal hygiene, and proper sewage disposal. During outbreak, it is pivotal to intensively investigate the suspected underlying cause and then

initiate targeted intervention to control and stop the outbreak [83]. Mass vaccination of HEV could be another effective strategy to control the outbreaks. Currently, an HEV vaccine has already been licensed for use in China [137] and give an insight that HEV is a vaccine-preventable disease [111]. Comparing the experience with HAV vaccination as an effective measure to control HAV outbreaks, the HEV vaccine holds promises to control large outbreaks. However, it is not known whether the current vaccine works fast enough to effectively protect the exposed population for clinical disease during an HEV outbreak and how long the protection will be afforded. Moreover, it is also not known whether the vaccine is safe and effective in pregnant women, the population in which a high fatality rate was seen during the outbreak [129]. In fact, there is disagreement among the HEV experts whether the current licensed vaccine is necessary to prevent outbreak following the recent earthquake in Nepal [138-140].

Limitation of the published literature

There are some limitations in the published literature of HEV outbreaks. First, the studies used different criteria to define suspected cases during HEV outbreak. Some studies only used physical symptoms of acute hepatitis (such as jaundice) [27]; whereas other studies included laboratory criteria such as liver enzyme (aspartate and alanine aminotransferase) [47, 62]. The differences in the criteria may then influences the different calculations of the attack rate. Second, the studies on HEV outbreak used different assays and diagnostic methods to confirm the presence of HEV as the causative agent of outbreak. Therefore, it is difficult to compare the validity of the reports. Third, the outbreaks studied varied in the proportion of suspected cases to be tested for HEV. Consequently, the proportion of confirmed HEV cases differs markedly between outbreaks. Moreover, these data also suggest that some of these outbreaks might have been caused not only by a single agent (HEV), but also another agent that may also spread by fecal-oral route, especially HAV. Finally, the outbreak studies used different epidemiological methods to investigate the outbreaks. Some of those outbreaks were investigated thoroughly, but some of them were not. The full versions of epidemiological investigations of several Indian epidemics were not available, even though the outbreaks involved a large scale, in which thousands of people were affected [13].

Conclusions

The available data suggest that HEV outbreaks occur repeatedly in many developing countries, especially in India and become a significant health problem in Asian and African continent, even before its identification. These outbreaks were mainly due to HEV genotype 1 and 2. Prevention of HEV outbreak in the future is therefore required to reduce the burden of HEV disease. The HEV vaccine, which has been licensed in China, could be potentially used in the control of HEV infection in the future. However, its safety (especially in pregnant women) and efficacy during the outbreak require further investigation. Meanwhile, the preventive measures of HEV outbreak would mainly depend upon improved sanitation and hygiene.

Acknowledgements

The author thank to the Indonesia Endowment Fund for Education (LPDP) for funding PhD fellowship to Mohamad S. Hakim; the Dutch Digestive Foundation (MLDS) for a career development grant (No. CDG 1304), the Daniel den Hoed Foundation for a Centennial Award fellowship and the Erasmus MC Mrace grant to Q. Pan; and the China Scholarship Council for funding PhD fellowship to W. Wang (201303250056).

Supplementary Information

<http://onlinelibrary.wiley.com/doi/10.1111/liv.13237/abstract>

Conflict of interest

The authors have none to declare.

References

1. Kamar N, Bendall R, Legrand-Abravanel F, *et al.* Hepatitis E. *Lancet* 2012; **379**: 2477-88.
2. Purcell RH, Emerson SU. Hepatitis E: an emerging awareness of an old disease. *J Hepatol* 2008; **48**: 494-503.
3. Teshale EH, Hu DJ. Hepatitis E: Epidemiology and prevention. *World J Hepatol* 2011; **3**: 285-91.
4. Kim JH, Nelson KE, Panzner U, Kasture Y, Labrique AB, Wierzbza TF. A systematic review of the epidemiology of hepatitis E virus in Africa. *BMC Infect Dis* 2014; **14**: 308.
5. Rein DB, Stevens GA, Theaker J, Wittenborn JS, Wiersma ST. The global burden of hepatitis E virus genotypes 1 and 2 in 2005. *Hepatology* 2012; **55**: 988-97.
6. Ahmad I, Holla RP, Jameel S. Molecular virology of hepatitis E virus. *Virus Res* 2011; **161**: 47-58.
7. Reyes GR, Purdy MA, Kim JP, *et al.* Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science* 1990; **247**: 1335-9.
8. Tam AW, Smith MM, Guerra ME, *et al.* Hepatitis E virus (HEV): Molecular cloning and sequencing of the full-length viral genome. *Virology* 1991; **185**: 120-31.
9. Teo CG. Fatal outbreaks of jaundice in pregnancy and the epidemic history of hepatitis E. *Epidemiol Infect* 2012; **140**: 767-87.
10. Wong DC, Purcell RH, Sreenivasan MA. Epidemic and endemic hepatitis in India: Evidence for a non-A, non-B hepatitis virus aetiology. *Lancet* 1980; **2**: 876-9.
11. Belabbes EH, Bouguermouh A, Benatallah A, Illoul G. Epidemic non-A, non-B viral hepatitis in Algeria: Strong evidence for its spreading by water. *J Med Virol* 1985; **16**: 257-63.
12. Khuroo MS. Study of an epidemic of non-A, non-B hepatitis. Possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. *Am J Med* 1980; **68**: 818-24.
13. Arankalle VA, Chadha MS, Tsarev SA, *et al.* Seroepidemiology of water-borne hepatitis in India and evidence for a third enterically-transmitted hepatitis agent. *Proc Natl Acad Sci U S A* 1994; **91**: 3428-32.
14. Viswanathan R. Infectious hepatitis in Delhi (1955-56): a critical study-epidemiology. 1957. *Natl Med J India* 2013; **26**: 362-77.
15. Corwin A, Jarot K, Lubis I, *et al.* Two years' investigation of epidemic hepatitis E virus transmission in West Kalimantan (Borneo), Indonesia. *Trans R Soc Trop Med Hyg* 1995; **89**: 262-5.
16. Jennings GB, Lubis I, Listiyaningsih E, Burans JP, Hyams KC. Hepatitis E virus in Indonesia. *Trans R Soc Trop Med Hyg* 1994; **88**: 57.
17. Sedyaningsih-Mamahit ER, Larasati RP, Laras K, *et al.* First documented outbreak of hepatitis E virus transmission in Java, Indonesia. *Trans R Soc Trop Med Hyg* 2002; **96**: 398-404.
18. Uchida T, Aye TT, Ma X, *et al.* An epidemic outbreak of hepatitis E in Yangon of Myanmar: Antibody assay and animal transmission of the virus. *Acta Pathol Jpn* 1993; **43**: 94-8.
19. Corwin AL, Khiem HB, Clayson ET, *et al.* A waterborne outbreak of hepatitis E virus transmission in southwestern Vietnam. *Am J Trop Med Hyg* 1996; **54**: 559-62.
20. Nakano Y, Yamauchi A, Yano T, *et al.* A diffuse outbreak of hepatitis E in Mie Prefecture, 2005. *Jpn J Infect Dis* 2006; **59**: 136-8.
21. Aye TT, Uchida T, Ma XZ, *et al.* Complete nucleotide sequence of a hepatitis E virus isolated from the Xinjiang epidemic (1986-1988) of China. *Nucleic Acids Res* 1992; **20**: 3512.
22. Gurley ES, Hossain MJ, Paul RC, *et al.* Outbreak of hepatitis E in urban Bangladesh resulting in maternal and perinatal mortality. *Clin Infect Dis* 2014; **59**: 658-65.
23. Harun-Or-Rashid M, Akbar SMF, Takahashi K, *et al.* Epidemiological and molecular analyses of a non-seasonal outbreak of acute icteric hepatitis E in Bangladesh. *J Med Virol* 2013; **85**: 1369-76.
24. Iqbal M, Ahmed A, Qamar A, *et al.* An outbreak of enterically transmitted non-A, non-B hepatitis in Pakistan. *Am J Trop Med Hyg* 1989; **40**: 438-43.

25. Bryan JP, Tsarev SA, Iqbal M, *et al.* Epidemic hepatitis E in Pakistan: Patterns of serologic response and evidence that antibody to hepatitis E virus protects against disease. *J Infect Dis* 1994; **170**: 517-21.
26. Bryan JP, Iqbal M, Tsarev S, *et al.* Epidemic of hepatitis E in a military unit in Abbottabad, Pakistan. *Am J Trop Med Hyg* 2002; **67**: 662-8.
27. Rab MA, Bile MK, Mubarik MM, *et al.* Water-borne hepatitis E virus epidemic in Islamabad, Pakistan: A common source outbreak traced to the malfunction of a modern water treatment plant. *Am J Trop Med Hyg* 1997; **57**: 151-7.
28. Siddiqui AR, Jooma RA, Smego Jr RA. Nosocomial outbreak of hepatitis E infection in Pakistan with possible parenteral transmission. *Clin Infect Dis* 2005; **40**: 908-9.
29. Tsarev SA, Emerson SU, Reyes GR, *et al.* Characterization of a prototype strain of hepatitis E virus. *Proc Natl Acad Sci U S A* 1992; **89**: 559-63.
30. Clayson ET, Vaughn DW, Innis BL, Shrestha MP, Pandey R, Malla DB. Association of hepatitis E virus with an outbreak of hepatitis at a military training camp in Nepal. *J Med Virol* 1998; **54**: 178-82.
31. Al-Nasrawi KK, Al-Diwan JK, Al-Hadithi TS, Saleh AM. Viral hepatitis E outbreak in Al-Sadr city, Baghdad, Iraq. *East Mediterr Health J* 2010; **16**: 1128-32.
32. Sharapov MB, Favorov MO, Yashina TL, *et al.* Acute viral hepatitis morbidity and mortality associated with hepatitis E virus infection: Uzbekistan surveillance data. *BMC Infect Dis* 2009; **9**: 35.
33. Albetkova A, Drobeniuc J, Yashina T, *et al.* Characterization of hepatitis E virus from outbreak and sporadic cases in Turkmenistan. *J Med Virol* 2007; **79**: 1696-702.
34. Chobe LP, Chadha MS, Banerjee K, Arankalle VA. Detection of HEV RNA in faeces, by RT-PCR during the epidemics of hepatitis E in India (1976-1995). *J Viral Hepat* 1997; **4**: 129-33.
35. Sreenivasan MA, Banerjee K, Pandya PG, *et al.* Epidemiological investigations of an outbreak of infectious hepatitis in Ahmedabad city during 1975-76. *Indian J Med Res* 1978; **67**: 197-206.
36. Skidmore SJ, Yarbough PO, Gabor KA, Reyes GR. Hepatitis E virus: The cause of a waterborne hepatitis outbreak. *J Med Virol* 1992; **37**: 58-60.
37. Sreenivasan MA, Sehgal A, Prasad SR, Dhorje S. A sero-epidemiologic study of a water-borne epidemic of viral hepatitis in Kolhapur City, India. *J Hyg* 1984; **93**: 113-22.
38. Dilawari JB, Singh K, Chawla YK, *et al.* Hepatitis E virus: epidemiological, clinical and serological studies of north Indian epidemic. *Indian J Gastroenterol* 1994; **13**: 44-8.
39. Jameel S, Durgapal H, Habibullah CM, Khuroo MS, Panda SK. Enteric non-A, non-B hepatitis: Epidemics, animal transmission, and hepatitis E virus detection by the polymerase chain reaction. *J Med Virol* 1992; **37**: 263-70.
40. Arankalle VA, Chadha MS, Mehendale SM, Tungatkar SP. Epidemic hepatitis E: serological evidence for lack of intrafamilial spread. *Indian J Gastroenterol* 2000; **19**: 24-8.
41. Risbud AR, Chadha MS, Kushwah SS, Arankalle VA, Rodrigues FM, Banerjee K. Non A non B hepatitis epidemic in Rewa district of Madhya Pradesh. *J Assoc Physicians India* 1992; **40**: 262-4.
42. Ray R, Aggarwal R, Salunke PN, Mehrotra NN, Talwar GP, Naik SR. Hepatitis E virus genome in stools of hepatitis patients during large epidemic in north India. *Lancet* 1991; **338**: 783-4.
43. Naik SR, Aggarwal R, Salunke PN, Mehrotra NN. A large waterborne viral hepatitis E epidemic in Kanpur, India. *Bull WHO* 1992; **70**: 597-604.
44. Aggarwal R, Naik SR. Hepatitis E: Intrafamilial transmission versus waterborne spread. *J Hepatol* 1994; **21**: 718-23.
45. Singh V, Singh V, Raje M, Nain CK, Singh K. Routes of transmission in the hepatitis E epidemic of Saharanpur. *Trop Gastroenterol* 1998; **19**: 107-9.
46. Singh J, Aggarwal NR, Bhattacharjee J, *et al.* An outbreak of viral hepatitis E: role of community practices. *J Commun Dis* 1995; **27**: 92-6.

47. Kar P, Gangwal P, Budhiraja S, *et al.* Analysis of serological evidence of different hepatitis viruses in acute viral hepatitis in prisoners in relation to risk factors. *Indian J Med Res* 2000; **112**: 128-32.
48. Aggarwal R, Kumar R, Pal R, Naik S, Semwal SN, Naik SR. Role of travel as a risk factor for hepatitis E virus infection in a disease-endemic area. *Indian J Gastroenterol* 2002; **21**: 14-8.
49. Banerjee A, Sahni AK, Rajiva, Nagendra A, Saiprasad GS. Outbreak of viral hepatitis E in a regimental training centre. *Med J Armed Forces India* 2005; **61**: 326-9.
50. Kumar S, Ratho RK, Chawla YK, Chakraborti A. Virological investigation of a hepatitis E epidemic in North India. *Singapore Med J* 2006; **47**: 769-73.
51. Singh PMP, Handa BSK, Banerjee A. Epidemiological investigation of an outbreak of viral hepatitis. *Med J Armed Forces India* 2006; **62**: 332-4.
52. Swain SK, Baral P, Hutin YJ, Rao TV, Murhekar M, Gupte MD. A hepatitis E outbreak caused by a temporary interruption in a municipal water treatment system, Baripada, Orissa, India, 2004. *Trans R Soc Trop Med Hyg* 2010; **104**: 66-9.
53. Das P, Adhikary KK, Gupta PK. An outbreak investigation of viral hepatitis E in south Dumdum Municipality of Kolkata. *Indian J Community Med* 2007; **32**: 84-5.
54. Sailaja B, Murhekar MV, Hutin YJ, *et al.* Outbreak of waterborne hepatitis E in Hyderabad, India, 2005. *Epidemiol Infect* 2009; **137**: 234-40.
55. Sarguna P, Rao A, Sudha Ramana K. Outbreak of acute viral hepatitis due to hepatitis E virus in Hyderabad. *Indian J Med Microbiol* 2007; **25**: 378-82.
56. Martolia HCS, Hutin Y, Ramachandran V, Manickam P, Murhekar M, Gupte M. An outbreak of hepatitis E tracked to a spring in the foothills of the Himalayas, India, 2005. *Indian J Gastroenterol* 2009; **28**: 99-101.
57. Bali S, Kar SS, Kumar S, Ratho RK, Dhiman RK, Kumar R. Hepatitis E epidemic with bimodal peak in a town of north India. *Indian J Public Health* 2008; **52**: 189-93.
58. Rai RR, Nijhawan S, Mathur A, Sharma MP, Udawat HP, Singh N. Seroepidemiology and role of polymerase chain reaction to detect viremia in an epidemic of hepatitis E in Western India. *Trop Gastroenterol* 2008; **29**: 202-6.
59. Shankar P, Subrat K, Reddy GMM, Ratho RK, Rajesh K. Investigation of viral hepatitis E outbreak in a town in Haryana. *J Commun Dis* 2008; **40**: 249-54.
60. Khuroo MS, Khuroo MS. Seroepidemiology of a second epidemic of hepatitis E in a population that had recorded first epidemic 30 years before and has been under surveillance since then. *Hepatol Int* 2010; **4**: 494-9.
61. Chauhan NT, Prajapati P, Trivedi AV, Bhagyalaxmi A. Epidemic investigation of the jaundice outbreak in Girdharnagar, Ahmedabad, Gujarat, India, 2008. *Indian J Community Med* 2010; **35**: 294-7.
62. Vivek R, Nihal L, Illiayaraja J, *et al.* Investigation of an epidemic of Hepatitis E in Nellore in south India. *Trop Med Int Health* 2010; **15**: 1333-9.
63. Majumdar M, Singh MP, Pujhari SK, Bhatia D, Chawla Y, Ratho RK. Hepatitis E virus antigen detection as an early diagnostic marker: Report from India. *J Med Virol* 2013; **85**: 823-7.
64. Tambe MP, Patil SP, Dravid M, Bhagwat VR. Investigation of an outbreak of hepatitis 'E' in a rural area of Dhule district in Maharashtra. *J Krishna Ins Med Sciences Univ* 2015; **4**: 109-14.
65. Awsathi S, Rawat V, Rawat CM, Semwal V, Bartwal SJ. Epidemiological investigation of the jaundice outbreak in Lalkuan, Nainital district, Uttarakhand. *Indian J Community Med* 2014; **39**: 94-7.
66. Shata MT, Daef EA, Zaki ME, *et al.* Protective role of humoral immune responses during an outbreak of hepatitis E in Egypt. *Trans R Soc Trop Med Hyg* 2012; **106**: 613-8.
67. Mast EE, Polish LB, Favorov MO, *et al.* Hepatitis E among refugees in Kenya: Minimal apparent person-to-person transmission, evidence for age-dependent disease expression, and new serologic assays. In *Viral Hepatitis and Liver Disease*. Edited by Nishioka K, Suzuki H, Mishiro S, Oda T. Japan: Springer; 1994: 375-8.

68. Ahmed JA, Moturi E, Spiegel P, *et al.* Hepatitis E outbreak, Dadaab refugee camp, Kenya, 2012. *Emerg Infect Dis* 2013; **19**: 1010-2.
69. McCarthy MC, He J, Hyams KC, El-Tigani A, Khalid IO, Carl M. Acute hepatitis E infection during the 1988 floods in Khartoum, Sudan. *Trans R Soc Trop Med Hyg* 1994; **88**: 177.
70. Guthmann JP, Klovstad H, Boccia D, *et al.* A large outbreak of hepatitis E among a displaced population in Darfur, Sudan, 2004: The role of water treatment methods. *Clin Infect Dis* 2006; **42**: 1685-91.
71. Boccia D, Guthmann JP, Klovstad H, *et al.* High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan. *Clin Infect Dis* 2006; **42**: 1679-84.
72. Anonim. Hepatitis E, Sudan--update. *Wkly Epidemiol Rec* 2004; **79**: 341-2.
73. Anonim. Hepatitis E: Chad, Sudan. *Wkly Epidemiol Rec* 2004; **79**: 321.
74. Rayis DA, Jumaa AM, Gasim GI, Karsany MS, Adam I. An outbreak of hepatitis E and high maternal mortality at Port Sudan, Eastern Sudan. *Pathog Global Health* 2013; **107**: 66-8.
75. Thomson K, Luis Dvorzak J, Lagu J, *et al.* Investigation of hepatitis E outbreak among refugees - Upper Nile, South Sudan, 2012-2013. *Morb Mortal Wkly Rep* 2013; **62**: 581-6.
76. Nicand E, Armstrong GL, Enouf V, *et al.* Genetic heterogeneity of hepatitis E virus in Darfur, Sudan, and neighboring Chad. *J Med Virol* 2005; **77**: 519-21.
77. Escriba JM, Nakoune E, Recio C, *et al.* Hepatitis E, Central African Republic. *Emerg Infect Dis* 2008; **14**: 681-3.
78. Goumba CM, Yandoko-Nakoune ER, Komas NP. A fatal case of acute hepatitis E among pregnant women, Central African Republic. *BMC Res Notes* 2010; **3**: 103.
79. Goumba AI, Konamna X, Komas NP. Clinical and epidemiological aspects of a hepatitis E outbreak in Bangui, Central African Republic. *BMC Infect Dis* 2011; **11**: 93.
80. Teshale EH, Howard CM, Grytdal SP, *et al.* Hepatitis E epidemic, Uganda. *Emerg Infect Dis* 2010; **16**: 126-9.
81. Teshale EH, Grytdal SP, Howard C, *et al.* Evidence of person-to-person transmission of hepatitis E virus during a large outbreak in northern Uganda. *Clin Infect Dis* 2010; **50**: 1006-10.
82. Howard CM, Handzel T, Hill VR, *et al.* Novel risk factors associated with hepatitis E virus infection in a large outbreak in Northern Uganda: Results from a case-control study and environmental analysis. *Am J Trop Med Hyg* 2010; **83**: 1170-3.
83. Cummings MJ, Wamala JF, Komakech I, *et al.* Hepatitis E in Karamoja, Uganda, 2009-2012: epidemiology and challenges to control in a setting of semi-nomadic pastoralism. *Trans R Soc Trop Med Hyg* 2014; **108**: 648-55.
84. Gerbi GB, Williams R, Bakamutumaho B, *et al.* Hepatitis E as a cause of acute jaundice syndrome in northern Uganda, 2010-2012. *Am J Trop Med Hyg* 2015; **92**: 411-4.
85. Coursaget P, Krawczynski K, Buisson Y, Nizou C, Molinie C. Hepatitis E and hepatitis C virus infections among French soldiers with non-A, non-B hepatitis. *J Med Virol* 1993; **39**: 163-6.
86. Van Cuyck-Gandre H, Caudill JD, Zhang HY, *et al.* Short report: Polymerase chain reaction detection of hepatitis E virus in north African fecal samples. *Am J Trop Med Hyg* 1996; **54**: 134-5.
87. Anonim. Hepatitis E, Chad. *Wkly Epidemiol Rec* 2004; **79**: 313.
88. Anonim. Outbreak news. *Wkly Epidemiol Rec* 2004; **79**: 329-31.
89. Van Cuyck-Gandre H, Zhang HY, Tsarev SA, *et al.* Characterization of hepatitis E virus (HEV) from Algeria and Chad by partial genome sequence. *J Med Virol* 1997; **53**: 340-7.
90. Coursaget P, Buisson Y, Enogat N, *et al.* Outbreak of enterically-transmitted hepatitis due to hepatitis A and hepatitis E viruses. *J Hepatol* 1998; **28**: 745-50.
91. Grandadam M, Tebbal S, Caron M, *et al.* Evidence for hepatitis E virus quasispecies. *J Gen Virol* 2004; **85**: 3189-94.
92. Isaacson M, Frean J, He J, Seriwatana J, Innis BL. An outbreak of hepatitis E in Northern Namibia, 1983. *Am J Trop Med Hyg* 2000; **62**: 619-25.
93. Maila HT, Bowyer SM, Swanepoel R. Identification of a new strain of hepatitis E virus from an outbreak in Namibia in 1995. *J Gen Virol* 2004; **85**: 89-95.

94. Benjelloun S, Bahbouhi B, Bouchrit N, *et al.* Seroepidemiological study of an acute hepatitis E outbreak in Morocco. *Res Virol* 1997; **148**: 279-87.
95. Meng J, Cong M, Dai X, *et al.* Primary structure of open reading frame 2 and 3 of the hepatitis E virus isolated from Morocco. *J Med Virol* 1999; **57**: 126-33.
96. Bile K, Isse A, Mohamud O, *et al.* Contrasting roles of rivers and wells as sources of drinking water on attack and fatality rates in a hepatitis E epidemic in Somalia. *Am J Trop Med Hyg* 1994; **51**: 466-74.
97. Mushahwar IK, Dawson GJ, Bile KM, Magnusius LO. Serological studies of an enterically transmitted non-A, non-B hepatitis in Somalia. *J Med Virol* 1993; **40**: 218-21.
98. Tsega E, Krawczynski K, Hansson BG, *et al.* Outbreak of acute hepatitis E virus infection among military personnel in northern Ethiopia. *J Med Virol* 1991; **34**: 232-6.
99. Robson SC, Adams S, Brink N, Woodruff B, Bradley D. Hospital outbreak of hepatitis E. *Lancet* 1992; **339**: 1424-5.
100. Maurice D, Abassora M, Marcelin NM, Richard N. First documented outbreak of hepatitis E in Northern Cameroon. *Ann Trop Med Public Health* 2013; **6**: 682-3.
101. Said B, Ijaz S, Kafatos G, *et al.* Hepatitis E outbreak on cruise ship. *Emerg Infect Dis* 2009; **15**: 1738-44.
102. Garbuglia AR, Scognamiglio P, Petrosillo N, *et al.* Hepatitis E virus genotype 4 outbreak, Italy, 2011. *Emerg Infect Dis* 2013; **19**: 110-4.
103. Anonim. Enterically transmitted non-A, non-B hepatitis--Mexico. *MMWR Morb Mortal Wkly Rep* 1987; **36**: 597-602.
104. Velazquez O, Stetler HC, Avila C, *et al.* Epidemic transmission of enterically transmitted non-A, non-B hepatitis in Mexico, 1986-1987. *J Am Med Assoc* 1990; **263**: 3281-5.
105. Favorov MO, Fields HA, Purdy MA, *et al.* Serologic identification of hepatitis E virus infections in epidemic and endemic settings. *J Med Virol* 1992; **36**: 246-50.
106. Villalba MDLCM, Lay LDLAR, Chandra V, *et al.* Hepatitis E virus genotype 1, Cuba. *Emerg Infect Dis* 2008; **14**: 1320-2.
107. Nishiura H. Household data from the Ugandan hepatitis E virus outbreak indicate the dominance of community infection. *Clin Infect Dis* 2010; **51**: 117-8.
108. Aggarwal R. Hepatitis E virus and person-to-person transmission. *Clin Infect Dis* 2010; **51**: 477-8; author reply 8-9.
109. Arankalle VA, Paranjape S, Emerson SU, Purcell RH, Walimbe AM. Phylogenetic analysis of hepatitis E virus isolates from India (1976-1993). *J Gen Virol* 1999; **80**: 1691-700.
110. Huang CC, Nguyen D, Fernandez J, *et al.* Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). *Virology* 1992; **191**: 550-8.
111. Aggarwal R. Hepatitis E: Historical, contemporary and future perspectives. *J Gastroenterol Hepatol* 2011; **26 Suppl 1**: 72-82.
112. Lay LDLAR, Quintana A, Villalba MCM, *et al.* Dual infection with hepatitis A and E viruses in outbreaks and in sporadic clinical cases: Cuba 1998-2003. *J Med Virol* 2008; **80**: 798-802.
113. Favorov MO, Khudyakov YE, Mast EE, *et al.* IgM and IgG antibodies to hepatitis E virus (HEV) detected by an enzyme immunoassay based on an HEV-specific artificial recombinant mosaic protein. *J Med Virol* 1996; **50**: 50-8.
114. Lu L, Drobeniuc J, Kobylnikov N, *et al.* Complete sequence of a Kyrgyzstan swine Hepatitis E Virus (HEV) isolated from a piglet thought to be experimentally infected with human HEV. *J Med Virol* 2004; **74**: 556-62.
115. Labrique AB, Thomas DL, Stoszek SK, Nelson KE. Hepatitis E: an emerging infectious disease. *Epidemiol Rev* 1999; **21**: 162-79.
116. Khuroo MS. Hepatitis E: the enterically transmitted non-A, non-B hepatitis. *Indian J Gastroenterol* 1991; **10**: 96-100.
117. Aggarwal R. Hepatitis E: Epidemiology and natural History. *J Clin Exp Hepatol* 2013; **3**: 125-33.
118. Langlois EV, Haines A, Tomson G, Ghaffar A. Refugees: towards better access to health-care services. *Lancet* 2016; **387**: 319-21.

119. Toole MJ, Waldman RJ. The public health aspects of complex emergencies and refugee situations. *Annu Rev Public Health* 1997; **18**: 283-312.
120. Shultz A, Omollo JO, Burke H, *et al.* Cholera outbreak in Kenyan refugee camp: risk factors for illness and importance of sanitation. *Am J Trop Med Hyg* 2009; **80**: 640-5.
121. Kaic B, Borcic B, Ljubicic M, Brkic I, Mihaljevic I. Hepatitis A control in a refugee camp by active immunization. *Vaccine* 2001; **19**: 3615-9.
122. Nimri LF, Hijazi S. Rotavirus-associated diarrhoea in children in a refugee camp in Jordan. *J Diarrhoeal Dis Res* 1996; **14**: 1-4.
123. Mahamud AS, Ahmed JA, Nyoka R, *et al.* Epidemic cholera in Kakuma Refugee Camp, Kenya, 2009: the importance of sanitation and soap. *J Infect Dev Ctries* 2012; **6**: 234-41.
124. Uddin Khan S, Atanasova KR, Krueger WS, Ramirez A, Gray GC. Epidemiology, geographical distribution, and economic consequences of swine zoonoses: a narrative review. *Emerg Microbes Infect* 2013; **2**: e92.
125. Lewis HC, Wichmann O, Duizer E. Transmission routes and risk factors for autochthonous hepatitis E virus infection in Europe: a systematic review. *Epidemiol Infect* 2010; **138**: 145-66.
126. Zhang L, Yan B, Xu A. A hepatitis E outbreak by genotype 4 virus in Shandong province, China. *Vaccine* 2016; **34**: 3715-8.
127. Khuroo MS, Teli MR, Skidmore S, Sofi MA, Khuroo MI. Incidence and severity of viral hepatitis in pregnancy. *Am J Med* 1981; **70**: 252-5.
128. Patra S, Kumar A, Trivedi SS, Puri M, Sarin SK. Maternal and fetal outcomes in pregnant women with acute hepatitis E virus infection. *Ann Intern Med* 2007; **147**: 28-33.
129. Labrique AB, Sikder SS, Krain LJ, *et al.* Hepatitis E, a vaccine-preventable cause of maternal deaths. *Emerg Infect Dis* 2012; **18**: 1401-4.
130. Sehgal R, Patra S, David P, *et al.* Impaired monocyte-macrophage functions and defective toll-like receptor signaling in hepatitis E virus-infected pregnant women with acute liver failure. *Hepatology* 2015; **62**: 1683-96.
131. Pal R, Aggarwal R, Naik SR, Das V, Das S, Naik S. Immunological alterations in pregnant women with acute hepatitis E. *J Gastroenterol Hepatol* 2005; **20**: 1094-101.
132. Navaneethan U, Al Mohajer M, Shata MT. Hepatitis E and pregnancy: Understanding the pathogenesis. *Liver Int* 2008; **28**: 1190-9.
133. Rakesh P, Sherin D, Sankar H, Shaji M, Subhagan S, Salila S. Investigating a community-wide outbreak of hepatitis A in India. *J Glob Infect Dis* 2014; **6**: 59-64.
134. Saha T, Murhekar M, Hutin YJ, Ramamurthy T. An urban, water-borne outbreak of diarrhoea and shigellosis in a district town in eastern India. *Natl Med J India* 2009; **22**: 237-9.
135. Bhunia R, Ramakrishnan R, Hutin Y, Gupte MD. Cholera outbreak secondary to contaminated pipe water in an urban area, West Bengal, India, 2006. *Indian J Gastroenterol* 2009; **28**: 62-4.
136. Panda M, Hutin YJ, Ramachandran V, Murhekar M. A fatal waterborne outbreak of pesticide poisoning caused by damaged pipelines, Sindhikela, Bolangir, Orissa, India, 2008. *J Toxicol* 2009; **2009**: 692496.
137. Zhu FC, Zhang J, Zhang XF, *et al.* Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. *Lancet* 2010; **376**: 895-902.
138. Cousins S. Experts disagree over necessity of hepatitis E vaccine in Nepal. *BMJ* 2015; **350**: h3393.
139. Basnyat B, Dalton HR, Kamar N, *et al.* Nepali earthquakes and the risk of an epidemic of hepatitis E. *Lancet* 2015; **385**: 2572-3.
140. Shrestha A, Lama TK, Gupta BP, *et al.* Hepatitis E virus outbreak in postearthquake Nepal: is a vaccine really needed? *J Viral Hepat* 2016.

Chapter 3

Immunity against hepatitis E virus infection: Implications for therapy and vaccine development

Mohamad S. Hakim^{1,2}, Aqsa Ikram^{1,3}, Jianhua Zhou^{1,4}, Wenshi Wang¹, Maikel P. Peppelenbosch¹, Qiuwei Pan¹

- 1 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, the Netherlands
- 2 Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
- 3 Atta-Ur-Rahman School of Applied Biosciences, National University of Science and Technology, Islamabad, Pakistan
- 4 State Key Laboratory of Veterinary Etiological Biology, National Foot-and-Mouth Disease Reference Laboratory, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu, P. R. China

Reviews in Medical Virology 2018; 28(2):e1964.

Summary

Hepatitis E virus (HEV) is the leading cause of acute viral hepatitis worldwide and an emerging cause of chronic infection in immunocompromised patients. As with viral infections in general, immune responses are critical to determine the outcome of HEV infection. Accumulating studies in cell culture, animal models and patients have improved our understanding of HEV immunopathogenesis and informed the development of new antiviral therapies and effective vaccines. In this review, we discuss the recent progress on innate and adaptive immunity in HEV infection, and the implications for the development of effective vaccines and immune-based therapies.

Keywords: hepatitis E virus; innate immunity; adaptive immunity; vaccine; therapy

Abbreviations:

DC, dendritic cells; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; IFNs, interferons; IRF, interferon regulatory factor; IL, interleukin; ISGs, interferon-stimulated genes; ISGF3, interferon stimulated gene factor 3; JAK-STAT, Janus kinase-signal transducer and activator of transcription; NK, natural killer cells; NKT, natural killer T cells; MDA5, melanoma differentiation associated gene 5; ORF, open reading frames; PRR, pattern-recognition receptor; PEG-IFN α , pegylated interferon α ; RIG-I, retinoic acid inducible gene-I; RIP1, receptor-interacting protein kinase 1; RBV, ribavirin; SIRP- α , signal regulator protein α ; Treg, regulatory T cells; TLR, Toll-like receptors; TNF α , tumor necrosis factor α ; TRADD, TNF receptor 1-associated death domain protein; TNFAIP3, TNF α -induced protein 3; VLP, virus-like particles

Introduction

Hepatitis E virus (HEV) infection is the predominant cause of acute hepatitis worldwide.¹ It is also the causative agent of many large hepatitis outbreaks and continuously poses a serious threat, especially in developing countries.² HEV was initially thought to only cause acute infection where most patients remain asymptomatic.¹ However, it has recently been demonstrated that HEV can cause chronic infection and rapidly lead to cirrhosis and liver failure, mainly in immunocompromised organ transplant recipients.³ Ribavirin (RBV) monotherapy may be effective as off-label treatment for chronic hepatitis E.⁴ However, treatment failure occurs in a subset of patients, partially related to the pre-existence or subsequent development of mutations in the viral genome.⁵ Several mutations in HEV genome, including G1634R and Y1320H, have been associated with RBV treatment failure.⁶ In addition, recurrences of HEV viremia were observed in a substantial proportion of patients following RBV treatment cessation.⁴ Thus, new effective therapies for HEV infection are urgently needed.

The host immune system is essential to eliminate invading viral pathogens. The recognition of viral components produces antiviral cytokines, including type I interferons (IFNs) and tumor necrosis factor α (TNF α), to provide a rapid response against the infection.⁷ These components of innate and non-specific antiviral immunity are also pivotal to promote the development of more specific adaptive immunity, including virus-specific B and T cells, to facilitate a complete eradication of the virus.⁸ Recent advances in HEV cell culture and animal models, as well as studies in patients, have improved our understanding of this complex virus-host immunopathogenesis. In this review, we summarise the current understanding of innate and adaptive immunity against HEV and its implications for the development of HEV vaccines and immune-based therapy.

Innate Immunity against HEV

IFN responses following viral infections

HEV is a small, non-enveloped and positive-strand RNA virus with one serotype but several genotypes. Its complete genome is 7.2 kb in length and contains 3 open reading frames

(ORF1, ORF2 and ORF3). Recently, a novel ORF (ORF4) is identified in HEV genotype 1 (**Figure 1**).^{1,9}

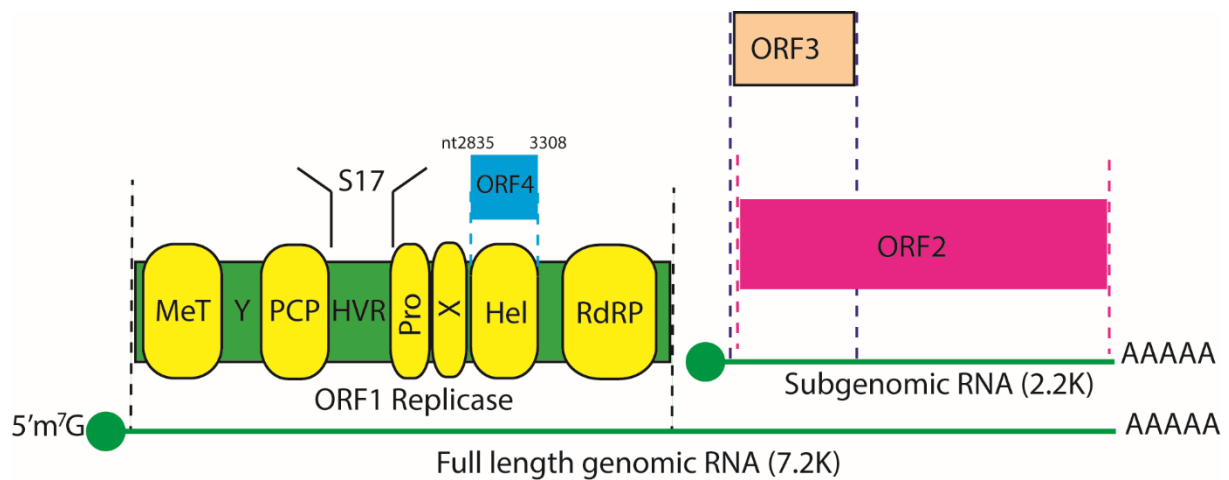


Figure 1. Molecular organization of HEV genome. The HEV genome is a single-stranded positive RNA, 7.2 kb in length. It has a 7-methylguanylate (m⁷G) at the 5' end and a poly-A tail at the 3' end. ORF1 is the largest open reading frame. It has several predicted domains, including methyltransferase (Met), Y domain (Y), papain-like cysteine protease (PCP), hypervariable region (HVR), proline-rich domain (Pro), X domain (X), helicase (Hel), and RNA-dependent RNA polymerase (RdRp). ORF2 and ORF3 are encoded by a subgenomic RNA (2.2 kb). ORF2 is the capsid protein. ORF3 partially overlaps with ORF2. An insertion of human sequences (S17) into the HVR domain increases cell culture adaptation of HEV genotype 3 Kernow-C1 p6 strain. The novel ORF4 protein (nt 2835-3308), which is overlapped with ORF1, is only identified in genotype 1.

Upon infection, HEV will likely be recognized by one or more pattern-recognition receptor (PRR) specific for RNA viruses. Foreign RNA sensors include RIG-I-like receptors (RLR) and Toll-like receptors (TLR) which sense the presence of viral RNA products in the cytoplasm and endosomal compartment, respectively. Among members of RLR are retinoic acid inducible gene-I (RIG-I, also known as DDX58) and melanoma differentiation associated gene 5 (MDA5, also known as IFIH1).⁷ Among 13 different known TLRs, TLR3, 7 and 8 are the main ones responsible for sensing virus-derived RNA.¹⁰ Viral recognition by RLR and TLR activates their downstream signaling pathways mediated by interferon regulatory factor 3 (IRF3), IRF7 and nuclear factor kappa-B (NF-κB). Ultimately, these signaling events increase production of IFNs and other pro-inflammatory cytokines.^{7,10}

Once produced, IFNs signal via autocrine and paracrine routes to combat the invading viruses. IFNs consist of three different types, type I (IFNα, IFNβ, IFNδ, and other sub-types), type II (IFNγ, the only member) and type III (IFNλ1, IFNλ2, IFNλ3 and IFNλ4).^{11,12} Binding of

IFNs to their corresponding receptors activates their common downstream signaling pathway, involving classical Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway. Following activation by type I and III IFNs, STAT1 and STAT2 proteins are phosphorylated and associate with IRF9. This complex, designated as interferon stimulated gene factor 3 (ISGF3), then translocates to the nucleus, to induce transcription of hundreds of IFN-stimulated genes (ISGs).^{11,12} ISGs are the ultimate effector antiviral molecules which cooperatively establish an antiviral state against diverse types of viruses.¹³ Given the vital role of IFNs in early defence mechanisms, many viruses, including HEV, have developed strategies to subvert IFN induction and also IFN-activated JAK-STAT signaling.

Induction and antagonism of IFNs by HEV

RIG-I and MDA5 are involved in the HEV-induced IFN response.¹⁴ HEV induces predominantly a type III IFN response, rather than a type I IFN, to control viral growth.¹⁴ Furthermore, lack of both RIG-I and TLR3 signaling facilitates HEV replication which can be reversed by reconstitution of RIG-I and TLR3.¹⁵ This suggests they have functional roles in HEV sensing and activation of the downstream anti-viral signaling pathway.¹⁵ Consistently, inhibition of IRF3 phosphorylation, the key downstream signaling pathway of TLR3, RIG-I and MDA5, notably increases HEV replication.^{14,15}

ORF3 of HEV genotype 4 induces signal regulator protein α (SIRP- α) expression which blocks phosphorylation of IRF3 and inhibits IFN β induction.¹⁶ This counteracting mechanism may explain the low induction of IFN β at the early phase of HEV infection, even though it is not effective at the later phases.¹⁷ Another mechanism involves the X domain of ORF1 which blocks poly(I•C)-induced phosphorylation of IRF3; while the PCP domain inhibits activation of RIG-I and TBK-1 via its deubiquitinase activity.¹⁸ Inhibition of IFN production may subsequently block the induction of ISG expression. However, *in vitro* cell culture studies^{14,19} as well as studies in mice with humanized liver²⁰ demonstrated ISG induction upon HEV infections. These results indicate that HEV has developed strategies to dampen IFN induction, but not a complete inhibition. Interestingly, studies in chimpanzees found that acute HEV showed a lower number and magnitude of ISG induction as compared to acute hepatitis C virus (HCV), suggesting that HCV is a more potent inducer of IFN response than HEV.²¹

Intriguingly, ORF3 protein of HEV genotype 1 and 3 has been shown to augment type I (IFN β) production. ORF3 protein increases the protein levels and activation of RIG-I.²² The exact role of this ORF3-mediated IFN β induction during various stages of HEV replication is unclear. It seems that HEV tightly controls the balance between inhibition and enhancement of IFN production at different stages of its life cycle to favour its existence in the infected host (**Figure 2**). These results also indicate that induction or inhibition of IFN production by HEV are genotype-specific.

The role of ISGs in HEV infection

In kidney transplant recipients with chronic HEV, cross-sectional microarray studies have identified the upregulation of 25 ISGs in blood samples. Surprisingly, a higher expression of these ISGs was associated with viral persistence²³, raising the intriguing question whether they serve as an indicator of active virus-host interaction or functionally combat the infection.

Many ISGs are also basally expressed at certain levels in homeostatic conditions without IFN stimulation. A prominent role of this basal IFN signaling has been described in restricting HEV replication. Gene silencing of JAK1 expression levels as well as the individual component of ISGF3 complex facilitates HEV replication.^{24,25} In these STAT1, STAT2 and IRF9 deficient cells, a notably lower ISG expression was found that may explain the increased HEV replication. Without IFN activation, the intact unphosphorylated ISGF3 complex is necessary to maintain basal ISG expression which subsequently provides a quick and timely immune response against invading viral pathogens, including HEV.²⁵

Many screening efforts have been performed to characterize individual antiviral ISGs against specific or a wide-spectrum of viruses, either via gene knockdown or ectopic over-expression approaches.¹³ Among the hundreds of ISGs, only a subset executed specific or broad antiviral effects.^{26,27} Profiling these well-known antiviral ISGs has identified RIG-I, MDA5 and IRF1 as potent anti-HEV effectors (**Figure 2**).^{28,29} Interestingly, both IRF1 and RIG-I activate a panel of ISGs without inducing IFN production.^{28,29} Thus, these ISGs likely activate a general host immune defence mechanism to indirectly combat HEV infection. ISG15 is induced upon HEV infection, but has no effect on viral replication. However, it negatively regulates the anti-HEV

effect of IFN treatment³⁰, illustrating the diversity and complexity of ISGs in regulating HEV infection.

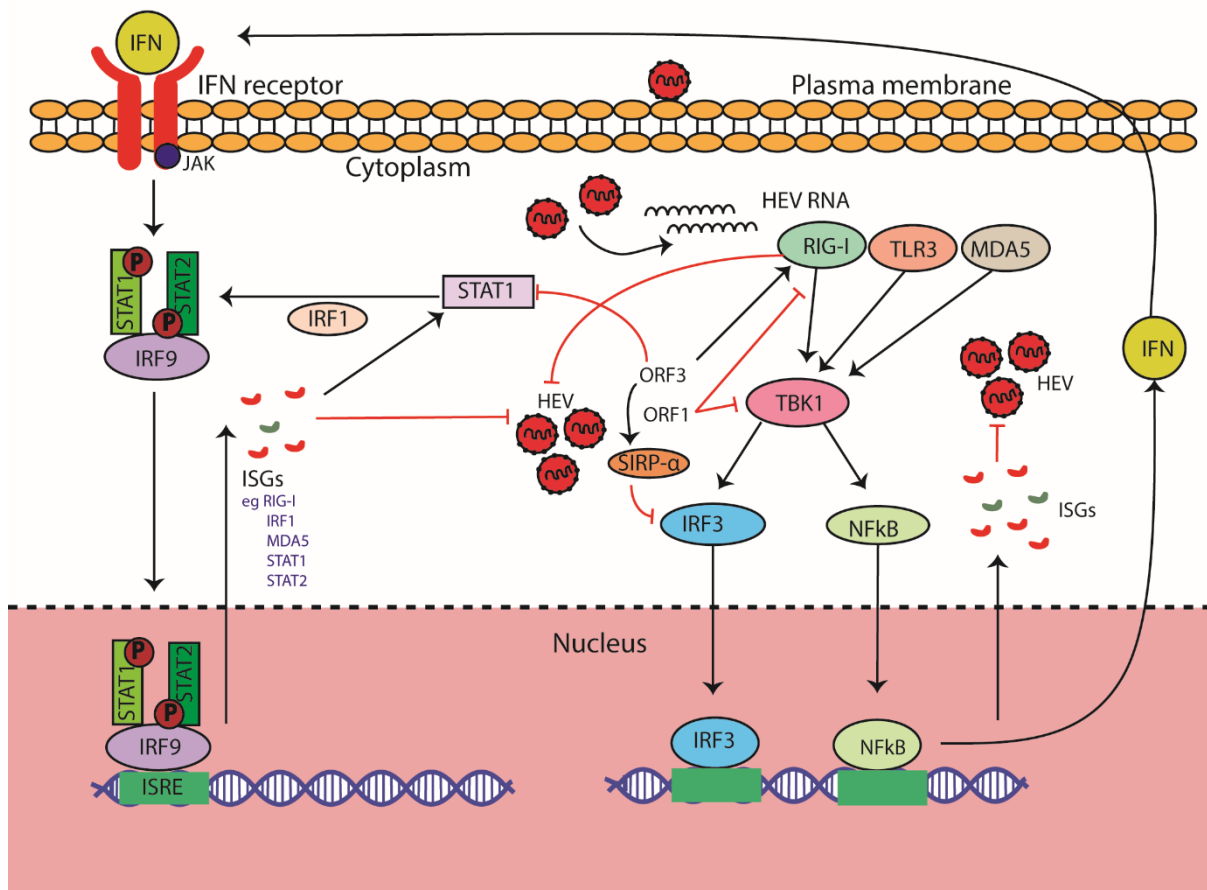


Figure 2. The interferon (IFN) pathway and HEV infection. Upon infection, HEV will likely be recognized by the host pattern-recognition receptor. There are several receptors, including RIG-I, MDA5 and TLR3, specific for RNA viruses. The recognition activates their downstream signaling pathway, involving IRF3 and NFκB and results in IFN production. The subsequent binding of IFNs to their corresponding receptors leads to STAT1 and STAT2 phosphorylation. Then, phosphorylated STAT1 and STAT2 binds IRF9, to form ISGF3 (IFN-stimulated gene factor 3) complex. This complex binds to a specific DNA promoter sequence, ISRE (IFN-stimulated response element) and induces the transcription of hundreds ISGs (IFN-stimulated genes). RIG-I, MDA5 and IRF1 have been identified as potent anti-HEV ISGs. IRF1 inhibits HEV via the activation of the JAK-STAT pathway. NF-κB, the key downstream signaling of TNFα, also has the ability to directly stimulate ISGs. On the other hand, HEV has developed strategies to circumvent this innate IFN response. ORF1 inhibits RIG-I and TBK-1 activation via its deubiquitinase activity. ORF3 blocks STAT1 phosphorylation and thereby inhibits IFN-initiated JAK-STAT signaling. ORF3 also induces SIRP-α that subsequently blocks IRF3 phosphorylation. At the same time, however, ORF3 enhances innate immune responses by a direct interaction with RIG-I.

TNF α and NF- κ B signaling pathway

Genetic polymorphisms in the promoter regions of the *TNF α* gene are associated with human susceptibility to HEV.³¹ A significantly higher level of *TNF α* was found in HEV-infected pregnant women and was associated with severe outcomes.³²

Transcriptional analysis of HEV-infected lung epithelial cell lines revealed upregulation of pro-inflammatory cytokines, including interleukin 6 (IL-6), IL-8 and *TNF α* .¹⁷ *TNF α* treatment moderately inhibits HEV replication, and the combination of *TNF α* and IFN α produces synergistic anti-HEV effects.³³ Interestingly, *TNF α* can independently induce ISG expression via NF- κ B complex, the key downstream component, explaining the possible mechanism of enhancing the antiviral effect of IFN.³³ Consistently, patients treated with anti-*TNF α* triggered exacerbation of HEV³⁴, indicating its important role in controlling HEV.

On the other hand, HEV has a capacity to subvert NF- κ B signaling. The P2 domain of ORF3 inhibits TLR3- and TLR4-induced NF- κ B signaling. Consequently, the production of pro-inflammatory cytokines, including *TNF α* , IL-8 and IL-1 β , is significantly reduced.^{35,36} Mechanistic studies revealed that these events are mediated via inhibition of TNF receptor 1-associated death domain protein (TRADD) and reduction of receptor-interacting protein kinase 1 (RIP1) ubiquitination.³⁵ Intriguingly, the P2 domain has also been reported to block *TNF α* -induced NF- κ B signaling via stimulation of *TNF α* -induced protein 3 (TNFAIP3, also known as A20), a negative regulator of NF- κ B signaling.³⁷ In addition, ORF2 protein impedes NF- κ B signaling by preventing I κ B α degradation.^{38,39} Thus, HEV has also evolved effective strategies to block TLR- and *TNF α* -induced NF- κ B signaling pathway.

The role of innate immune cells

Although cellular innate immunity has been extensively investigated in HEV infection, the role of different types of innate immune cells has been rarely studied in the context of HEV infection. The frequencies of natural killer (NK) and natural killer T (NKT) cells in the peripheral blood of acute HEV patients are lower than in healthy controls.⁴⁰ However, a considerably higher proportion of those cells express activation markers in acute HEV patients. During the convalescent phases, these changes become normalized.⁴⁰ It is not clear whether the reduced frequency of NK and NKT cells in the blood is the consequence of their

migration into the inflamed liver, and their function in this context remains unknown. Thus, future research is required to delineate the role of these innate immune cells in HEV infection, in particular on dendritic cells (DC) that are the key players of innate immunity against viral and bacterial infections.

Adaptive Immunity against HEV

Understanding of the importance of adaptive immunity in HEV elimination primarily derives from organ transplant patients receiving immunosuppressive therapy. These medications are aimed to prevent organ rejection mainly by inhibiting T cells. The majority of these patients fails to spontaneously clear HEV infection and develop chronic infection.^{3,41} The use of tacrolimus has been associated with chronic progression, probably due to the potent immunosuppressive effect.^{41,42} Furthermore, viral clearance was achieved after dose reduction of immunosuppressants in a subset of patients, confirming the essential role of host immunity in HEV clearance.⁴²

Humoral immune responses

Studies in non-human primates indicated that anti-HEV specific antibodies, whether due to prior infection or after passive and active immunization, protected the animals against subsequent challenges with HEV.⁴³⁻⁴⁷ It is well-known that HEV infection can induce the development of specific antibodies early during infection that play a role in the clinical outcome of acute HEV infection in humans.⁴⁸ In a typical acute HEV infection, anti-HEV IgM reaches a maximum level at 6-8 weeks after infection and becomes undetectable after 5-6 months. A gradual increase of anti-HEV IgG is shown during the acute and convalescent phases and may persist for life to provide immunity to the host.¹

As a non-enveloped virus, the ORF2-encoded capsid is the main target of anti-HEV neutralizing antibodies and a number of immunodominant epitopes have been identified in this region.^{49,50} However, a quasi-enveloped virion is identified in HEV-infected patients. HEV virions found in bile and feces are non-enveloped, while those in the blood and culture supernatant are associated with lipids derived from the cellular membrane, thus resembling

enveloped viruses.⁵¹ These lipid-associated HEV virions are more resistant to anti-HEV antibodies⁵², suggesting HEV strategies to evade humoral immune responses.

The protective role of naturally-acquired antibody responses has been demonstrated in outbreak and non-outbreak settings.⁵³⁻⁵⁶ Similarly, passive anti-HEV IgG immunization is also protective for pregnant women in endemic settings.⁵⁷ Persistence of anti-HEV IgG has been observed at 5-14 years after the occurrence of an outbreak.^{58,59} However, a gradual substantial loss of anti-HEV IgG has been observed in other studies.^{60,61} This suggests that repeated exposure to natural infection maintains herd immunity against HEV. Of note, the progressive loss of HEV-specific antibodies may be responsible for repeated outbreaks in the same areas.⁶¹

HEV-specific T cell response

The activation of HEV-specific CD4⁺ and CD8⁺ T cell responses has been detected in patients acutely infected with HEV. The functionality of these HEV-specific T cell responses has been demonstrated *in vitro*, as characterized by proliferation and cytokine production, including TNF α and IFN γ .⁶²⁻⁷¹ Noteworthy, the frequency as well as the overall strength and breadth of these responses vary between studies, reflecting the different assays and HEV-derived proteins (peptides) used for *in vitro* stimulation.

A longitudinal analysis of acute-resolving HEV patients demonstrated a rapid decline of HEV-specific T cell responses during the first few weeks after primary infection.^{64,72} Importantly, HEV-specific T cell responses have been detected years after HEV clearance both in humans and chimpanzee, indicating the successful generation of long-lived HEV-specific memory T cells.^{63,68,72,73}

Infiltration of HEV-specific T cells into the liver could also contribute to the liver damage due to their cytotoxic (perforin and granzyme) activity. Indeed, a prominent infiltration of CD8⁺ T cells was found in the liver biopsy from HEV-induced acute liver failure.⁷⁴ Similarly, infiltration of CD8⁺ T cells has been shown in the liver biopsies of HEV patients dying of fulminant hepatic failure.⁷⁵ In these patients, gene expression profiles demonstrated a significant overexpression of genes associated with cytotoxic activity.⁷⁵ In addition, fewer IFN γ - and TNF α -producing CD4⁺ T cells were found in HEV-induced fulminant hepatic failure

compared to acute-resolving HEV patients.⁶⁵ However, it is not clear whether these weaker responses are the causes or consequences of disease progression. Collectively, these results suggest that T cell responses are associated with HEV immunopathogenesis in the infected liver.

Several studies mapped the dominant epitopes eliciting T cell responses in HEV-infected patients. Specific regions in ORF2 are commonly targeted by HEV-specific T cells.^{63,66,68,72,76} These targeted epitopes are mostly located at relatively conserved regions of the HEV genome.⁷² However, HEV-specific T cells against ORF1 have also been demonstrated.⁷¹ Interestingly, HEV genotype 3-infected patients mount detectable responses upon stimulation with genotype 1-derived peptides and *vice versa*, suggesting cross-genotype protection.^{68,72} The generation of HEV-specific B and T cell responses, along with anti-HEV treatments, likely exert a selective pressure towards HEV genome variability, leading to an increased virus quasispecies and subsequently influence HEV adaptation and pathogenesis.⁷⁷⁻⁷⁹

T cell exhaustion in chronic hepatitis E

A continuously high antigen stimulation may lead to T cell exhaustion, as characterized by impaired proliferation, cytokine production and cytotoxic activity. This phenomenon has been well described in several chronic viral infections, including hepatitis B virus (HBV), HCV and human immunodeficiency virus (HIV).⁸⁰ Several factors contribute to the dysfunctional state of T cells in chronic viral infections, such as negative regulation by regulatory T cells (Treg) and IL10 as well as upregulation of inhibitory receptors, i.e. PD-1 and CTLA-4. T cell exhaustion may limit immunopathology in the infected host, however, it favours viral growth and facilitates viral persistence.⁸¹

Dysfunctional HEV-specific T cell responses have been found in chronic HEV-infected patients and are associated with high HEV-antigen levels.^{71,73} It needs to be further investigated whether these dysfunctional HEV-specific T cell responses were specifically due to chronic HEV or due to immunosuppressive therapy in general. In contrast, a detectable proliferation and IFN γ production of HEV-specific T cell responses were observed in those who resolved the HEV infection.⁷³ Interestingly, in chronic HEV patients who achieved viral

clearance, these T cell responses were improved⁷³, even though this phenomenon was not observed in another study.⁷¹ These phenomena were probably associated with different treatments received by these patients, including dose-reduction of immunosuppressive medications and antiviral RBV therapy.

Following PBMC stimulation with HEV-derived pools, higher IL-10 production was observed in HEV-infected organ transplant recipients who progressed into chronic HEV, as compared to those who cleared the infection⁷³, suggesting a role for IL-10 in regulation of T cell function. An increased frequency of CD4⁺ CD25⁺ Foxp3⁺ and an elevated level of IL-10 were found in the peripheral blood of acute HEV patients.⁸² Future studies are encouraged to further investigate the role of negative regulation by Treg and IL-10 in acute and chronic HEV patients.

Development of HEV Vaccines

The protective role of HEV-specific antibodies provides the foundation for vaccine development. A safe and efficacious vaccine is crucial in public health community to reduce the burden of HEV diseases globally. It has been successfully demonstrated in clinical trials that anti-HEV antibodies can be induced by vaccination. In a large phase III clinical trial in China, virus-like particles (VLP) HEV239 vaccine (Hecolin®), which is derived from aa. 368-606 pORF2 of genotype 1, showed a high efficacy to prevent HEV infection.⁸³ Follow up studies have demonstrated that this high efficacy is maintained to effectively protect the population against HEV infection.⁸⁴ It is predicted that HEV239 vaccine-induced IgG is detectable to provide protection for nearly a lifetime.^{85,86} Interestingly, the study area is endemic for HEV genotype 4, suggesting that the vaccine-induced antibodies conferred cross-genotype protection, consistent with the findings from the previous animal studies.^{46,87} This vaccine has now been approved by the China Food and Drug Administration.⁸⁸ Another vaccine candidate, a genotype 1 recombinant protein (rHEV), is also efficacious against HEV in a phase II clinical trial conducted in Nepal.⁸⁹ In addition, some novel HEV vaccines, including genotype 4-derived peptide vaccines, are now in different stages of development.⁹⁰⁻⁹³

Exploiting the role of HEV-specific CD4⁺ and CD8⁺ T cells in HEV clearance should contribute to the development of either prophylactic or therapeutic HEV vaccines. Since Hecolin[®] mainly induce humoral responses, there is no evidence of the involvement of HEV-specific T cells in Hecolin[®]-induced protective immunity. Therefore, it would be valuable to carefully examine HEV-specific T cell responses in Hecolin[®]-vaccinated individuals.

In fact, only one single serotype of HEV is recognized despite the presence of different genotypes. However, since there is no definitive evidence that Hecolin[®] could confer protection against HEV genotypes 2 or 3, further studies are required to assess the vaccine efficacy in different endemic areas. In addition, it is important to study the safety and efficacy of HEV vaccine in pregnant women⁹⁴, the population in which a high case fatality rate was observed during many HEV outbreaks. The trial is currently conducted in a rural area in Bangladesh, involving more than 20,000 women of childbearing age by administration of Hecolin[®] at day 0, 1 month and 6 month (NCT02759991).

Large and prolonged HEV outbreaks are frequently seen in Asian and African countries.² It has been suggested that Hecolin[®] could be valuable in preventing severe morbidity and mortality in this epidemic setting, such as in a recent outbreak in Nepal.^{95,96} However, the effectiveness of HEV vaccine is not known to provide a quick protection for the exposed populations during HEV outbreaks. Therefore, other HEV experts argue against its usefulness for mass vaccination during HEV outbreaks.⁹⁷

Immune-based Therapies for HEV

IFN treatment

In cell culture, the response of HEV infection to IFN treatment is moderate.^{14,19,24,98,99} Only IFN α exerts a considerable anti-HEV effect^{24,100}; whereas other types, including IFN β , IFN γ , IFN λ 1, IFN λ 2, and IFN λ 3, do not show clear antiviral activity against HEV.²⁴ HEV blocks IFN α -induced ISG expression via inhibition of STAT1 phosphorylation mediated by ORF3 protein.⁹⁸ Interestingly, recent studies in humanized mice models have documented a rapid clearance of HEV genotype 1 and 3 upon pegylated IFN α (PEG-IFN α) treatment.¹⁰¹

In the clinic, several retrospective case series and case reports have documented an efficacy of PEG-IFN α to treat chronic HEV patients.^{4,24,102} However, it is associated with severe adverse events, including graft rejection and thrombocytopenia. Therefore, PEG-IFN α is not recommended as the first-line therapy in chronic HEV patients.^{4,103}

Novel immune-based therapeutic strategies

Dissecting immune responses responsible for HEV clearance provides proof-of-concept for designing novel anti-HEV therapy, which ideally should circumvent the limitations of IFN α treatment.⁴ The identification of specific anti-HEV ISGs, i.e. RIG-I, potentially guides the development of new effective antiviral therapies against HEV with limited side effects.²⁸ A number of RIG-I agonists are currently in various phases of clinical trials to treat viral infections¹⁰⁴, deserving its evaluation as novel anti-HEV therapies.

Importantly, manipulation of T cell immunity is a potential strategy for immune-based interventions in chronic viral infections.⁸¹ It has been shown that restoration of HEV-specific T cells is achieved by blocking inhibitory pathways through PD-1 and CTLA-4.⁷³ However, the responses to individual inhibitory receptor blockade are patient-specific and therefore challenges the development of personalized immunotherapy by manipulating inhibitory receptor pathway.⁷³

Another way to induce a powerful immune response is via T cell-based vaccines. Several strategies have been developed to induce specific T cell responses, including peptide-based CD8⁺ T cell vaccines or CD4⁺ helper T cell-targeted vaccines.^{105,106} Such approaches have been employed for treating cancer and chronic HBV infection.¹⁰⁵⁻¹⁰⁷ Identification and optimization of the target epitopes are essential in designing peptide-based T cell vaccines against HEV.^{68,71,72} These vaccine strategies may be useful for chronic HEV patients. However, the major drawback of their application in HEV is currently limited understanding of T cell responses against HEV as well as a limited interest in developing novel anti-HEV therapy by pharmaceutical industries.

Conclusions and Future Perspective

Significant progress has been achieved in understanding innate and adaptive immunity to HEV infection, but many gaps remain. Future studies to improve our insight of HEV immunopathogenesis in association with HEV clearance and distinct clinical outcomes are encouraged. Characterisation of cellular innate immunity, including NK cells, DC and macrophages, is highly important since these cells are the key effectors that initiate immune responses against HEV and govern the establishment of antiviral B and T cell immunity.

Previous studies on T cell immunity were predominantly performed in the peripheral blood of HEV-infected patients. However, HEV is a hepatotropic virus and its clinical progression is determined by immune-mediated liver pathology. It is highly possible that the liver microenvironment will determine the strength and quality of immune responses against HEV. Therefore, the immune responses to HEV in the liver should be comprehensively studied.

Although a commercial vaccine for HEV prevention is currently available in China, the limited availability restrains its potential role to reduce the global burden of HEV infection.¹⁰⁸ Therefore, further development and particularly implementation of HEV vaccines as well as novel antiviral strategies are essential for better control of the disease.

Acknowledgements

The authors thank for financial support provided by the Indonesia Endowment Fund for Education (LPDP) for funding PhD fellowship to Mohamad S. Hakim; the Dutch Digestive Foundation (MLDS) for a career development grant (No. CDG 1304) and the Daniel den Hoed Foundation for a Centennial Award fellowship and the Erasmus MC Mrace grant to Q. Pan.

Conflict of interest

The authors declare no conflict of interest.

ORCID

Qiuwei Pan: <http://orcid.org/0000-0001-9982-6184>

References

- 1 Hoofnagle JH, Nelson KE, Purcell RH. Hepatitis E. *N Engl J Med*. 2012; 367: 1237-1244. doi:10.1056/NEJMra1204512.
- 2 Hakim MS, Wang W, Bramer WM *et al*. The global burden of hepatitis E outbreaks: a systematic review. *Liver Int*. 2017; 37: 19-31. doi:10.1111/liv.13237.
- 3 Zhou X, de Man RA, de Knecht RJ *et al*. Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol*. 2013; 23: 295-304. doi:10.1002/rmv.1751.
- 4 Peters van Ton AM, Gevers TJ, Drenth JP. Antiviral therapy in chronic hepatitis E: a systematic review. *J Viral Hepat*. 2015; 22: 965-973. doi:10.1111/jvh.12403.
- 5 Debing Y, Gisa A, Dallmeier K *et al*. A mutation in the hepatitis E virus RNA polymerase promotes its replication and associates with ribavirin treatment failure in organ transplant recipients. *Gastroenterology*. 2014; 147: 1008-1011 e1007; quiz e1015-1006. doi:10.1053/j.gastro.2014.08.040.
- 6 van Tong H, Hoan NX, Wang B *et al*. Hepatitis E virus mutations: functional and clinical relevance. *EBioMedicine*. 2016; 11: 31-42. doi:10.1016/j.ebiom.2016.07.039.
- 7 Wu J, Chen ZJ. Innate immune sensing and signaling of cytosolic nucleic acids. *Annu Rev Immunol*. 2014; 32: 461-488. doi:10.1146/annurev-immunol-032713-120156.
- 8 Takeuchi O, Akira S. Innate immunity to virus infection. *Immunol Rev*. 2009; 227: 75-86. doi:10.1111/j.1600-065X.2008.00737.x.
- 9 Nair VP, Anang S, Subramani C *et al*. Endoplasmic reticulum stress induced synthesis of a novel viral factor mediates efficient replication of genotype-1 hepatitis E virus. *PLoS Pathog*. 2016; 12: e1005521. doi:10.1371/journal.ppat.1005521.
- 10 Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 2011; 34: 637-650. doi:10.1016/j.immuni.2011.05.006.
- 11 Syedbash M, Egli A. Interferon lambda: modulating immunity in infectious diseases. *Front Immunol*. 2017; 8: 119. doi:10.3389/fimmu.2017.00119.
- 12 Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol*. 2005; 5: 375-386. doi:10.1038/nri1604.
- 13 Schneider WM, Chevillotte MD, Rice CM. Interferon-stimulated genes: a complex web of host defenses. *Annu Rev Immunol*. 2014; 32: 513-545. doi:10.1146/annurev-immunol-032713-120231.
- 14 Yin X, Li X, Ambardekar C *et al*. Hepatitis E virus persists in the presence of a type III interferon response. *PLoS Pathog*. 2017; 13: e1006417. doi:10.1371/journal.ppat.1006417.
- 15 Devhare PB, Desai S, Lole KS. Innate immune responses in human hepatocyte-derived cell lines alter genotype 1 hepatitis E virus replication efficiencies. *Sci Rep*. 2016; 6: 26827. doi:10.1038/srep26827.
- 16 Huang F, Yang C, Yu W *et al*. Hepatitis E virus infection activates signal regulator protein alpha to down-regulate type I interferon. *Immunol Res*. 2016; 64: 115-122. doi:10.1007/s12026-015-8729-y.
- 17 Devhare PB, Chatterjee SN, Arankalle VA, Lole KS. Analysis of antiviral response in human epithelial cells infected with hepatitis E virus. *PLoS One*. 2013; 8: e63793. doi:10.1371/journal.pone.0063793.
- 18 Nan Y, Yu Y, Ma Z *et al*. Hepatitis E virus inhibits type I interferon induction by ORF1 products. *J Virol*. 2014; 88: 11924-11932. doi:10.1128/JVI.01935-14.
- 19 Zhang F, Qi Y, Harrison TJ *et al*. Hepatitis E genotype 4 virus from feces of monkeys infected experimentally can be cultured in PLC/PRF/5 cells and upregulate host interferon-inducible genes. *J Med Virol*. 2014; 86: 1736-1744. doi:10.1002/jmv.24014.
- 20 Sayed IM, Verhoye L, Cocquerel L *et al*. Study of hepatitis E virus infection of genotype 1 and 3 in mice with humanised liver. *Gut*. 2017; 66: 920-929. doi:10.1136/gutjnl-2015-311109.

- 21 Yu C, Boon D, McDonald SL *et al.* Pathogenesis of hepatitis E virus and hepatitis C virus in chimpanzees: similarities and differences. *J Virol.* 2010; 84: 11264-11278. doi:10.1128/JVI.01205-10.
- 22 Nan Y, Ma Z, Wang R *et al.* Enhancement of interferon induction by ORF3 product of hepatitis E virus. *J Virol.* 2014; 88: 8696-8705. doi:10.1128/JVI.01228-14.
- 23 Moal V, Textoris J, Ben Amara A *et al.* Chronic hepatitis E virus infection is specifically associated with an interferon-related transcriptional program. *J Infect Dis.* 2013; 207: 125-132. doi:10.1093/infdis/jis632.
- 24 Zhou X, Xu L, Wang W *et al.* Disparity of basal and therapeutically activated interferon signalling in constraining hepatitis E virus infection. *J Viral Hepat.* 2016; 23: 294-304. doi:10.1111/jvh.12491.
- 25 Wang W, Yin Y, Xu L *et al.* Unphosphorylated ISGF3 drives constitutive expression of interferon-stimulated genes to protect against viral infections. *Sci Signal.* 2017; 10. doi:10.1126/scisignal.aah4248.
- 26 Schoggins JW, Wilson SJ, Panis M *et al.* A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature.* 2011; 472: 481-485. doi:10.1038/nature09907.
- 27 Schoggins JW, MacDuff DA, Imanaka N *et al.* Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature.* 2014; 505: 691-695. doi:10.1038/nature12862.
- 28 Xu L, Wang W, Li Y *et al.* RIG-I is a key antiviral interferon-stimulated gene against hepatitis E virus regardless of interferon production. *Hepatology.* 2017; 65: 1823-1839. doi:10.1002/hep.29105.
- 29 Xu L, Zhou X, Wang W *et al.* IFN regulatory factor 1 restricts hepatitis E virus replication by activating STAT1 to induce antiviral IFN-stimulated genes. *FASEB J.* 2016; 30: 3352-3367. doi:10.1096/fj.201600356R.
- 30 Sooryanarain H, Rogers AJ, Cao D *et al.* ISG15 modulates type-I interferon signaling and antiviral response during hepatitis E virus replication. *J Virol.* 2017. doi:10.1128/JVI.00621-17.
- 31 Mishra N, Arankalle VA. Association of polymorphisms in the promoter regions of TNF-alpha (-308) with susceptibility to hepatitis E virus and TNF-alpha (-1031) and IFN-gamma (+874) genes with clinical outcome of hepatitis E infection in India. *J Hepatol.* 2011; 55: 1227-1234. doi:10.1016/j.jhep.2011.03.023.
- 32 Kumar A, Devi SG, Kar P *et al.* Association of cytokines in hepatitis E with pregnancy outcome. *Cytokine.* 2014; 65: 95-104.
- 33 Wang W, Xu L, Brandsma JH *et al.* Convergent transcription of interferon-stimulated genes by TNF-alpha and IFN-alpha augments antiviral activity against HCV and HEV. *Sci Rep.* 2016; 6: 25482. doi:10.1038/srep25482.
- 34 Behrendt P, Luth S, Dammermann W *et al.* Exacerbation of hepatitis E virus infection during anti-TNFalpha treatment. *Joint Bone Spine.* 2017; 84: 217-219. doi:10.1016/j.jbspin.2016.09.017.
- 35 He M, Wang M, Huang Y *et al.* The ORF3 protein of genotype 1 hepatitis E virus suppresses TLR3-induced NF-kappaB signaling via TRADD and RIP1. *Sci Rep.* 2016; 6: 27597. doi:10.1038/srep27597.
- 36 Lei Q, Li L, Cai J *et al.* ORF3 of Hepatitis E virus inhibits the expression of proinflammatory cytokines and chemotactic factors in LPS-stimulated human PMA-THP1 cells by inhibiting NF-kappaB pathway. *Viral Immunol.* 2016; 29: 105-111. doi:10.1089/vim.2015.0107.
- 37 Xu J, Wu F, Tian D *et al.* Open reading frame 3 of genotype 1 hepatitis E virus inhibits nuclear factor-kappa B signaling induced by tumor necrosis factor-alpha in human A549 lung epithelial cells. *PLoS One.* 2014; 9: e100787. doi:10.1371/journal.pone.0100787.
- 38 Surjit M, Varshney B, Lal SK. The ORF2 glycoprotein of hepatitis E virus inhibits cellular NF-kappaB activity by blocking ubiquitination mediated proteasomal degradation of IkappaBalpha in human hepatoma cells. *BMC Biochem.* 2012; 13: 7. doi:10.1186/1471-2091-13-7.

- 39 Tian Y, Huang W, Yang J *et al.* Systematic identification of hepatitis E virus ORF2 interactome reveals that TMEM134 engages in ORF2-mediated NF-kappaB pathway. *Virus Res.* 2017; 228: 102-108. doi:10.1016/j.virusres.2016.11.027.
- 40 Srivastava R, Aggarwal R, Bhagat MR, Chowdhury A, Naik S. Alterations in natural killer cells and natural killer T cells during acute viral hepatitis E. *J Viral Hepat.* 2008; 15: 910-916. doi:10.1111/j.1365-2893.2008.01036.x.
- 41 Kamar N, Garrouste C, Haagsma EB *et al.* Factors associated with chronic hepatitis in patients with hepatitis E virus infection who have received solid organ transplants. *Gastroenterology.* 2011; 140: 1481-1489. doi:10.1053/j.gastro.2011.02.050.
- 42 Kamar N, Abravanel F, Selves J *et al.* Influence of immunosuppressive therapy on the natural history of genotype 3 hepatitis-E virus infection after organ transplantation. *Transplantation.* 2010; 89: 353-360. doi:10.1097/TP.0b013e3181c4096c.
- 43 Uchida T, Aye TT, Ma X *et al.* An epidemic outbreak of hepatitis E in Yangon of Myanmar: antibody assay and animal transmission of the virus. *Acta Pathol Jpn.* 1993; 43: 94-98.
- 44 Tsarev SA, Tsareva TS, Emerson SU *et al.* Successful passive and active immunization of cynomolgus monkeys against hepatitis E. *Proc Natl Acad Sci U S A.* 1994; 91: 10198-10202.
- 45 Arankalle VA, Favorov MO, Chadha MS, Phule DM, Banerjee K. Rhesus monkeys infected with hepatitis E virus (HEV) from the former USSR are immune to subsequent challenge with an Indian strain of HEV. *Acta Virol.* 1993; 37: 515-518.
- 46 Huang W, Zhang H, Harrison TJ *et al.* Cross-protection of hepatitis E virus genotypes 1 and 4 in rhesus macaques. *J Med Virol.* 2008; 80: 824-832. doi:10.1002/jmv.21140.
- 47 Tsarev SA, Tsareva TS, Emerson SU *et al.* ELISA for antibody to hepatitis E virus (HEV) based on complete open-reading frame-2 protein expressed in insect cells: identification of HEV infection in primates. *J Infect Dis.* 1993; 168: 369-378.
- 48 Zhu FC, Huang SJ, Wu T *et al.* Epidemiology of zoonotic hepatitis E: a community-based surveillance study in a rural population in China. *PLoS One.* 2014; 9: e87154. doi:10.1371/journal.pone.0087154.
- 49 Zhang J, Li SW, Wu T *et al.* Hepatitis E virus: neutralizing sites, diagnosis, and protective immunity. *Rev Med Virol.* 2012; 22: 339-349. doi:10.1002/rmv.1719.
- 50 Tang ZM, Tang M, Zhao M *et al.* A novel linear neutralizing epitope of hepatitis E virus. *Vaccine.* 2015; 33: 3504-3511. doi:10.1016/j.vaccine.2015.05.065.
- 51 Nagashima S, Takahashi M, Kobayashi T *et al.* Characterization of the quasi-enveloped hepatitis E virus particles released by the cellular exosomal pathway. *J Virol.* 2017; 91. doi:10.1128/JVI.00822-17.
- 52 Chapuy-Regaud S, Dubois M, Plisson-Chastang C *et al.* Characterization of the lipid envelope of exosome encapsulated HEV particles protected from the immune response. *Biochimie.* 2017; 141: 70-79. doi:10.1016/j.biochi.2017.05.003.
- 53 Bryan JP, Tsarev SA, Iqbal M *et al.* Epidemic hepatitis E in Pakistan: patterns of serologic response and evidence that antibody to hepatitis E virus protects against disease. *J Infect Dis.* 1994; 170: 517-521.
- 54 Bryan JP, Iqbal M, Tsarev S *et al.* Epidemic of hepatitis E in a military unit in Abbotabad, Pakistan. *Am J Trop Med Hyg.* 2002; 67: 662-668.
- 55 Shata MT, Daef EA, Zaki ME *et al.* Protective role of humoral immune responses during an outbreak of hepatitis E in Egypt. *Trans R Soc Trop Med Hyg.* 2012; 106: 613-618. doi:10.1016/j.trstmh.2012.07.004.
- 56 Zhang J, Zhang XF, Zhou C *et al.* Protection against hepatitis E virus infection by naturally acquired and vaccine-induced immunity. *Clin Microbiol Infect.* 2014; 20: O397-405. doi:10.1111/1469-0691.12419.
- 57 Arankalle VA, Chadha MS, Dama BM *et al.* Role of immune serum globulins in pregnant women during an epidemic of hepatitis E. *J Viral Hepat.* 1998; 5: 199-204.
- 58 Khuroo MS, Kamili S, Dar MY, Moecklii R, Jameel S. Hepatitis E and long-term antibody status. *Lancet.* 1993; 341: 1355.

- 59 Chadha MS, Walimbe AM, Arankalle VA. Retrospective serological analysis of hepatitis E patients: a long-term follow-up study. *J Viral Hepat.* 1999; 6: 457-461.
- 60 Corwin A, Jarot K, Lubis I *et al.* Two years' investigation of epidemic hepatitis E virus transmission in West Kalimantan (Borneo), Indonesia. *Trans R Soc Trop Med Hyg.* 1995; 89: 262-265.
- 61 Khuroo MS, Khuroo MS. Seroepidemiology of a second epidemic of hepatitis E in a population that had recorded first epidemic 30 years before and has been under surveillance since then. *Hepatol Int.* 2010; 4: 494-499. doi:10.1007/s12072-009-9159-5.
- 62 Naik S, Aggarwal R, Naik SR *et al.* Evidence for activation of cellular immune responses in patients with acute hepatitis E. *Indian J Gastroenterol.* 2002; 21: 149-152.
- 63 Shata MT, Barrett A, Shire NJ *et al.* Characterization of hepatitis E-specific cell-mediated immune response using IFN-gamma ELISPOT assay. *J Immunol Methods.* 2007; 328: 152-161. doi:10.1016/j.jim.2007.08.014.
- 64 Wu T, Zhang J, Su ZJ *et al.* Specific cellular immune response in hepatitis E patients. *Intervirology.* 2008; 51: 322-327. doi:10.1159/000172627.
- 65 Srivastava R, Aggarwal R, Sachdeva S *et al.* Adaptive immune responses during acute uncomplicated and fulminant hepatitis E. *J Gastroenterol Hepatol.* 2011; 26: 306-311. doi:10.1111/j.1440-1746.2010.06356.x.
- 66 Husain MM, Aggarwal R, Kumar D, Jameel S, Naik S. Effector T cells immune reactivity among patients with acute hepatitis E. *J Viral Hepat.* 2011; 18: e603-608. doi:10.1111/j.1365-2893.2011.01489.x.
- 67 Tripathy AS, Das R, Rathod SB, Arankalle VA. Cytokine profiles, CTL response and T cell frequencies in the peripheral blood of acute patients and individuals recovered from hepatitis E infection. *PLoS One.* 2012; 7: e31822. doi:10.1371/journal.pone.0031822.
- 68 Gisa A, Suneetha PV, Behrendt P *et al.* Cross-genotype-specific T-cell responses in acute hepatitis E virus (HEV) infection. *J Viral Hepat.* 2016; 23: 305-315. doi:10.1111/jvh.12495.
- 69 Srivastava R, Aggarwal R, Jameel S *et al.* Cellular immune responses in acute hepatitis E virus infection to the viral open reading frame 2 protein. *Viral Immunol.* 2007; 20: 56-65. doi:10.1089/vim.2006.0053.
- 70 TrehanPati N, Sukriti S, Geffers R *et al.* Gene expression profiles of T cells from hepatitis E virus infected patients in acute and resolving phase. *J Clin Immunol.* 2011; 31: 498-508. doi:10.1007/s10875-010-9506-2.
- 71 Al-Ayoubi J, Behrendt P, Bremer B *et al.* Hepatitis E virus ORF 1 induces proliferative and functional T-cell responses in patients with ongoing and resolved hepatitis E. *Liver Int.* 2017. doi:10.1111/liv.13521.
- 72 Brown A, Halliday JS, Swadling L *et al.* Characterization of the specificity, functionality, and durability of host T-cell responses against the full-length hepatitis E virus. *Hepatology.* 2016; 64: 1934-1950. doi:10.1002/hep.28819.
- 73 Suneetha PV, Pischke S, Schlaphoff V *et al.* Hepatitis E virus (HEV)-specific T-cell responses are associated with control of HEV infection. *Hepatology.* 2012; 55: 695-708. doi:10.1002/hep.24738.
- 74 Prabhu SB, Gupta P, Durgapal H *et al.* Study of cellular immune response against hepatitis E virus (HEV). *J Viral Hepat.* 2011; 18: 587-594. doi:10.1111/j.1365-2893.2010.01338.x.
- 75 Naik A, Goel A, Agrawal V *et al.* Changes in gene expression in liver tissue from patients with fulminant hepatitis E. *World J Gastroenterol.* 2015; 21: 8032-8042. doi:10.3748/wjg.v21.i26.8032.
- 76 Aggarwal R, Shukla R, Jameel S *et al.* T-cell epitope mapping of ORF2 and ORF3 proteins of human hepatitis E virus. *J Viral Hepat.* 2007; 14: 283-292. doi:10.1111/j.1365-2893.2006.00796.x.
- 77 Grandadam M, Tebbal S, Caron M *et al.* Evidence for hepatitis E virus quasispecies. *J Gen Virol.* 2004; 85: 3189-3194. doi:10.1099/vir.0.80248-0.

- 78 Ikram A, Hakim MS, Zhou JH *et al.* Genotype-specific acquisition, evolution and adaptation of characteristic mutations in hepatitis E virus. *Virulence*. 2017; 1-12. doi:10.1080/21505594.2017.1358349.
- 79 Lhomme S, Abravanel F, Dubois M *et al.* Hepatitis E virus quasispecies and the outcome of acute hepatitis E in solid-organ transplant patients. *J Virol*. 2012; 86: 10006-10014. doi:10.1128/JVI.01003-12.
- 80 Shin EC, Sung PS, Park SH. Immune responses and immunopathology in acute and chronic viral hepatitis. *Nat Rev Immunol*. 2016; 16: 509-523. doi:10.1038/nri.2016.69.
- 81 Hakim MS, Spaan M, Janssen HL, Boonstra A. Inhibitory receptor molecules in chronic hepatitis B and C infections: novel targets for immunotherapy? *Rev Med Virol*. 2014; 24: 125-138.
- 82 Tripathy AS, Das R, Rathod SB, Gurav YK, Arankalle VA. Peripheral T regulatory cells and cytokines in hepatitis E infection. *Eur J Clin Microbiol Infect Dis*. 2012; 31: 179-184. doi:10.1007/s10096-011-1291-1.
- 83 Zhu FC, Zhang J, Zhang XF *et al.* Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. *Lancet*. 2010; 376: 895-902. doi:10.1016/S0140-6736(10)61030-6.
- 84 Zhang J, Shih JW, Xia NS. Long-term efficacy of a hepatitis E vaccine. *N Engl J Med*. 2015; 372: 2265-2266. doi:10.1056/NEJMc1504302.
- 85 Chen S, Zhou Z, Wei FX *et al.* Modeling the long-term antibody response of a hepatitis E vaccine. *Vaccine*. 2015; 33: 4124-4129. doi:10.1016/j.vaccine.2015.06.050.
- 86 Su YY, Huang SJ, Guo M *et al.* Persistence of antibodies acquired by natural hepatitis E virus infection and effects of vaccination. *Clin Microbiol Infect*. 2017; 23: 336 e331-336 e334. doi:10.1016/j.cmi.2016.10.029.
- 87 Li SW, Zhang J, Li YM *et al.* A bacterially expressed particulate hepatitis E vaccine: antigenicity, immunogenicity and protectivity on primates. *Vaccine*. 2005; 23: 2893-2901. doi:10.1016/j.vaccine.2004.11.064.
- 88 Park SB. Hepatitis E vaccine debuts. *Nature*. 2012; 491: 21-22. doi:10.1038/491021a.
- 89 Shrestha MP, Scott RM, Joshi DM *et al.* Safety and efficacy of a recombinant hepatitis E vaccine. *N Engl J Med*. 2007; 356: 895-903. doi:10.1056/NEJMoa061847.
- 90 Cao YF, Tao H, Hu YM *et al.* A phase 1 randomized open-label clinical study to evaluate the safety and tolerability of a novel recombinant hepatitis E vaccine. *Vaccine*. 2017. doi:10.1016/j.vaccine.2017.05.072.
- 91 Kulkarni SP, Thanapati S, Arankalle VA, Tripathy AS. Specific memory B cell response and participation of CD4+ central and effector memory T cells in mice immunized with liposome encapsulated recombinant NE protein based Hepatitis E vaccine candidate. *Vaccine*. 2016; 34: 5895-5902. doi:10.1016/j.vaccine.2016.10.046.
- 92 Wen J, Behloul N, Dai X *et al.* Immunogenicity difference between two hepatitis E vaccines derived from genotype 1 and 4. *Antiviral Res*. 2016; 128: 36-42. doi:10.1016/j.antiviral.2016.02.002.
- 93 Xia M, Wei C, Wang L *et al.* Development and evaluation of two subunit vaccine candidates containing antigens of hepatitis E virus, rotavirus, and astrovirus. *Sci Rep*. 2016; 6: 25735. doi:10.1038/srep25735.
- 94 Wu T, Zhu FC, Huang SJ *et al.* Safety of the hepatitis E vaccine for pregnant women: a preliminary analysis. *Hepatology*. 2012; 55: 2038. doi:10.1002/hep.25522.
- 95 Basnyat B, Dalton HR, Kamar N *et al.* Nepali earthquakes and the risk of an epidemic of hepatitis E. *Lancet*. 2015; 385: 2572-2573. doi:10.1016/S0140-6736(15)61110-2.
- 96 Nelson KE, Shih JW, Zhang J *et al.* Hepatitis E vaccine to prevent morbidity and mortality during epidemics. *Open Forum Infect Dis*. 2014; 1: ofu098. doi:10.1093/ofid/ofu098.
- 97 Shrestha A, Lama TK, Gupta BP *et al.* Hepatitis E virus outbreak in postearthquake Nepal: is a vaccine really needed? *J Viral Hepat*. 2016; 23: 492. doi:10.1111/jvh.12505.
- 98 Dong C, Zafrullah M, Mixson-Hayden T *et al.* Suppression of interferon-alpha signaling by hepatitis E virus. *Hepatology*. 2012; 55: 1324-1332. doi:10.1002/hep.25530.

- 99 Todt D, Francois C, Anggakusuma *et al.* Antiviral activities of different interferon types and subtypes against hepatitis E virus replication. *Antimicrob Agents Chemother.* 2016; 60: 2132-2139. doi:10.1128/AAC.02427-15.
- 100 Debing Y, Emerson SU, Wang Y *et al.* Ribavirin inhibits in vitro hepatitis E virus replication through depletion of cellular GTP pools and is moderately synergistic with alpha interferon. *Antimicrob Agents Chemother.* 2014; 58: 267-273. doi:10.1128/AAC.01795-13.
- 101 van de Garde MDB, Pas SD, van Oord GW *et al.* Interferon-alpha treatment rapidly clears Hepatitis E virus infection in humanized mice. *Sci Rep.* 2017; 7: 8267. doi:10.1038/s41598-017-07434-y.
- 102 Dalton HR, Keane FE, Bendall R, Mathew J, Ijaz S. Treatment of chronic hepatitis E in a patient with HIV infection. *Ann Intern Med.* 2011; 155: 479-480. doi:10.7326/0003-4819-155-7-201110040-00017.
- 103 Mazzola A, Tran Minh M, Charlotte F *et al.* Chronic hepatitis E viral infection after liver transplantation: a regression of fibrosis after antiviral therapy. *Transplantation.* 2017; 101: 2083-2087. doi:10.1097/TP.0000000000001766.
- 104 Junt T, Barchet W. Translating nucleic acid-sensing pathways into therapies. *Nat Rev Immunol.* 2015; 15: 529-544. doi:10.1038/nri3875.
- 105 Kumai T, Fan A, Harabuchi Y, Celis E. Cancer immunotherapy: moving forward with peptide T cell vaccines. *Curr Opin Immunol.* 2017; 47: 57-63. doi:10.1016/j.coi.2017.07.003.
- 106 Melssen M, Slingluff CL, Jr. Vaccines targeting helper T cells for cancer immunotherapy. *Curr Opin Immunol.* 2017; 47: 85-92. doi:10.1016/j.coi.2017.07.004.
- 107 Li J, Bao M, Ge J *et al.* Research progress of therapeutic vaccines for treating chronic hepatitis B. *Hum Vaccin Immunother.* 2017; 13: 986-997. doi:10.1080/21645515.2016.1276125.
- 108 Wu X, Chen P, Lin H, Hao X, Liang Z. Hepatitis E virus: Current epidemiology and vaccine. *Hum Vaccin Immunother.* 2016; 12: 2603-2610. doi:10.1080/21645515.2016.1184806.

Chapter 4

Distinct Antiviral Potency of Sofosbuvir against Hepatitis C and E Viruses

Wenshi Wang^{1#}, **Mohamad S. Hakim**^{1,2#}, Vidya P. Nair³, Petra E. de Ruiter⁴, Fen Huang⁵, Dave Sprengers¹, Luc J. W. Van Der Laan⁴, Maikel P. Peppelenbosch¹, Milan Surjit³, Qiuwei Pan¹

- 1 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, the Netherlands
 - 2 Department of Microbiology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia
 - 3 Virology Laboratory, Vaccine and Infectious Disease Research Centre, Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad, Haryana, India
 - 4 Department of Surgery, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, the Netherlands
 - 5 Medical Faculty, Kunming University of Science and Technology, Kunming, PR China
- # Both authors contributed equally

Gastroenterology 2016; 151(6): 1251-1253.

Dear Editor,

Sofosbuvir (SOF), the direct-acting anti-hepatitis C virus (HCV) drug (targeting HCV RdRp; RNA-dependent RNA polymerase), has been recently reported to be a potential anti-HEV drug candidate.¹ However, some debates emerged whether SOF is a promising drug candidate for treating hepatitis E.² Given the important potential clinical implications, this study has comparatively assessed the antiviral efficacy of SOF in both HCV and HEV models. We believe that the anti-HEV and anti-HCV potency of SOF should be comparatively assessed, before proposing its clinical application for treating HEV-infected patients.

In our study, the potential anti-HEV effect of SOF was investigated in HEV replication models with concentrations ranging from 0.01 μ M to 10 μ M (Supplementary Figure 1A), which is comparable to the previous study.¹ In this model, human hepatoma Huh7 cells were transfected with a subgenomic construct of HEV coding sequence derived from genotype 1 (Sar55/S17/luc) and genotype 3 (Kernow-C1, p6-luc), in which the 5' portion of open reading frame 2 (ORF2) coding sequence was replaced with a gene encoding a secreted form of Gaussia luciferase. To normalize for non-specific effects of SOF on luciferase signals, Huh7 cells stably expressing a non-secreted firefly luciferase under control of the human phosphoglycerate kinase (PGK) promotor (PGK-Luc) was used. In addition, Huh7 cells harboring a subgenomic HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET; Huh7-ET) was used as control of anti-HCV activity.³ We demonstrated that SOF significantly reduced and even eliminated HCV-driven luciferase activity at 24 and 48 hours of treatments, but did not affect the PGK-driven luciferase activity. However, an antiviral effect of SOF was not observed in both HEV genotype 1- and genotype 3-based subgenomic replicon either at 24 and 48 hours of treatment (Figure 1A).

We further evaluated the anti-HEV effect of SOF in the full-length (Kernow-C1, p6) infectious models of HEV genotype 3. The infectious HCV model, containing full-length JFH1-derived genome, was used as the control of antiviral activity.⁴ In this model, only a modest effect was observed after the treatment of SOF at the highest concentration (10 μ M) for 48 hours (Figure 1B). In contrast, SOF strongly inhibited HCV infection. At concentration of 1 μ M, SOF completely eliminated HCV RNA (Figure 1B). These results showed the highly specific effect of SOF against HCV replication.

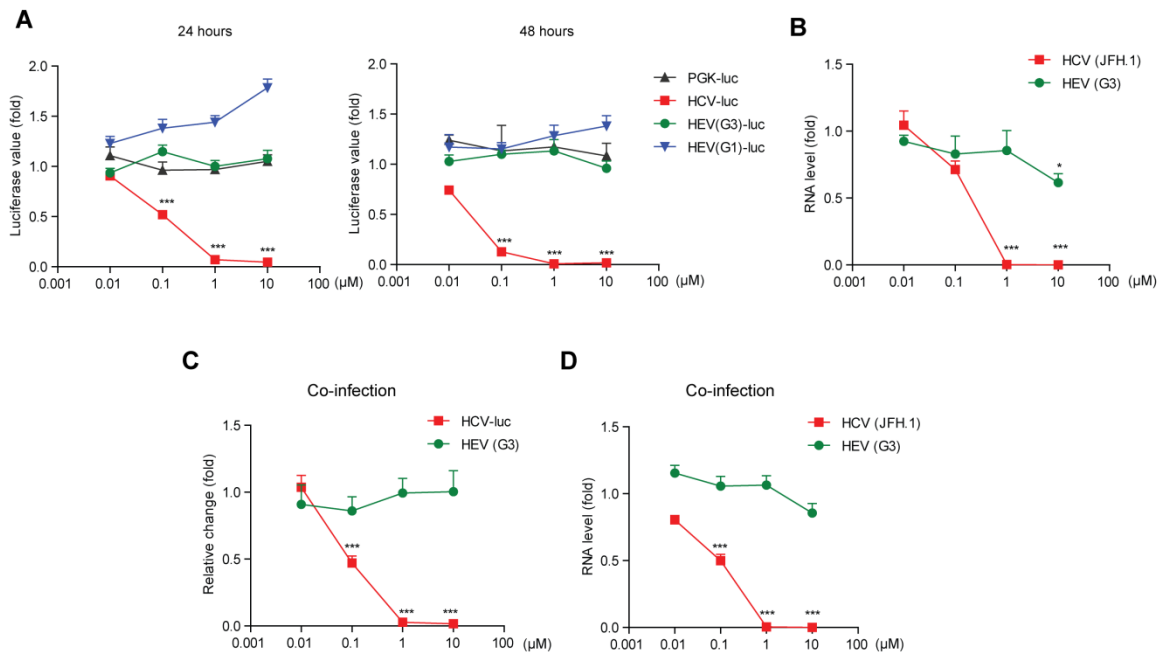


Figure 1. The antiviral effect of SOF was investigated on various HEV and HCV models. (A) In the Huh7 cell-based subgenomic HCV replicon, treatment with SOF dose-dependently decreased and even eliminated HCV replication-related luciferase activity. While SOF exerted no effects on HEV replication in both HEV genotype 1- and 3-based subgenomic replicon either at 24 and 48 hours of treatment. PGK-Luc was used to assess non-specific effects of SOF on luciferase signals ($n = 3$ independent experiments with 2–3 replicates each). **(B)** SOF strongly inhibited HCV in Huh7 based full-length JFH1-infectious model, while a modest effect on HEV was observed in the full-length p6 infectious models after the treatment of SOF at the highest concentration (10 μ M) for 48 hours ($n = 2$ independent experiments with 2 replicates each). **(C)** Huh7 cell-based subgenomic HCV replicon was infected with infectious HEV p6 particles. SOF exerted no significant anti-HEV effect, while retained its strong effect against HCV replication (measured at 48 hours) ($n = 4$). **(D)** Huh7 cells were coinfectd with both infectious HEV (p6) and HCV (JFH.1) particles. SOF led to the elimination of HCV at the concentration of 1 μ M, while exerting no significant effect on HEV (measured at 48 hours) ($n = 4$).

We then examined whether SOF could directly inhibit the activity of HEV RdRp enzyme using an *in vitro* RdRp assay.⁵ Huh7 purified RdRp-Flag was used in the presence of increasing dose of SOF. As template, an *in vitro* transcribed RNA containing 130 bases from 5'-end and 210 bases from 3'-end of HEV genotype 1 was employed. Addition of SOF at any of the concentrations tested did not inhibit HEV RdRp activity as measured by the level of double stranded RNA intermediate level (680 bases) (Supplementary Figure 1B).

Acute and chronic HEV patients may develop extra-hepatic manifestations such as neurological and kidney complications.⁶ Therefore, we established HEV genotype 3-based

infectious and replication models in human embryonic kidney epithelial cell line HEK 293T cells and human glioblastoma cell line U-87 MG cells. In line with the results observed in Huh7-based HEV replication model, we did not observe any effect of SOF on both HEK 293T and U-87 MG based HEV replication models (Supplementary Figure 1C). Furthermore, in both HEK 293T cell and U-87 MG cell based HEV infectious models, only a moderate effect was observed after the treatment of SOF at the highest concentration (10 μ M) for 48 hours (Supplementary Figure 1D). This is consistent with the result in Huh7 based HEV infectious model. The similar results we obtained from both hepatic and extra-hepatic cell lines further emphasized the highly specific effect of SOF against HCV relative to HEV.

In clinical settings, coinfection of HCV and HEV could be found.⁷ To clarify whether SOF could inhibit viral replication of both viruses in this specific setting, Huh7-ET cells infected with infectious HEV particles (Kernow-C1, p6) were used as an *in vitro* HCV and HEV coinfection model. Surprisingly, SOF lost its modest effect on HEV, while retained its strong antiviral effect against HCV replication (Figure 1C). This observation was further supported by the coinfection of both infectious HEV (Kernow-C1, p6) and HCV particles (JFH.1) in Huh7 cells. In this model, SOF led to the elimination of HCV virus at the concentration of 1 μ M/mL, while exerting no significant effect on HEV even at the concentration of 10 μ M (Figure 1D). This results underscored the highly specific anti-viral effect of SOF against HCV.

Based on evaluation in chronic HCV patients, the geometric mean steady state of SOF concentration was 828 ng•hr/mL when coadministered with ribavirin.⁸ The concentration was much lower than the concentration of SOF we used *in vitro* at which SOF showed modest effect on HEV (10 μ M of SOF is equivalent to 5294.5 ng/mL). Therefore, we suggest that SOF is likely not valuable to be used in the clinical settings for treating HEV-infected patients. Nevertheless, targeting HEV polymerase is a potential strategy to develop new antiviral drugs against HEV.

References

1. Dao Thi VL, et al. Gastroenterology 2016; 150: 82-85.
2. Wang W, et al. Gastroenterology 2016; 150: 1690.
3. Frese M, et al. Hepatology 2002; 35: 694-703.
4. **Wakita T, Pietschmann T**, et al. Nat Med 2005; 11: 791-796.
5. Nair VP, et al. PLoS Pathog 2016; 12: e1005521.
6. Kamar N, et al. Lancet 2012; 379: 2477-2488.
7. Wu KT, et al. J Med Virol 2009; 81: 1734-1742.
8. Summers BB, et al. J Pharm Pharmacol 2014; 66: 1653-1666.

Author names in bold designate shared co-first authorship.

Supplementary Materials and Methods

Anti-viral Agents

Sofosbuvir (SOF) was purchased from Selleckchem (Houston, TX). Stocks of SOF were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM.

Cell Culture

Human hepatoma cell line Huh7, human embryonic kidney epithelial cell line HEK 293T cells, and human glioblastoma cell line U-87 MG cells were cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 IU/mL streptomycin.

Stable luciferase expressing cells were generated by transducing naïve Huh7 with a lentiviral vector expressing the firefly luciferase gene under control of the human phosphoglycerate kinase (PGK) promoter (LV-PGK-Luc). LV-PGK-Luc was used as household luciferase activity for normalization and to determine the specific effects on viral replication-related luciferase activity.

Hepatitis E Virus (HEV) Cell Culture Models

A plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) and a construct containing subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-Luc) were used to generate HEV genomic RNA by using the Ambion mMESSAGE mMACHINE in vitro RNA transcription Kit (Life Technologies Corporation, Carlsbad, CA). For HEV genotype 1, we used Sar55/S17/luc subgenomic replicons coupled with a Gaussia luciferase reporter gene.

The Huh7 cells, HEK 293T cells, and U-87 MG cells were collected and centrifuged for 5 min, 1500 rpm, 4 °C. Supernatant was removed and washed with 4 mL Optimem by centrifuging for 5 min, 1500 rpm, 4 °C. The cell pellet was re-suspended in 100 µL Optimem and mixed with p6 full-length HEV RNA or p6-Luc subgenomic RNA. Electroporation was performed to generate infectious or replication models, respectively.

Hepatitis C Virus (HCV) Cell Culture Models

HCV subgenomic replicon model (Huh7-ET) was based on Huh7 cells containing a subgenomic HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET) which contains the non-structural coding sequence of HCV and the firefly luciferase gene. Huh7-ET cells were cultured in the presence of 250 µg/mL G418 (Sigma). As an infectious model, Huh7 cells harboring the full-length JFH-1 derived HCV genome was used.

Measurement of Luciferase Activity

To quantify the HEV replication models, the activity of secreted gaussia luciferase in the cell culture medium was measured using BioLux *Gaussia* Luciferase Flex Assay Kit (New England Biolabs, Ipswich, MA). Huh7-ET and PGK-Luc firefly luciferase activity was quantified by adding luciferin potassium salt (100 mM, Sigma) to the cells and then incubating for 30 minutes at 37 °C. Both gaussia and firefly luciferase activities were quantified with a LumiStar Optima luminescence counter (BMG labTech, Offenburg, Germany).

Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (Takara Bio Inc). The cDNA of HEV, HCV, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and human retinitis pigmentosa 2 (RP2) were amplified by 50 cycles and quantified with a SYBRGreen-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to the manufacturer's instructions. GAPDH and RP2 were considered as reference genes to normalize gene expression. The qPCR primer sequences listed as follows: HEV-F 5'-ATTGGCCAGAAGTTGGTTTTTCAC-3'; HEV-R 5'-CCGTGGCTATAATTGTGGTCT-3'; HCV-F 5'-GTCTAGCCATGGCGTTAGTATGAG-3'; HCV-R 5'-AGATGTTTCAGGCACTTGCGG-3'; GAPDH-F 5'-TGTCCCCACCCCAATGTATC-3'; GAPDH-R 5'-CTCCGATGCCTGCTTCACTACCTT-3'; RP2-F 5'-GTCAGAGACAGAAGAGCAGCGA-3'; RP2-R 5'-GGACACTTCCTTTGTCTGAACTAG-3'

MTT assays

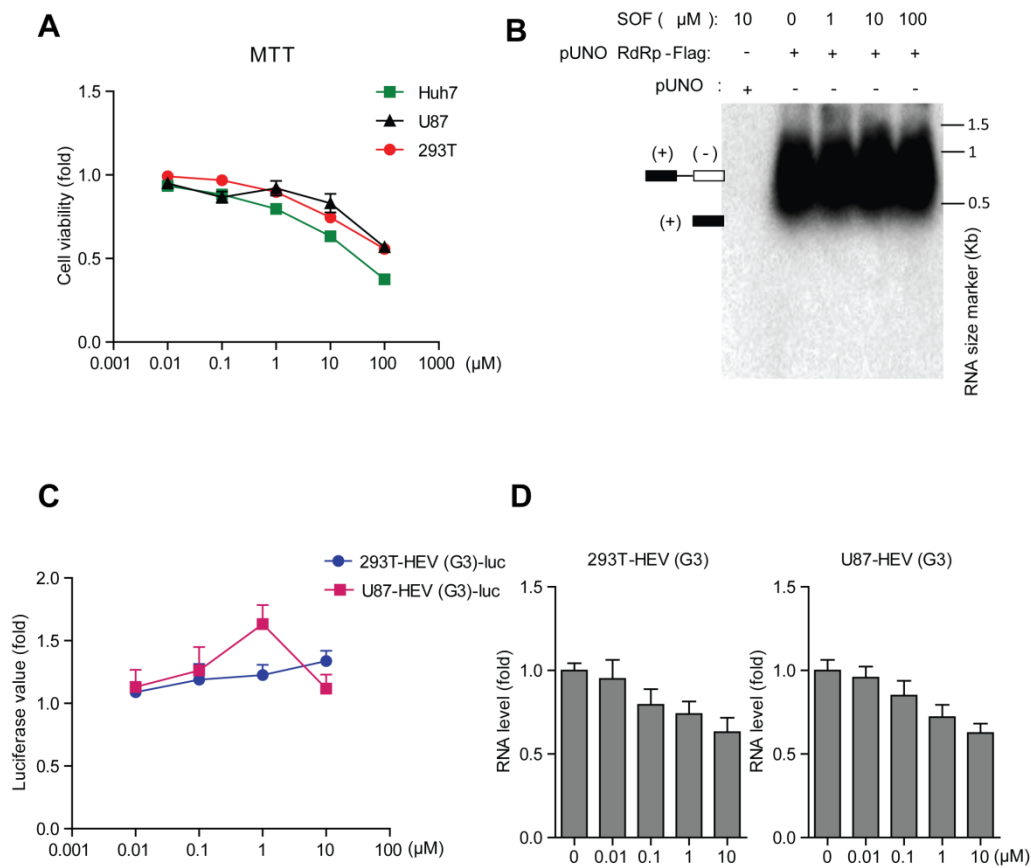
10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to cells seeded in 96-well plates. The cells were incubated at 37 °C with 5% CO₂ for 3 h. The culture medium was then removed and 100 µl of DMSO was added to each well. The absorbance of each well was read on the microplate absorbance readers (BIO-RAD) at wavelength of 490 nm. All measurements were performed in triplicates.

HEV RNA-dependent RNA-polymerase (RdRp) Assay

HEV RdRp assay was performed as described previously (see reference no. 5 in the main document). SOF was added to the reaction mixture at the indicated final concentrations (1, 10, 100 µM).

Statistical Analysis

Statistical analysis was performed using the nonpaired, nonparametric test (Mann-Whitney test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). P values <.05 were considered statistically significant.



Supplementary figure 1. The antiviral effect of SOF was evaluated on extra-hepatic cell based HEV models. (A) The effects of SOF on hepatic and extra-hepatic cell lines were determined by MTT assay. SOF showed relatively strong cytotoxicity at the concentration of 100 μM. **(B)** Effect of SOF on HEV RdRp activity *in vitro*. RdRp assay was performed using purified HEV RdRp and a 340 base HEV RNA template. Schematic illustrates the position of ds RNA (+,-) and ss RNA. **(C)** SOF exerted no antiviral effect on both HEK 293T and U-87 MG based HEV replication models (n = 3 independent experiments with 2–3 replicates each). **(D)** In both HEK 293T cell and U-87 MG cell based HEV infectious models, a moderate anti-HEV effect was observed after the treatment of SOF for 48 hours (n = 2 independent experiments with 2 replicates each).

Chapter 5

Genotype-specific acquisition, evolution and adaptation of characteristic mutations in hepatitis E virus

Aqsa Ikram^{1,2}, **Mohamad S. Hakim**^{1,3}, Jianhua Zhou^{1,4}, Wenshi Wang¹, Maikel P. Peppelenbosch¹, Qiuwei Pan¹

- 1 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, the Netherlands
- 2 Atta Ur Rahman School of Applied Biosciences, National University of Science and Technology, Islamabad, Pakistan
- 3 Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
- 4 State Key Laboratory of Veterinary Etiological Biology, National Foot-and-Mouth Disease Reference Laboratory, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu, P.R. China

Virulence 2018; 9(1): 121-132.

Abstract

Hepatitis E virus (HEV) infection is a major cause of acute hepatitis but also provokes chronic infection in immunocompromised patients. Although the pathogenesis and treatment outcome involve complex interplay between the virus and host, the nature of adaptive responses of HEV to the host immune system remain obscure at best. In this study, we employed large-scale proteomic bioinformatics to profile characteristic mutations in human HEV isolates associated to ribavirin treatment failure, chronic hepatitis, hepatic failure or altered immunoreactivity. The prevalence of specific mutations was examined in a large number of protein sequences of ORF1 and ORF2 regions of the three major human-derived HEV genotypes (1, 3 and 4). By analyzing potential B, CD4⁺ and CD8⁺ T cell epitopes, we found that many of these mutations overlap with the predicted epitopes and are frequently present among the three HEV genotypes. These overlapping mutations mediate reduced antigenicity. Finally, by delineation of diversification and evolution of the underlying epitopes, we observe that most of these variants apparently evolved earlier in genotype 1 when compared to genotypes 3 and 4. These results indicate that HEV is under substantial evolutionary pressure to develop mutations enabling evasion of the host immune response and resistance to antiviral treatment. This indicates the existence of an ongoing evolutionary arms race between human immunity, antiviral medication and HEV.

Key words: B and T cells; epitope; evolution; hepatitis E virus; mutation

Introduction

Hepatitis E virus (HEV) is a non-enveloped, single-stranded positive sense RNA virus which mainly infects the liver.¹ It causes over three million acute cases and 57,000 deaths every year.² Although eight HEV genotypes are now recognized, there are 4 well-defined genotypes infecting humans, including genotype 1, 2, 3 and 4. Genotypes 1 and 2 are found only in humans and are responsible for most cases of infection in the developing countries. Genotypes 3 and 4 circulate in several animals (e.g. pigs, wild boars and deer) and are the main cause of sporadic infection in the developed countries.^{3,4} Although no FDA-approved treatment is available, pegylated interferon- α (PEG-IFN- α) or ribavirin has been used as off-label treatment for some cases of HEV infection.^{5,6}

As an RNA virus, HEV possesses a high mutation rate. It was indirectly estimated from clinical isolates that the mutation rate of HEV was ~ 1.5 base substitutions per site per year, which is quite similar to that reported for hepatitis C virus (HCV).⁷ The viral RNA-dependent RNA polymerase (RdRp), which lacks the proof-reading capacity, is an important factor contributing to the high rate of mutations in the HEV genome.⁸ Furthermore, the selection pressure imposed by the host immune responses may also contribute to the variability of HEV genome. Recently, studies have hinted at the acquisition by the HEV genome of certain mutations associated with ribavirin treatment failure, chronic hepatitis, hepatic failure and reduced immunoreactivity.⁹ The observation that different mutations change the HEV proteome with respect to not only the therapeutic response but also the immunoreactivity highlights the importance of studying HEV mutations and their effects on the host immune responses.

Only limited knowledge is available on the contribution of HEV genome variants towards susceptibility, pathogenesis and therapeutic responses. To elucidate these processes, we have used a large-scale proteomic bioinformatics to profile human HEV characteristic mutations. In the present study, we have comprehensively investigated the proteomic variation of the open reading frame 1 (ORF1) and ORF2 regions among the major HEV genotypes 1, 3 and 4, by retrieving a large dataset of HEV sequences. We first mapped the presence and abundance of the mutations related to ribavirin treatment failure, chronic hepatitis, hepatic failure or reduced immunoreactivity. Furthermore, we examined the

overlap of these mutations with predicted B cell, CD4⁺ and CD8⁺ T cell epitopes, and assessed their antigenicity. Interestingly, we have observed that these overlapped mutations evolved earlier in genotype 1 when compared with genotype 3 and 4. Thus, the acquisition and evolution of these characteristic mutations may help the virus to evade host immune response, develop resistance to antiviral treatment, and facilitate its adaptation in human population.

Results

Identification of HEV characteristic mutations

Several HEV variants were previously reported both *in vitro* and *in vivo*. Using a combination of phrases/keywords in PubMed (**Table S1**), we explored clinical and *in vitro* data reporting mutations within the ORF1 and ORF2 of HEV. In our analysis, 20 mutations (**Table S2**) were identified in these regions, 17 in ORF1 and 3 in ORF2 (**Figure 1**). Among these mutations, one was related to chronic hepatitis, nine with hepatic failure, eight with ribavirin treatment failure, and two with altered immunoreactivity (**Table S2**).

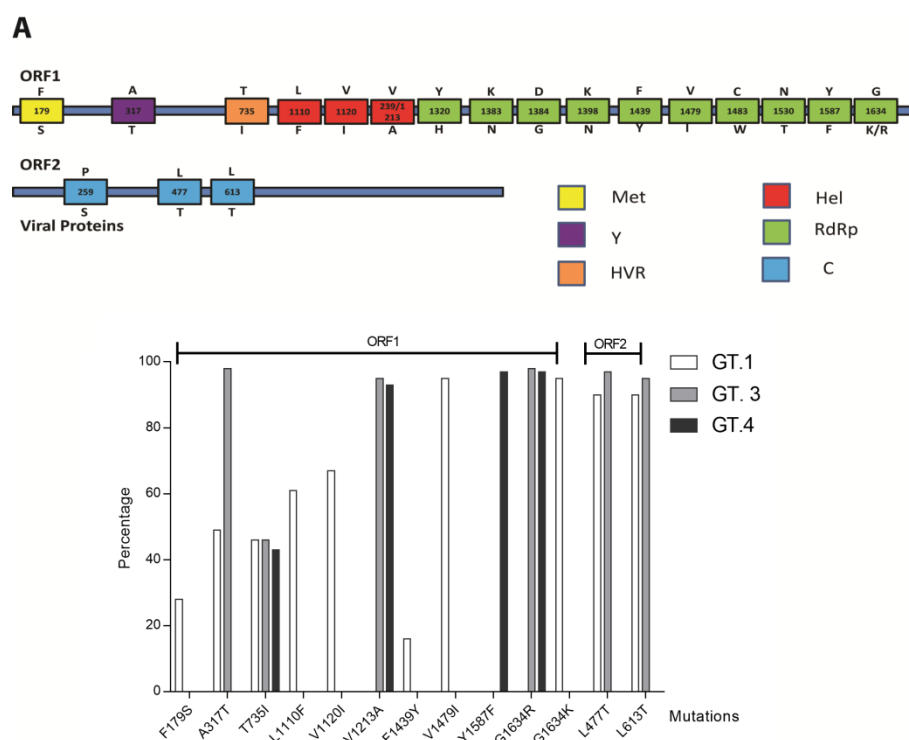


Figure 1. (A). Identified mutations of ORF1 and ORF2 regions of HEV. The number within the box represents the amino acid position; the letter(s) above the box refer to the wild type amino acid, and the letter below the box are relevant mutations reported in previous studies. (Met: methyltransferase; Y: Y-domain; HVR: hypervariable regions; Hel: RNA helicase; RdRP: RNA-dependent RNA polymerase; C: capsid protein) **(B).** The prevalence of mutations within HEV genotype 1 (n = 81), 3 (n = 182) and 4 (n = 143). Amino acid diversity was measured as the proportion of sequences that varies from the consensus sequence.

Frequency of mutations within ORF1 and ORF2

To evaluate the relevance of these reported characteristic mutations, we first evaluated their presence in the circulating strains based on the HEV sequences deposited in the GenBank. We have searched the 4 main genotypes identified from the human host. However, limited sequences are available for genotype 2. Thus, we retrieved 57 ORF1, 51 ORF2 (genotype 1), 131 ORF1, 131 ORF2 (genotype 3) and 99 ORF1, 96 ORF2 (genotype 4) of HEV sequences (retrieved in January 2017). The selected sequences represent all major genotypes (1, 3 and 4). The full-length sequences used in this study based on each ORF and genotype are provided in **Table S3**.

After removal of closely related and redundant sequences, 406 full length ORF1 and ORF2 sequences were finally selected for further analysis. **Table 1, Table S4** and **Figure 1** show the number of all possible and experimentally confirmed amino acid variants and frequency of their variation for each HEV genotype. The number of variants found in ORF1 region was higher (17 variants) than that of ORF2 region (three variants). Out of 17 variants in ORF1, eight were related to hepatic failure; one with chronic hepatitis; and eight with ribavirin treatment failure. While in ORF2, one variation was associated with hepatic failure and two with immunoreactivity. When analyzing HEV genotypes, it was revealed that genotype 1 possesses a higher number of mutations (eight) with considerable frequency when compared with genotype 3 and 4 which possesses only six and three mutations, respectively (**Table 1 and Figure 1B**). This observation further suggests the high level of polymorphism in HEV genotype 1.

	Mutations	Percentage		
		GT1 ORF1 (57)	GT3 ORF1 (131)	GT4 ORF1 (99)
ORF1	F179S	16 (28%)	0	0
	A317T	28 (49%)	128 (98%)	0
	T735I	26 (46%)	60 (46%)	43 (43%)
	L1110F	35 (61%)	0	0
	V1120I	38 (67%)	0	0
	V1213A	0	125 (95%)	93 (93%)
	Y1320H	0	0	0
	K1383N	0	0	0
	D1384G	0	0	0
	K1398N	0	0	0
	F1439Y	9 (16%)	0	0
	V1479I	54 (95%)	0	0
	C1483W	0	0	0
	N1530T	0	0	0
	Y1587F	0	0	96 (97%)
	G1634R	0	128 (98%)	96 (97%)
	G1634K	54 (95%)	0	0
		GT1 ORF2 (51)	GT3 ORF2 (131)	GT4 ORF2 (96)
ORF2	P259S	0	0	0
	L477T	46 (90%)	127 (97%)	0
	L613T	46 (90%)	125 (95%)	0

Table 1. All possible and experimentally confirmed (*in vivo* and *in vitro*) mutations and their prevalence in the major HEV genotypes (1, 3, 4). Different colors indicate the mutations related to ribavirin treatment failure (yellow), chronic hepatitis (purple), hepatic failure (red) or altered immunoreactivity (blue).

Mutations within genotype 1, 3 and 4

In genotype 1, nine out of 17 reported mutations (V1213A, Y1320H, K1383N, D1384G, K1398N, C1483W, N1530T, Y1587F, G1634R) in ORF1 and one (P259S) out of three in ORF2, were found to be conserved (i.e. not present in our analyzed sequences), while others have shown considerable variations (**Table 1**). Among these variable mutations, T735I and G1634R/K were observed to be frequent among all selected genotype (1, 3 and 4). Another mutation (A317T) was found in two genotypes (1 and 3). Four mutations (ORF1 = 2; ORF2 = 2) reached the frequency of >90% (**Table 1, Figure 1B**). Among these, two mutations were

related to ribavirin treatment failure (G1634K and V1479I) and two were associated with altered immunoreactivity [L477T (ORF2; genotype 1, 3) and L613T (ORF2; genotype 1, 3)].

In genotype 3, we found that the reported mutation sites [13 in ORF1 (F179S, L1110F, V1120I, Y1320H, K1383N, D1384G, K1398N, F1439Y, V1479I, C1483W, N1530T, Y1587F, G1634K) and one (P259S) in ORF2] were conserved (i.e. not present in our analyzed sequences) (**Table 1, Figure 1B**). Mutations of ORF1, i.e. V1213A and G1634R, were found with a considerable frequency in genotypes 3 and 4. The most important variants were A317T, V1213A and G1634R which showed a high prevalence in genotype 3. Five mutations approached a frequency of >90%, where one was related to chronic hepatitis (V1213A), one with hepatic failure (A317T), one with ribavirin treatment failure (G1634R) and two with immunoreactivity (L477T, L613T) (**Table 1, Figure 1B**).

In genotype 4, 13 sites in ORF1 (F179S, A317T, L1110F, V1120I, V1213A, Y1320H, K1383N, K1398N, D1384G, F1439Y, V1479I, C1483W, N1530T) and three in ORF2 (P259S, L477T, L613T) were found to be conserved. Mutation Y1587F was frequent in genotype 4. Two mutations reached the frequency of >90% (Y1587F, G1634R) (**Table 1, Figure 1B**). Both mutations have been reported to be related to ribavirin treatment failure (Y1587F, G1634R).

Mutations as a missense SNPs and their effects on protein structural stability

In our analysis, four in silico SNP prediction algorithms were employed to predict the selected mutations as neutral or deleterious. According to predicted results, most of the selected mutations with high prevalence in HEV infected population are deleterious (**Table S5**). Next, most of these mutations are predicted to cause destabilization of the protein as calculated using three web servers (**Table S6**), suggesting the importance of these mutations. Because the crystal structure of HEV ORF2 (2ZTN-amino acid 129-606) is available (**Figure 3A**), we have modeled the effect of two mutations that are located within this region on structural stability of the protein. We found that both P259S (**Figure 3B**) and L477T (**Figure 3C**) are predicted to cause destabilization of ORF2 protein.

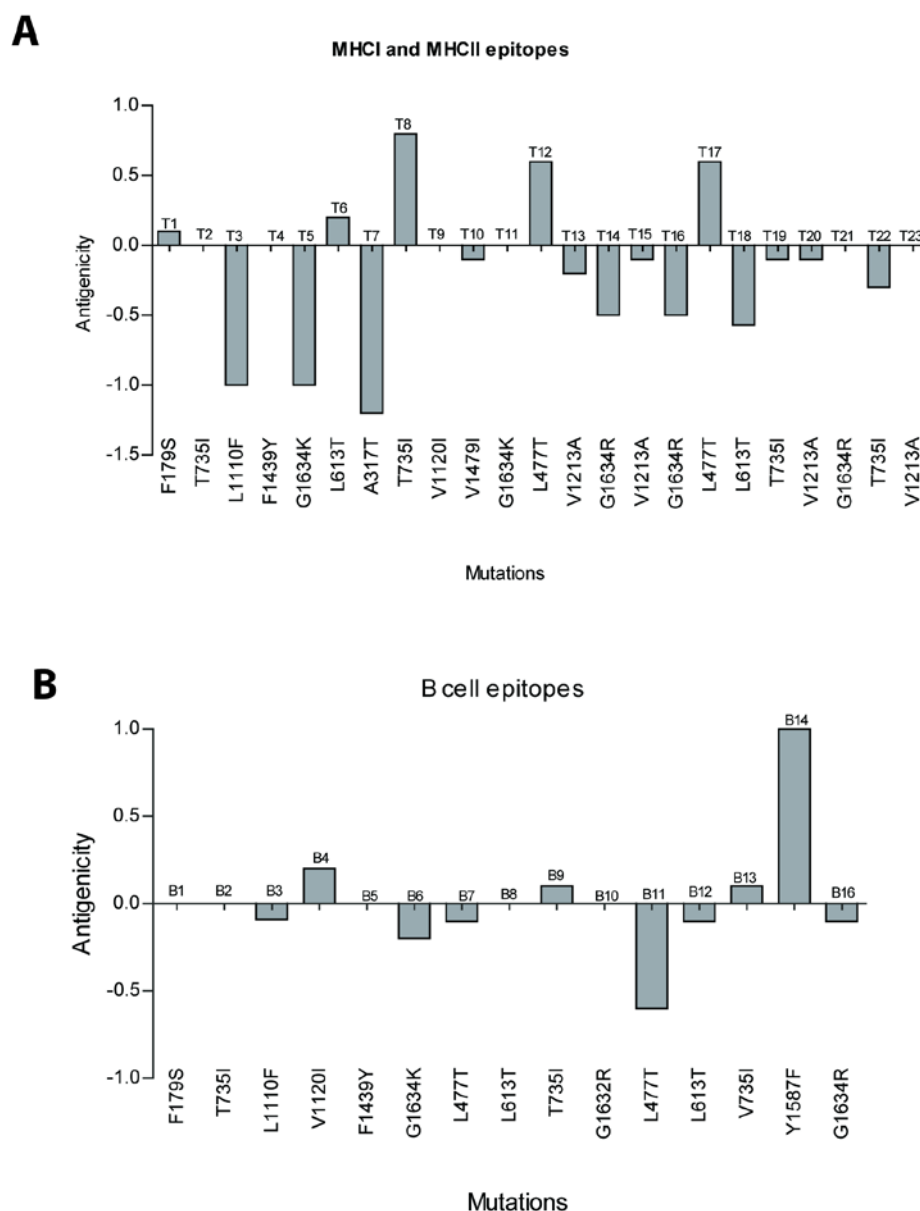


Figure 2. Antigenicity difference between wild-type and mutated T cell (MHCI and MHCII) (A) and B cell epitopes (B).

Overlap of characteristic mutations within the predicted B cell, CD4+ and CD8+ T cell epitopes

The consensus sequence of ORF1 and ORF2 of genotypes 1, 3 and 4 (respectively) was used to predict B cells, major histocompatibility complex class I (MHCI) and class II (MHCII) T cell epitopes using IEDB, ProPred-1 and ProPred, respectively.¹⁰⁻¹² In case of MHCI and II, epitope's binding to maximum number of alleles and binding capacity of <500 mM were selected. The predicted epitopes were further confirmed by BLASTp¹³ to avoid considering the epitopes that have a homology with human proteins.

The predicted epitopes were then evaluated for the presence or absence of the reported mutations (**Table 1**). We found that many of these characteristic mutations reported in ORF1 and ORF2 regions overlap with the predicted epitopes and were also frequently observed among selected HEV genotypes (1, 3 and 4) (**Table 2 and Table 3**). In our analysis, all epitopes possessing mutations that are present in our analysed HEV sequences were considered. **Table 2** and **Table 3** show mutations in the predicted epitopes of ORF1 and ORF2 (consensus genotype 1, 3 and 4) against B cells, MHC-I and MHC-II T cells. It was observed that ORF1 and ORF2 of genotype 1 possess more epitopes with reported mutations when compared with genotype 3 and 4 (**Table 2 and 3**). Most of these overlapped mutations were related to hepatic failure (HF), followed by ribavirin treatment failure (RTF), altered immunoreactivity (AI) and chronic hepatitis (CH) (HF>RTF>AI>CH).

Alteration of epitope antigenicity by characteristic mutations

We hypothesize that HEV may alter its epitopes by acquiring mutations to evade the immune recognition by both B and T cells.¹⁴ The online tool VaxiJen¹⁵ was used to detect the effect of each mutation on the antigenicity of the epitopes (wild type and mutation containing epitopes) (**Table 2 and Table 3**). Interestingly, in many cases, mutated epitopes have a reduced antigenicity when compared to the wild-type epitope (**Table 2, Table 3, and Figure 2**). Some mutated epitopes have a sustained antigenicity, while only few mutated epitopes have an increased antigenicity. These findings suggest that most of the reported mutations within predicted epitopes have a reduced antigenicity. Consequently, they are less recognized by both B and T cells and thus, decreasing the effective roles of the adaptive immune cells to clear the infections.

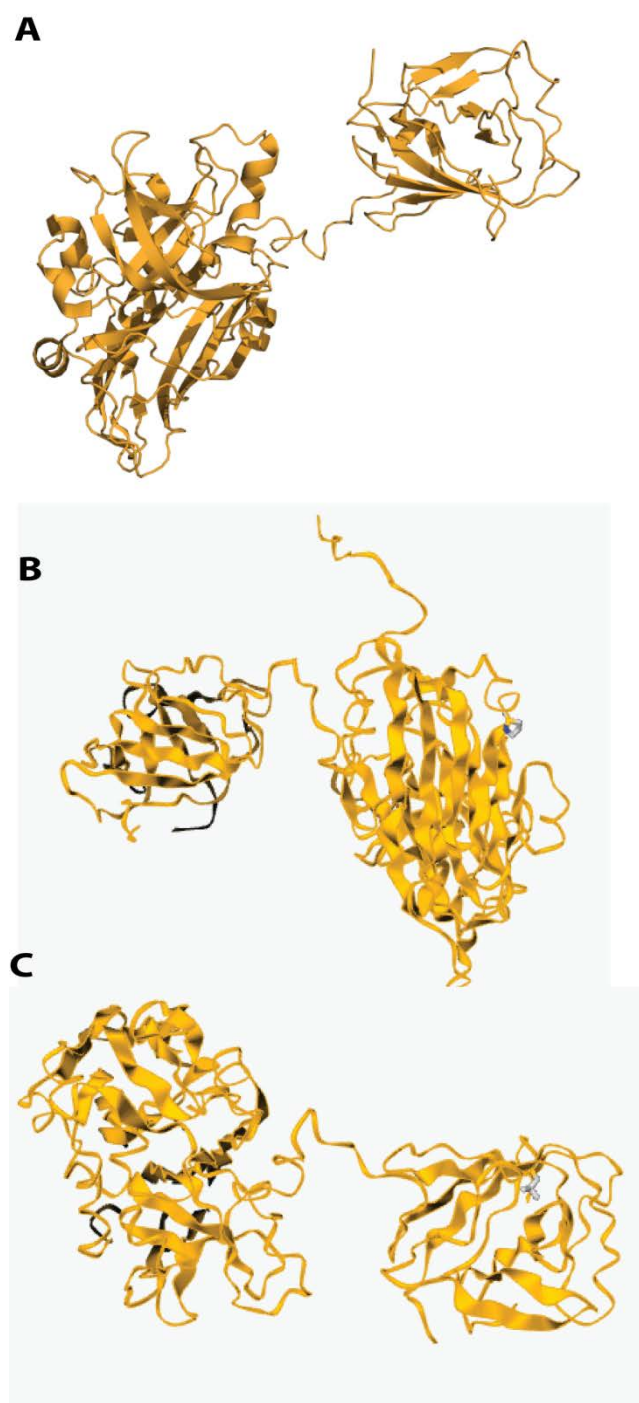


Figure 3. The effects of mutations (P259S, L477T) on the structural stability of ORF2 region of HEV predicted by DUET web server. Mutations are shown in blue color ribbon. (A) Crystal structure without mutation of ORF2 (2ZTN-amino acid 129-606). (B). P259S; feature: destabilizing; secondary structure: loop or irregular. (C). L477T; feature: destabilizing; secondary structure: extended beta-strand.

Region/ Genotype	Epitopes name	Position	Wild-type epitope	Antigenicity	Mutated epitope	Antigenicity
MHCI						
ORF1/GT1	T1	F179S	MFRHGMTRL	0.1	MF /SRHGMTRL	0.2
	T2	T735I	ATPTPAAPL	0.2	ATPT/ IP AAPL	0.2
	T3	L1110F	TTSRVLRSL	0.4	TTSRV L /FRSL	-0.6
	T4	F1439Y	FYGDAFDDT	0.2	FYGDA F /YDDT	0.2
	T5	G1634K	AVSDFLRGL	0.4	AVSDF LRG /KL	-0.6
ORF2/GT1	T6	L613T	SALALLEDL	0.3	SALALLED L /T	0.5
MHCII						
ORF1/GT1	T7	A317T	FHAVPAHIW	0.5	FHAV PA /THIW	-0.7
	T8	T735I	IPSRAATPT	0.2	IPSRAATPT/ I	1
	T9	V1120I	FWGEPAVGQ	0.2	FWGEP AV /IGQ	0.2
	T10	V1479I	LGLECAVME	0.8	LGLECA V /IME	0.7
	T11	G1634K	LRGLTNVAQ	0.7	LR G /KLTNVAQ	0.7
ORF2/GT1	T12	L477T	WLSLLAAEY	1.1	WLS LL /TAAEY	1.7
MHCI						
ORF1/GT3	T13	V1213A	VIVNNFFLV	0.1	VIVNNFF L V /A	-0.1
	T14	G1634R	LGLAVCDFL	0.2	LG /RLAVCDFL	-0.3
MHCII						
ORF1/GT3	T15	V1213A	LVGGEVGH	1.1	LV /AGGEVGH	1
	T16	G1634R	LGLAVCDFL	0.2	LG /RLAVCDFL	-0.3
ORF2/GT3	T17	L477T	WLSLLAAEY	1.1	WLS LL /TAAEY	1.7
	T18	L613T	VLEDLIDYP	0.4	VLE D L /TIDYP	-0.17
MHCI						
ORF1/GT4	T19	T735I	AEADTPVAV	0.2	AEAD T /IPVAV	0.1
	T20	V1213A	REVGISDAI	0.8	REVGIS D V /AI	0.7
	T21	G1634R	SERAEQLRL	0.3	SE G /RAEQLRL	0.3
MHCII						
ORF1/GT4	T22	T735I	VATDVPPPA	0.4	VAT/ ID VPPPA	0.1
	T23	V1213A	VIVNNFFLS	0.03	V /AIVNNFFLS	0.02

Table 2. Antigenicity evaluation of wild-type and mutated epitopes. The effect of mutations on the antigenicity (threshold level = 0.4) of predicted T cell epitopes (MHCI and MHCII). Bold letters (one-letter amino acid code) represent mutations within the predicted epitopes.

Region/ Genotype	Epitopes name	Position	Wild-type B cell epitopes	Antigenicity	Mutated B cell epitopes	Antigenicity
ORF1/GT1	B1	F179S	FRHGMTRLY	0.1	F /SRHGMTRLY	0.1
	B2	T735I	TPAAPLPPP	0.1	T /IPAAPLPPP	0.1
	B3	L1110F	TTSRVLRSL	0.1	TTSRV L /FRSL	0.01
	B4	V1120I	WGEPAVGQK	0.8	WGEPAV I GQK	1
	B5	F1439Y	FYGDAFDDT	0.2	FYGDA F /YDDT	0.2
	B6	G1634K	SDFLRKLTN	0.1	SDFLR G /KLTN	-0.1
ORF2/GT1	B7	L477T	SLTAAEYDQ	1.1	SLL /TAAEYDQ	1.0
	B8	L613T	LLDYPARAH	0.4	L /TLDYPARAH	0.4
ORF1/GT3	B9	T735I	LPHPTPPVS	0.5	LPHPT I PPVS	0.6
	B10	G1632R	ERAEQLRLA	0.1	ERAEQL G /RLA	0.1
ORF2/GT3	B11	L477T	SLTAAEYDQ	1.1	SLL /TAAEYDQ	0.5
	B12	L613T	LIDYPARAH	0.5	L /TIDYPARAH	0.4
ORF1/GT4	B13	V735I	TPVAVDVPP	0.5	TPVAV I /IDVPP	0.6
	B14	Y1587F	CGLKLVVDY	0.7	CGLKLV DY /F	1.7
	B15	G1634R	KNWGPSEG	1.8	KNWGPSEG R	1.7

Table 3. Antigenicity evaluation of wild-type and mutated epitopes. The effect of mutations on the antigenicity (threshold level = 0.4) of predicted B cells epitopes. Bold letters (one-letter amino acid code) represent mutations within the predicted epitopes.

Evolution of the characteristic mutations

To visualize the mutation evolution within the predicted epitopes of each genotype, a heat map was generated for each reported mutation (**Figure 4**). The concept of evolving mutations among the HEV genotypes within antigenic area will be helpful in determining their role in both immune and therapeutic responses. Different trends of evolving mutations have been observed in our analysis. The mutations A317T, T735I, L1110F, V1120I, V1479I and G1634K which were frequently observed in genotype 1 ORF1 have an evolving period of 3 years (1998-2001), 6 years (1998-2003), 8 years (1992-1999) 7 years (1992-1998), 5 years (1983-1987) and 4 years (1983-1986), respectively (**Figure 4 and Figure 5**). A commonly observed variation in all selected genotypes (1, 3 and 4), i.e. T735I, was found to be evolved

earlier in ORF1 of genotype 1 (1998-2003) when compared with genotype 3 and 4. Another common variation G1634K/R evolved earlier in ORF1 of genotype 1 (1983-1986) as compared to genotype 3 (1993-1997) and genotype 4 (1995-1998) (**Figure 4 and Figure 5**). Similarly, the mutations in ORF2 (L477T and L613T) appear to evolve earlier in genotype 1 compared to genotype 3 (**Figure 4 and Figure 5**). These data collectively indicate an earlier acquisition of several characteristic mutations in genotype 1 as compared to genotype 3 and 4.

Discussion

Ribavirin monotherapy has been widely used for treatment of chronic hepatitis E.⁵ It is in general very effective. However, for a subset of patients, ribavirin treatment fails.^{16, 17} Mutations in the viral polymerase have been noted before or during therapy in these patients.¹⁸ Furthermore, many mutations related to hepatic failure, chronic hepatitis and immunoreactivity have also been reported, conferring the importance of these mutations in HEV pathogenesis and treatment responses. In support of this, several studies have shown that proteomic variations in certain epitopes that are associated with these mutations can critically influence the outcome of the immune responses.¹⁹ These antigenic variations have been observed among HEV strains using genotype- and strain-specific monoclonal antibodies.²⁰ Even though a number of studies have reported frequencies of mutations in the HEV genome, the global prevalence of these characteristic mutations has not been comprehensively studied. Thus, this study has evaluated the HEV ORF1 and ORF2 variability in major genotypes (1, 3 and 4) by a systematic retrieval of human-derived HEV sequences. Furthermore, important co-occurrence of B and T cell (CD4⁺ and CD8⁺) epitope mutations was revealed, suggesting adaptation of viruses to escape immune surveillance.

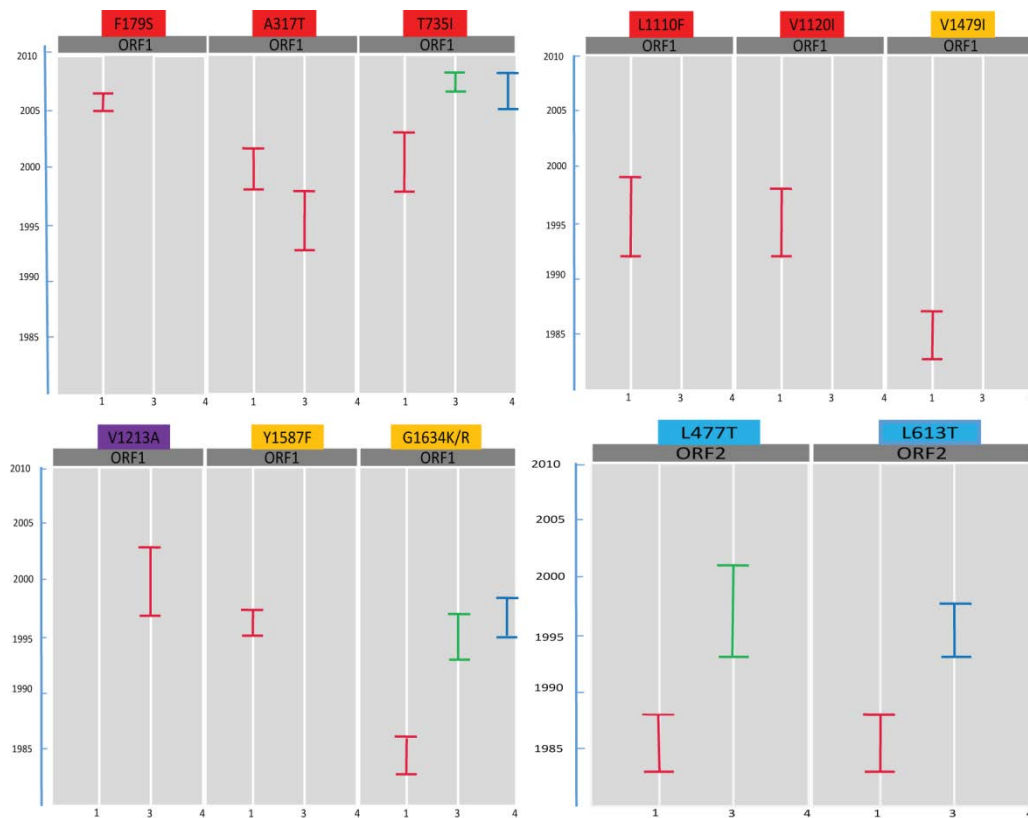


Figure 5. Evolution period of overlapped mutations. Each bar represents the evolving period (years) of each mutations.

By exploring the intra-genotypic diversity from representative human HEV genotypes, we have demonstrated that genotype 1 produces the largest number of intra-genotypic variants (**Table 1**). These variants were found to be evolved with a period of on average ~4 years but varied from 3 to 5 years in the human population (**Figure 4 and Figure 5**). Surprisingly, some reported mutations, including Y1320H, K1383N, D1384G and K1398N (related to ribavirin treatment failure) and C1483W, N1530T and P259S (related to hepatic failure), were hardly present in our retrieved large set of sequences, suggesting their insignificance. In contrast, two mutations T735I (related to hepatic failure) and G1634R (related to ribavirin treatment failure) were found in all selected genotypes (1, 3 and 4) with a considerably high frequency. Among these mutations, G1634R/K was identified in patients as a baseline mutation that affects ribavirin treatment response.²¹ This mutation was also observed in a patient with chronic hepatitis E, experiencing ribavirin treatment failure with a completely resistant phenotype.¹⁶ However, the exact clinical relevance of such mutations in ribavirin treatment failure is uncertain since findings from *in vitro* studies show that some of these mutations facilitate HEV replication but paradoxically seem to increase ribavirin sensitivity,¹⁶ thus

requiring further investigation. Furthermore, effective new antiviral therapy is needed for HEV patients with ribavirin treatment failure.²² Two immunoreactivity-related mutations (L477T and L613T) were mainly found in genotype 1 and 3 highlighting the role of such mutations in affecting host immune response (**Table 1**).

To investigate the implication of these characteristic mutations in the host immune response, we have profiled their overlap with predicted B and T cell epitopes. We found that the hotspot sites where mutations and predicted epitopes overlap are frequently present among many HEV genotypes (**Table 1 and Figure 2**). The subsequent analysis of these mutations showed that this overlap mostly decreases and in few cases, sustains or enhances the antigenicity of the mutated epitopes (**Table 2, Table 3 and Figure 2**). Experimental studies have demonstrated that only antibodies recognizing conformational epitopes are neutralizing, and the aa residues Leu477 and Leu613 in the capsid protein are important in forming a neutralization-sensitive epitope representing the importance of these mutations in epitope deformation.²³ In patients, HEV-specific T cells target relatively conserved HEV peptides, and those are predominantly located in the ORF2 capsid protein. The T-cell responses persist over years after resolution of HEV infection, suggesting the role in both clearance of primary infection and protective immune response against secondary infection.²⁴ Based on our study, we propose a model of immune evasion by HEV (**Figure 6**). In this model, mutated epitopes will result in escape recognition by B and T cells. Furthermore, we have mapped the evolution of these mutations in all selected genotypes. We found that most of the overlapped mutations are more abundantly present in genotype 1 as compared to genotype 3 and 4. Interestingly, the common mutations evolved earlier in genotype 1 than genotype 3 and 4.

In summary, our study represents a comprehensive analysis of the characteristic mutations in the major HEV genotypes. Some of these mutations overlap with predicted B and T cell epitopes that are expected to affect the antigenicity. We further revealed the evolution of these mutations among the three major genotypes. These results indicate that HEV is under substantial evolutionary pressure to develop mutations enabling evasion of the host immune response and resistance to antiviral treatment. This indicates the existence of an ongoing evolutionary arms race between human immunity, antiviral medication and the HEV.

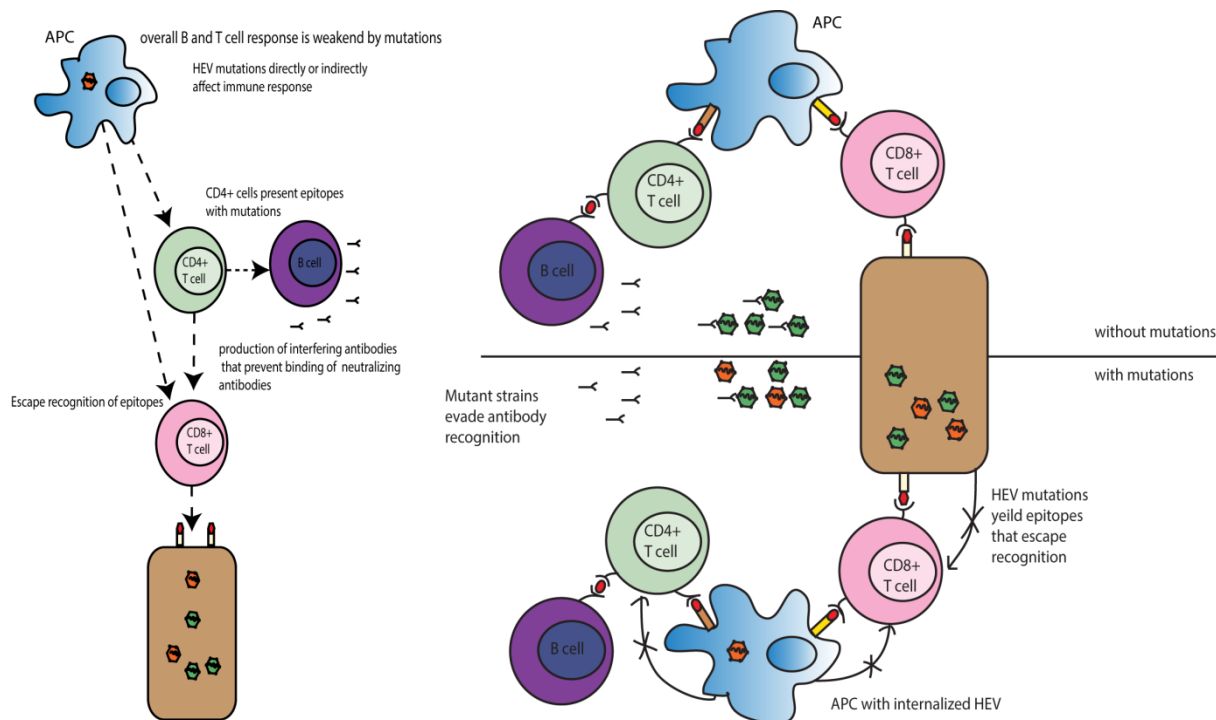


Figure 6. Possible mechanisms of immune evasion by hepatitis E virus. Main routes by which HEV mutations may result in evasion of the host immune responses. Mutated epitopes presented by antigen presenting cells, B and T cells will result in escape recognition of the epitopes.

Material and Methods

Data collection

A database on reported mutations within the ORF1 and ORF2 regions of HEV is currently not available. We reviewed earlier studies (through January 2017), using a combination of the key words that are listed in **Table S1**, and evaluated mutations in HEV-infected individuals, as well as in HEV replicons. These studies were searched from the PubMed (ncbi.nlm.nih.gov/pubmed), EMBASE, and Cochrane Library databases.

Retrieval of sequences

We retrieved 57 ORF1, 51 ORF2 (genotype 1), 131 ORF1, 131 ORF2 (genotype 3) and 99 ORF1, 96 ORF2 (genotype 4) of HEV protein sequences from the GenBank²⁵ (accessed on January 2017). The selected protein sequences represent all major genotypes (1, 3 and 4). The GenBank accession numbers of HEV protein sequences used in this study against each

ORF and genotype are provided in **Table S3**. The sequences were trimmed manually and analyzed using the reference protein sequences of selected genotypes [AF185822 (genotype 1), AB291960 (genotype 3), AB200239 (genotype 4)]. Different quality control measures were performed for the sequences, and many sequences were disqualified for further analysis based on the following two conditions: (a) if the sequence derived from a non-human host; and (b) if the sequence was a clonal sequence from the same patient. Sequences were also annotated by the year of sampling. In some cases where the source did not provide the sampling year, we used the submission date to the GenBank as the sampling year.

Consensus sequence and mutations analysis

The HEV ORF1 and ORF2 sequences were aligned using ClustalW, BioEdit and CLC Workbench 7 (<http://www.clcbio.com>). A consensus analysis for each HEV genotype was performed to observe the presence or absence of mutation at each site. These mutations were selected on the basis of published data reporting all experimentally proven mutations (*in vitro* and *in vivo*). The prevalence of each mutation was then measured within the selected regions (ORF1 and ORF2) of each genotype (1, 3 and 4).

Analysis of mutations as a missense SNPs and their effects on structural stability

To validate selected mutations as missense SNPs in ORF1 and ORF2 regions of HEV genotype 1, 3 and 4, computational analysis was performed using four tools PROVEAN (Protein Variation Effect Analyzer),²⁶ nsSNP analyser,²⁷ SNPs & GO and PMUT.²⁸ These tools describe missense SNPs as damaging or neutral to function and structure. To predict the change in protein stability due to these SNPs, DUET,²⁹ I-Mutant version 2.0,³⁰ and STRUM³¹ web servers were used. As an input in I-Mutant and STRUM servers, FASTA sequences of ORF1, ORF2 regions of selected genotypes were used. PDB files of 3D structures (2ZTN) of ORF2 were used as an input in DUET web server.

Prediction of the B and T cell epitopes and comparison with the host proteome

Nine-mer B cell epitopes were predicted against HEV genotype (1, 3 and 4) from their consensus sequence by using online tool, the Immune Epitope Database (IEDB).³² Similarly, nine-mer T cell epitopes (MHC class I and II), following the same criteria as B cell epitopes, were predicted by online T cell epitope prediction tools, including ProPred-I (MHCI) and ProPred (MHCII).^{33, 34} ProPred-I and ProPred identify and predict 47 types of MHCI and 57 types of MHCII allele specific binding peptides in a provided protein, respectively.^{33, 34} The predicted T cell epitopes were also confirmed by IEDB tool.³⁵ The selected epitopes were analyzed for comparisons with the human proteome using the Protein Blast program (BLASTp).¹³ This was performed to validate that these epitopes will not trigger an autoimmune response.

Overlapping sites of mutations and predicted epitopes

A comprehensive exploration was performed to find out any reported mutations positioned in a predicted epitope. Predicted B cells and T cells epitopes were mapped to ORF1 and ORF2 regions that contain most of the reported mutation sites. Finally, the incidence of these overlapped mutations was determined among the selected genotypes (1, 3 and 4) by using percentage formula.

Prediction of antigenicity of epitopes

To investigate the antigenic properties of epitopes before and after mutations, Vaxijen tool was used.¹⁵ The analysis was performed to find out whether these mutations lead to decreased, enhanced or sustained antigenicity of the specific epitopes.

Evolutionary analysis of mutations

To visualize the evolution of amino acid mutation related to chronic hepatitis, hepatic failure, ribavirin treatment failure or immunoreactivity within each genotype, a heat map was developed by GraphPad Prism version 7 (GraphPad Software, La Jolla California, USA) to calculate the number of amino acid and the isolation year differences between three

individual genotypes. Isolation years were extracted from the strain-annotated information. The difference values were added into a matrix where the y-axis represents the isolation year differences and the x-axis represents the amino acid differences.

Abbreviations

AI	Altered immunoreactivity
CH	Chronic hepatitis
HCV	Hepatitis C virus (HCV)
HEV	Hepatitis E virus
HF	Hepatic failure
MHCI	Major histocompatibility complex class I
MHCII	Major histocompatibility complex class II
PEG-IFN- α	Pegylated interferon- α
ORF	Open reading frame
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RTF	Ribavirin treatment failure

Acknowledgements

The authors thank to the Higher Education Commission of Pakistan for financial support to A. Ikram; the Indonesia Endowment Fund for Education (LPDP) for PhD scholarship to Mohamad S. Hakim; the China Scholarship Council for funding PhD fellowship to W. Wang (201303250056); the Dutch Digestive Foundation (MLDS) for a career development grant (No. CDG 1304), the Daniel den Hoed Foundation for a Centennial Award fellowship and the Erasmus MC Mrace grant to Q. Pan.

References

1. Kamar N, Bendall R, Legrand-Abravanel F, Xia NS, Ijaz S, Izopet J, et al. Hepatitis E. *Lancet* 2012; 379:2477-88.
2. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; 380:2095-128.
3. Teshale EH, Hu DJ. Hepatitis E: epidemiology and prevention. *World J Hepatol* 2011; 3:285-91.
4. Hakim MS, Wang W, Bramer WM, Geng J, Huang F, de Man RA, et al. The global burden of hepatitis E outbreaks: a systematic review. *Liver Int* 2017; 37:19-31.
5. Kamar N, Mallet V, Izopet J. Ribavirin for chronic hepatitis E virus infection. *N Engl J Med* 2014; 370:2447-8.
6. Wedemeyer H, Pischke S, Manns MP. Pathogenesis and treatment of hepatitis E virus infection. *Gastroenterology* 2012; 142:1388-97 e1.
7. Takahashi K, Toyota J, Karino Y, Kang JH, Maekubo H, Abe N, et al. Estimation of the mutation rate of hepatitis E virus based on a set of closely related 7.5-year-apart isolates from Sapporo, Japan. *Hepatol Res* 2004; 29:212-5.
8. Lhomme S, Garrouste C, Kamar N, Saune K, Abravanel F, Mansuy JM, et al. Influence of polyproline region and macro domain genetic heterogeneity on HEV persistence in immunocompromised patients. *J Infect Dis* 2014; 209:300-3.
9. van Tong H, Hoan NX, Wang B, Wedemeyer H, Bock CT, Velavan TP. Hepatitis E virus mutations: functional and clinical relevance. *EBioMedicine* 2016; 11:31-42.
10. Singh R, Kaul R, Kaul A, Khan K. A comparative review of HLA associations with hepatitis B and C viral infections across global populations. *World J Gastroenterol* 2007; 13:1770-87.
11. Sturniolo T, Bono E, Ding J, Raddizzani L, Tuereci O, Sahin U, et al. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat Biotechnol* 1999; 17:555-61.
12. Vita R, Overton JA, Greenbaum JA, Ponomarenko J, Clark JD, Cantrell JR, et al. The immune epitope database (IEDB) 3.0. *Nucleic Acids Res* 2015; 43:D405-12.
13. Lavigne R, Seto D, Mahadevan P, Ackermann HW, Kropinski AM. Unifying classical and molecular taxonomic classification: analysis of the Podoviridae using BLASTP-based tools. *Res Microbiol* 2008; 159:406-14.
14. Gu Y, Tang X, Zhang X, Song C, Zheng M, Wang K, et al. Structural basis for the neutralization of hepatitis E virus by a cross-genotype antibody. *Cell Res* 2015; 25:604-20.
15. Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinformatics* 2007; 8:4.
16. Debing Y, Ramiere C, Dallmeier K, Piorkowski G, Trabaud MA, Lebosse F, et al. Hepatitis E virus mutations associated with ribavirin treatment failure result in altered viral fitness and ribavirin sensitivity. *J Hepatol* 2016; 65:499-508.
17. Debing Y, Gisa A, Dallmeier K, Pischke S, Bremer B, Manns M, et al. A mutation in the hepatitis E virus RNA polymerase promotes its replication and associates with ribavirin treatment failure in organ transplant recipients. *Gastroenterology* 2014; 147:1008-11 e7; quiz e15-6.
18. Todt D, Walter S, Brown RJ, Steinmann E. Mutagenic effects of ribavirin on hepatitis E virus-Viral extinction versus Selection of fitness-Enhancing mutations. *Viruses* 2016; 8.
19. Liang JH, Dai X, Dong C, Meng JH. A single amino acid substitution changes antigenicity of ORF2-encoded proteins of hepatitis E virus. *Int J Mol Sci* 2010; 11:2962-75.
20. Liang J-H, Dai X, Dong C, Meng J-H. A single amino acid substitution changes antigenicity of ORF2-encoded proteins of hepatitis E virus. *Int J Mol Sci* 2010; 11:2962-75.
21. Lhomme S, Kamar N, Nicot F, Ducos J, Bismuth M, Garrigue V, et al. Mutation in the hepatitis E virus polymerase and outcome of ribavirin therapy. *Antimicrob Agents Chemother* 2016; 60:1608-14.

22. Kamar N, Wang W, Dalton HR, Pan Q. Direct-acting antiviral therapy for hepatitis E virus? *Lancet Gastroenterol Hepatol* 2017; 2:154-5.
23. Zhang H, Dai X, Shan X, Meng J. The Leu477 and Leu613 of ORF2-encoded protein are critical in forming neutralization antigenic epitope of hepatitis E virus genotype 4. *Cell Mol Immunol* 2008; 5:447-56.
24. Brown A, Halliday JS, Swadling L, Madden RG, Bendall R, Hunter JG, et al. Characterization of the specificity, functionality, and durability of host T-cell responses against the full-length hepatitis E virus. *Hepatology* 2016; 64:1934-50.
25. Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, et al. GenBank. *Nucleic Acids Res* 2013; 41:D36-42.
26. Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics* 2015; 31:195.
27. Bao L, Zhou M, Cui Y. nsSNPAnalyzer: identifying disease-associated nonsynonymous single nucleotide polymorphisms. *Nucleic Acids Res* 2005; 33:W480-W2.
28. Ferrer-Costa C, Gelpí JL, Zamakola L, Parraga I, De La Cruz X, Orozco M. PMUT: a web-based tool for the annotation of pathological mutations on proteins. *Bioinformatics* 2005; 21:3176-8.
29. Pires DE, Ascher DB, Blundell TL. DUET: a server for predicting effects of mutations on protein stability using an integrated computational approach. *Nucleic Acids Res* 2014; 42:gku411.
30. Capriotti E, Fariselli P, Casadio R. I-Mutant2. 0: predicting stability changes upon mutation from the protein sequence or structure. *Nucleic Acids Res* 2005; 33:W306-W10.
31. Quan L, Lv Q, Zhang Y. STRUM: structure-based prediction of protein stability changes upon single-point mutation. *Bioinformatics* 2016; 32:2936-46.
32. Vita R, Zarebski L, Greenbaum JA, Emami H, Hoof I, Salimi N, et al. The immune epitope database 2.0. *Nucleic Acids Res* 2010; 38:D854-62.
33. Singh H, Raghava GP. ProPred: prediction of HLA-DR binding sites. *Bioinformatics* 2001; 17:1236-7.
34. Singh H, Raghava GP. ProPred1: prediction of promiscuous MHC class-I binding sites. *Bioinformatics* 2003; 19:1009-14.
35. Kim Y, Ponomarenko J, Zhu Z, Tamang D, Wang P, Greenbaum J, et al. Immune epitope database analysis resource. *Nucleic Acids Res* 2012; 40:W525-30.

Supplementary information

Table S1. Search terms used in this study.

Organism	Mutations	Method of introducing mutations
Hepatitis E virus	Mutations	Engineered
HEV ORF1	Mutagenesis	Site directed
HEV ORF2	Site directed mutations	Wild type
HEV genotype 1	Transductions	Induced
HEV genotype 3	Mutants	
HEV genotype 4	Codon	

Table S2. Schematic illustration of the study.

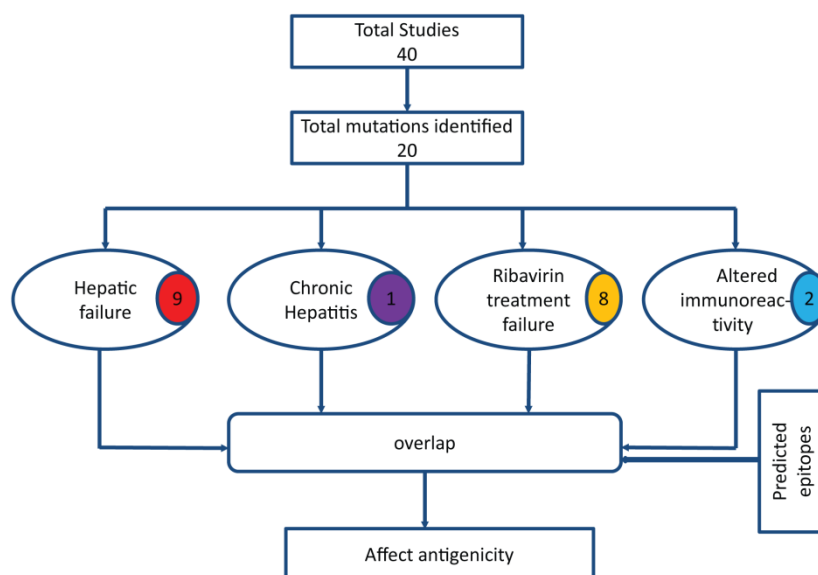


Table S3. The GenBank accession numbers of HEV sequences used in this study.

Available online: <https://www.tandfonline.com/doi/suppl/10.1080/21505594.2017.1358349>

Table S4. HEV mutations analysed in this study.**A. Twenty main mutations analyzed in the present study.**

Mutations	Effect	Reference(s)
Y1320H; G1634R/K, K1383N, D1384G; K1398R; V1479I; Y1587F	Ribavirin treatment failure	[1-3]
L477T; L613T	Influence the immunoreactivity of HEV by affecting the neutralization epitope	[4]
F179S, A317T, T735I, L1110F, V1120I, F1439Y, C1483W, N1530T, P259S	Fulminant hepatic failure	[5-7]
V1213A	Chronic hepatitis	[8]

B. Other studies analyzed to see the effect of specific mutations.

Studies for HEV mutation evaluations	Reference(s)
H443L; C457A; C459A; C471A; C472A; C481A; C483A; H497L; H590L	[9, 10]
Insertion/deletion	[11]
Mutational analysis	[12]
Insertion/deletion of a 24 bp RdRp-derived fragment	[1]
N809A; H812L; G816A/V; G817A/V	[13, 14]
L1110F; V1120I	[15]
Effect of mutagenesis on enzyme activity	[16]
K1383N	[1]
F51L	[11, 17]
T59A	[17, 18]
S390L	[17, 18]
N137Q; N310Q; N311Q	[19]
N562Q/D/P/Y	[19]
A5145C; A5178C; A5190C; G5676T; T5690G	[20]
CGC5148–5150AGA	[20]
A5108Δ; T5109C; C5112U; TCT5116–5118AGC; T5121C	[20]
S80A (V66G)	[20]
G6574C; C6570G; G7106T/A; G7097A; C7144A	[21-23]
C1816, U3148, C5907	[24, 25]
186 bp insertion	[26]
90 bp insertion	[27]
171 bp insertion	[28]

174 bp insertion	[28]
117 bp insertion	[29]
246 bp deletion	[30]
Recombination	[31]
Mutations in HEV	[32, 33]
Deletion	[34]
HEV adaptations	[35, 36]
HEV mutations (review)	[37]
Ribavirin-induced mutagenesis	[3]
Role of asparagine at position 562	[38]
Role of truncated ORF2 region in immunoreactivity	[39]
HEV pathogenesis in pregnancy	[40]

Table S5. Missense SNPs in ORF1 and ORF2 regions of HEV predicted to be deleterious using nsSNP Analyzer, PROVEAN, PMUT and SNPs & GO.

Mutations	Provean	PMUT	SNPs&Go	nsSNP analyser	Provean	PMUT	SNPs&Go	nsSNP analyser	Provean	PMUT	SNPs&Go	nsSNP analyser
ORF1	GT1				GT3				GT4			
F179S	-	-	-	-	-	-	-	-	-	-	-	-
A317T	-	-	-	-	-	-	-	-	-	-	-	-
T735I	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
L1110F	-	-	-	-	-	-	-	-	-	-	-	-
V1120I	-	-	-	-	-	-	-	-	-	-	-	-
V1213A	-	-	-	-	-	-	-	-	-	-	-	-
Y1320H	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
K1383N	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
D1384G	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
K1398N	-	-	-	-	-	-	-	-	-	-	-	-
F1439Y	-	-	-	-	-	-	-	-	-	-	-	-
V1479I	-	-	-	-	-	-	-	-	-	-	-	-
C1483W	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
N1530T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Y1587F	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
G1634R	-	-	-	-	-	-	-	-	-	-	-	-
G1634K	-	✓	✓	-	-	✓	✓	-	-	✓	✓	-
ORF2												
P259S	-	-	-	-	-	-	-	-	-	-	-	-
L477T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
L613T	-	-	-	-	-	-	-	-	-	-	-	-

Table S6. Stability analysis of the mutations by using I-Mutant web server. Tick mark in the table shows that a mutation is found to be destabilizing by the server.

Mutations	I-Mutant	I-Mutant	I-Mutant
ORF1	GT1	GT3	GT4
F179S	✓	✓	✓
A317T	✓	✓	✓
T735I	✓	✓	✓
L1110F	✓	✓	✓
V1120I	✓	✓	✓
V1213A	✓	✓	✓
Y1320H	✓	✓	✓
K1383N	✓	✓	✓
D1384G	✓	✓	✓
K1398N	✓	✓	✓
F1439Y	✓	✓	✓
V1479I	✓	✓	✓
C1483W	✓	✓	✓
N1530T	✓	✓	✓
Y1587F	✓	✓	✓
G1634R	✓	✓	✓
G1634K			
ORF2			
P259S	-	-	-
L477T	✓	✓	✓
L613T	✓	✓	✓

Reference

1. Debing, Y., et al., Hepatitis E virus mutations associated with ribavirin treatment failure result in altered viral fitness and ribavirin sensitivity. *Journal of Hepatology*, 2016. **65**(3): p. 499-508.
2. Debing, Y., et al., A mutation in the hepatitis E virus RNA polymerase promotes its replication and associates with ribavirin treatment failure in organ transplant recipients. *Gastroenterology*, 2014. **147**(5): p. 1008-1011. e7.
3. Todt, D., et al., In vivo evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome. *Gut*, 2016. **65**(10): p. 1733-1743.
4. Zhang, H., et al., The Leu477 and Leu613 of ORF2-encoded protein are critical in forming neutralization antigenic epitope of hepatitis E virus genotype 4. *Cellular & Molecular Immunology*, 2008. **5**(6): p. 447.
5. Borkakoti, J., et al., Novel molecular alterations in the ORF 2 capsid gene of hepatitis E virus in patients with acute liver failure in North India. *Archives of Virology*, 2014. **159**(12): p. 3391-3394.
6. Mishra, N., A.M. Walimbe, and V.A. Arankalle, Hepatitis E virus from India exhibits significant amino acid mutations in fulminant hepatic failure patients. *Virus Genes*, 2013. **46**(1): p. 47-53.
7. Borkakoti, J., G. Ahmed, and P. Kar, Report of a novel c1483w mutation in the hepatitis E virus polymerase in patients with acute liver failure. *Infection, Genetics and Evolution*, 2016. **44**: p. 51-54.
8. Takahashi, K., Virulent strain of hepatitis E virus genotype 3, Japan. *Emerging Infectious Disease*, 2009. **15**(5): p. 704-709.
9. Parvez, M.K. and M.S. Al-Dosari, Evidence of MAPK-JNK1/2 activation by hepatitis E virus ORF3 protein in cultured hepatoma cells. *Cytotechnology*, 2015. **67**(3): p. 545-550.
10. Parvez, M.K. and A.A. Khan, Molecular modeling and analysis of hepatitis E virus (HEV) papain-like cysteine protease. *Virus Research*, 2014. **179**: p. 220-224.
11. Pudupakam, R., et al., Deletions of the hypervariable region (HVR) in open reading frame 1 of hepatitis E virus do not abolish virus infectivity: evidence for attenuation of HVR deletion mutants in vivo. *Journal of Virology*, 2009. **83**(1): p. 384-395.
12. Pudupakam, R., et al., Mutational analysis of the hypervariable region of hepatitis E virus reveals its involvement in the efficiency of viral RNA replication. *Journal of Virology*, 2011. **85**(19): p. 10031-10040.
13. Parvez, M.K., The intergenic-junction variant (genotype 2 isolate) of hepatitis E virus restores the CREX 'stem-loop' structural integrity, essential for viral life cycle. *Gene*, 2015. **559**(2): p. 149-154.
14. Parvez, M.K., Molecular characterization of hepatitis E virus ORF1 gene supports a papain-like cysteine protease (PCP)-domain activity. *Virus Research*, 2013. **178**(2): p. 553-556.
15. Devhare, P., et al., Analysis of helicase domain mutations in the hepatitis E virus derived from patients with fulminant hepatic failure: effects on enzymatic activities and virus replication. *Virus Research*, 2014. **184**: p. 103-110.
16. Mhaindarkar, V., K. Sharma, and K.S. Lole, Mutagenesis of hepatitis E virus helicase motifs: effects on enzyme activity. *Virus Research*, 2014. **179**: p. 26-33.
17. Córdoba, L., et al., Three amino acid mutations (F51L, T59A, and S390L) in the capsid protein of the hepatitis E virus collectively contribute to virus attenuation. *Journal of Virology*, 2011. **85**(11): p. 5338-5349.
18. Huang, Y., et al., Capped RNA transcripts of full-length cDNA clones of swine hepatitis E virus are replication competent when transfected into Huh7 cells and infectious when intrahepatically inoculated into pigs. *Journal of Virology*, 2005. **79**(3): p. 1552-1558.
19. Graff, J., et al., Mutations within potential glycosylation sites in the capsid protein of hepatitis E virus prevent the formation of infectious virus particles. *Journal of Virology*, 2008. **82**(3): p. 1185-1194.
20. Graff, J., et al., The open reading frame 3 gene of hepatitis E virus contains a cis-reactive element and encodes a protein required for infection of macaques. *Journal of Virology*, 2005. **79**(11): p. 6680-6689.
21. Emerson, S., et al., Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a cis-reactive element. *Proceedings of the National Academy of Sciences*, 2001. **98**(26): p. 15270-15275.
22. Emerson, S.U., et al., An essential RNA element resides in a central region of hepatitis E virus ORF2. *Journal of General Virology*, 2013. **94**(Pt 7): p. 1468-1476.
23. Graff, J., et al., In vitro and in vivo mutational analysis of the 3'-terminal regions of hepatitis E virus genomes and replicons. *Journal of Virology*, 2005. **79**(2): p. 1017-26.
24. Inoue, J., et al., Analysis of the full-length genome of genotype 4 hepatitis E virus isolates from patients with fulminant or acute self-limited hepatitis E. *Journal of Medical Virology*, 2006. **78**(4): p. 476-484.

25. Inoue, J., et al., Nucleotide substitutions of hepatitis E virus genomes associated with fulminant hepatitis and disease severity. *The Tohoku Journal of Experimental Medicine*, 2009. **218**(4): p. 279-284.
26. Johne, R., et al., An ORF1-rearranged hepatitis E virus derived from a chronically infected patient efficiently replicates in cell culture. *Journal of Viral Hepatitis*, 2014. **21**(6): p. 447-456.
27. Legrand-Abravanel, F., Hepatitis E virus genotype 3 diversity, France. *Emerging Infectious Disease*, 2009. **15**(1): p. 110-114.
28. Shukla, P., et al., Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus–host recombinant. *Proceedings of the National Academy of Sciences*, 2011. **108**(6): p. 2438-2443.
29. Nguyen, H., et al., A naturally occurring human/hepatitis E recombinant virus predominates in serum but not in faeces of a chronic hepatitis E patient and has a growth advantage in cell culture. *Journal of General Virology*, 2012. **93**(3): p. 526-530.
30. Reyes, G.R., et al., Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science*, 1990. **247**(4948): p. 1335.
31. Wang, H., et al., Recombination analysis reveals a double recombination event in hepatitis E virus. *Virology Journal*, 2010. **7**(1): p. 129.
32. Sanjuan, R., et al., Viral mutation rates. *Journal of Virology*, 2010. **84**(19): p. 9733-9748.
33. Cao, D. and X.J. Meng, Molecular biology and replication of hepatitis E virus. *Emerg Microbes Infect*, 2012. **1**(8): p. e17.
34. Ray, R., et al., Indian hepatitis E virus shows a major deletion in the small open reading frame. *Virology*, 1992. **189**(1): p. 359-362.
35. Purdy, M.A., J. Lara, and Y.E. Khudyakov, The hepatitis E virus polyproline region is involved in viral adaptation. *PLOS One*, 2012. **7**(4): p. e35974.
36. Dalton, H.R., et al., Locally acquired hepatitis E in chronic liver disease. *Lancet*, 2007. **369**(9569): p. 1260.
37. van Tong, H., et al., Hepatitis E virus mutations: functional and clinical relevance. *EBioMedicine*, 2016. **1**: p. 31-42.
38. Xu, M., et al., Role of asparagine at position 562 in dimerization and immunogenicity of the hepatitis E virus capsid protein. *Infection, Genetics and Evolution*, 2016. **37**: p. 99-107.
39. Zhou, Y.-H., R.H. Purcell, and S.U. Emerson, A truncated ORF2 protein contains the most immunogenic site on ORF2: antibody responses to non-vaccine sequences following challenge of vaccinated and non-vaccinated macaques with hepatitis E virus. *Vaccine*, 2005. **23**(24): p. 3157-3165.
40. Navaneethan, U., M. Al Mohajer, and M.T. Shata, Hepatitis E and pregnancy: understanding the pathogenesis. *Liver International*, 2008. **28**(9): p. 1190-1199.

Part II.

Rotavirus and Norovirus

Chapter 6

Basal interferon signaling and therapeutic use of interferons in controlling rotavirus infection in human intestinal cells and organoids

Mohamad S. Hakim^{1,2}, Sunrui Chen¹, Shihao Ding¹, Yuebang Yin¹, Aqsa Ikram^{1,3}, Xiaoxia Ma⁴, Wenshi Wang¹, Maikel P. Peppelenbosch¹, Qiuwei Pan¹

- 1 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, the Netherlands
- 2 Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
- 3 Atta-Ur-Rahman School of Applied Biosciences, National University of Science and Technology, Islamabad, Pakistan
- 4 Key Laboratory of Bioengineering and Biotechnology of State Ethnic Affairs Commission; Engineering and Technology Research Center for Animal Cell; College of Life Science and Engineering, Northwest University for Nationalities; Lanzhou, China

Scientific Reports 2018; 8(1): 8341.

Abstract

Rotavirus (RV) primarily infects enterocytes and results in severe diarrhea, particularly in children. It is known that the host immune responses determine the outcome of viral infections. Following infections, interferons (IFNs) are produced as the first and the main anti-viral cytokines to combat the virus. Here we showed that RV predominantly induced type III IFNs (IFN- λ 1), and to a less extent, type I IFNs (IFN- α and IFN- β) in human intestinal cells. However, it did not produce detectable IFN proteins and thus, was not sufficient to inhibit RV replication. In contrast, we revealed the essential roles of the basal IFN signaling in limiting RV replication by silencing *STAT1*, *STAT2* and *IRF9* genes. In addition, exogenous IFN treatment demonstrated that RV replication was able to be inhibited by all types of IFNs, both in human intestinal Caco2 cell line and in primary intestinal organoids. In these models, IFNs significantly upregulated a panel of well-known anti-viral IFN-stimulated genes (ISGs). Importantly, inhibition of the JAK-STAT cascade abrogated ISG induction and the anti-RV effects of IFNs. Thus, our study shall contribute to better understanding of the complex RV-host interactions and provide rationale for therapeutic development of IFN-based treatment against RV infection.

Keywords: immune responses; interferons; interferon-stimulated genes; JAK-STAT pathway; rotavirus

Introduction

Rotavirus (RV) is a member of the *Reoviridae* family that primarily infects mature enterocytes of the small intestinal villi. However, it can spread systemically to cause viremia and infection of multiple organs¹. RV is the most frequent agent of severe dehydrating diarrhea episodes in children under five years of age². Before introduction of RV vaccines, RV caused 9.8 billion of severe diarrhea episodes and 1.9 billion diarrhea-related deaths worldwide, with the highest burden in southeast Asian and African countries³. The incidence is lower especially in countries that have introduced oral RV vaccination⁴.

Intestinal innate immune responses are the first line of defense to battle RV infection⁵. Recognition of RV viral proteins and double-stranded RNA by the host induces the production of cytokines, including interferons (IFNs)⁶. IFNs are potent anti-viral cytokines classified into three different groups, type I (IFN- α , IFN- β , IFN- δ , and others), type II (IFN- γ) and type III (IFN- λ 1, IFN- λ 2 and IFN- λ 3) IFNs^{7,8}. Some members are widely used in the clinic for treating viral infections or malignancy; whereas others are at stages of clinical development. Even though they bind to distinct receptors, they signal through a common, classical Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway^{8,9}.

Once activated, STAT1 and STAT2 are phosphorylated and bind IFN regulatory factor 9 (IRF9) to form IFN stimulated gene factor 3 complex (ISGF3). ISGF3 subsequently translocates to the nucleus, inducing transcription of hundreds IFN-stimulated genes (ISGs) which cooperatively establish an anti-viral state that protects against various types of viruses¹⁰. Furthermore, IFN induction following RV recognition is essential to promote the development of adaptive, B-cell-mediated immune responses¹¹. On the other hand, however, RV has developed effective strategies to evade the host immune response¹². RV can inhibit IFN production in the infected cells¹³ and also block the action of STAT1 and STAT2 proteins¹⁴. Viral nonstructural protein NSP1-mediated IFN inhibition has been shown to be associated with different levels of RV replication in primary mouse cells¹⁵.

Detectable levels of IFN- α ¹⁶ and IFN- γ ^{17,18} were documented in children with acute RV diarrhea, suggesting a role for these IFNs in disease pathogenesis. Indeed, early *in vitro*¹⁹⁻²¹ and animal studies in calves²² and piglets²³ demonstrated anti-RV effects of both type I and II

IFNs. However, in the murine models of homologous RV infection, administration of type I (IFN- α and IFN- β) and II (IFN- γ) IFNs failed to protect the mice against RV infection²⁴. In addition, mice deficient in type I or II IFN receptor signaling controlled RV infection as well as wild-type mice^{24,25}. These results suggest a minor role of type I and II IFNs in controlling RV infection in mice. Interestingly, a more prominent function of type III IFNs in constraining viral infection was suggested by experimentation in mouse models^{25,26}. Administration of IFN- λ conferred better protection against RV infection than IFN- α/β ²⁵.

Because animal models do not always recapitulate the responsiveness in human, we therefore comprehensively assessed the role of endogenous and the therapeutic IFNs on RV infection in human intestinal cell line and primary intestinal organoids. We found that the basal JAK-STAT cascade is effective in restraining RV infection. Furthermore, RV is sensitive to inhibition by all three types of IFNs in both models. Our results strengthen the evidence of essential roles of IFN pathway in protecting the host against viral infection.

Results

RV infection modulates *IFN* gene expression

First, we investigated whether RV SA11 modulates the expression of the three types of *IFN* genes. Human intestinal Caco2 cells were infected with RV SA11 for 48 hours. An effective replication was shown by an increase in intracellular RNA level as well as secreted rotavirus in culture medium (Supplementary Fig. S1). In addition, immunofluorescence staining showed VP6-positive Caco2 cells at 48 hours after infection, indicating productive replications (Supplementary Fig. S2).

Relative RNA levels of *ifna*, *ifnb*, *ifng*, *il29* (IFN- λ 1) and *il28* (IFN- λ 2/IFN- λ 3) genes were examined and compared to uninfected cells at 6, 24, 36 and 48 hours post infection. As shown in Fig. 1, RV infection had no major effect on the gene expression at 6 and 24 hours post-infection. At 36 hours after infection, only *il29* gene expression was notably increased by 3.4 ± 1.0 ($P < 0.05$) fold. Importantly, at 48 hours after infection, the expression of *ifna* and *ifnb* genes were significantly increased by 2.8 ± 0.6 ($P < 0.001$) and 2.8 ± 0.5 ($P < 0.01$) fold, respectively. A profound upregulation was observed on *il29* by 29.6 ± 10.7 fold ($P <$

0.001). No difference was found on *il28* gene expression. The expression level of *ifng* gene was undetectable (data not shown). Together, our findings showed that RV SA11 infection preferentially induced *il29* (IFN- λ 1) gene expression in Caco2 cells.

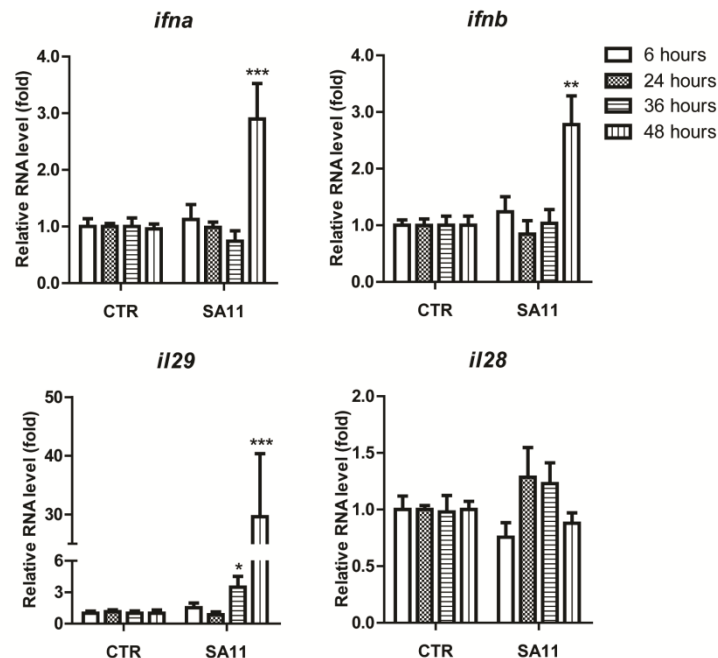


Figure 1. RV infection modulates IFN gene expression in Caco2 cells. Caco2 cells were infected with RV SA11. Relative RNA levels of *ifna*, *ifnb*, *il29* (IFN- λ 1) and *il28* (IFN- λ 2 and IFN- λ 3) genes were examined at 6, 24, 36 and 48 hours post infection as compared to uninfected cells. Data were normalized to GAPDH and presented as means \pm SEM. (n = 3 independent experiments with each of 3-4 replicates; **P < 0.01; ***P < 0.0001)

The increased expression of IFN genes does not result in production of detectable IFN protein and was not sufficient to limit RV SA11 replication

To examine whether the increased expression of IFN genes result in IFN production in our cell culture system, we collected the conditioned medium (supernatant) derived from control and SA11-infected Caco2 cells at 48 hours post-infection. Then, we performed IFN production bioassay by adding the conditioned medium into two highly IFN sensitive cell lines, Huh7-based ISRE-luciferase and HCV-luciferase reporter cell lines. As shown in Fig. 2A, the supernatant from SA11-infected Caco2 cells was not capable of stimulating ISRE reporter, while as low as 1 U/mL of IFN- α significantly induced ISRE-luc by 1.8 ± 0.2 fold ($P < 0.001$). Consistently, it was not able to diminish HCV replication, although as low as 0.1 IU/mL of IFN- α considerably reduced HCV-luc by $40 \pm 7\%$ fold ($P < 0.001$) (Fig. 2B).

The induced expression of IFN- α and IFN- λ 1 proteins can signal via autocrine and paracrine manner to stimulate ISGs expression. Therefore, to confirm our previous findings from ISRE-luciferase cell lines, we also examined ISG expression in SA11-infected Caco2 cells. Despite a clear induction of IFN genes, we observed no upregulation of ISGs, both at 24 and 48 hours post-infection. At 48 hours, some ISGs were significantly downregulated by SA11 infection, including IRF1, IRF9, MX1 and IFIT3 (Fig. 2C).

To rule out the possibility that endogenous IFN produced (if any) following RV infection is sufficient to restrict RV replication, we investigated whether the inhibition of JAK proteins, the downstream elements of IFN receptor, influences RV replication. Treatment of JAK I inhibitor at 5 and 10 μ M had no effect on RV replication (Fig. 2D). At those concentrations, the drug did not influence the cell viability as determined by MTT assay (Fig. 2E). Collectively, our results demonstrate that the increased expression of IFN genes during RV infection does not result in IFN production and consequently appears relatively unimportant for constraining RV replication in intestinal epithelial cells.

Basal IFN signaling is necessary to restrict RV replication

As no functional production of IFN is observed following RV infection, we investigated the possibility that constitutive ligand-independent IFN signal transduction defends intestinal epithelial cells against RV replication. ISGF3 that consists of STAT1, STAT2 and IRF9 is a central complex in IFN signal transduction. We have previously reported that in the absence of IFN stimulation, unphosphorylated ISGF3 drives constitutive ISG expression in homeostatic conditions and is critical to provide immunity against hepatitis C (HCV) and E (HEV) virus infections²⁷. It is thus possible that constitutive IFN signal transduction is important for counteracting RV in intestinal cells as well.

To test this possibility we first transduced Caco2 cells with a lentiviral vector expressing STAT1- and STAT2-specific shRNA. A successful knockdown is shown in Fig. 3A and 3C. Supplementary Fig. S3 shows the quantification of knockdown efficiency. Importantly, shRNA-mediated STAT1 and STAT2 knockdown resulted in an increased RV replication by 3.8 ± 0.6 fold ($P < 0.01$) (Fig. 3B) and 13 ± 4.6 ($P < 0.001$) (Fig. 3D), respectively.

Next we investigated the role of IRF9. Two of three tested IRF9-specific shRNA (sh-2 and sh-3) exert a potent gene silencing capacity (Fig. 3E and Supplementary Fig. S3). Consistently, IRF9 KD led to 4.8 ± 1.0 fold elevation of RV replication as compared to sh-CTR transfected cells ($P < 0.001$) (Fig. 3F). Altogether, these results indicate that the integrity of ISGF3 complex is required to provide basal immunity against RV infections.

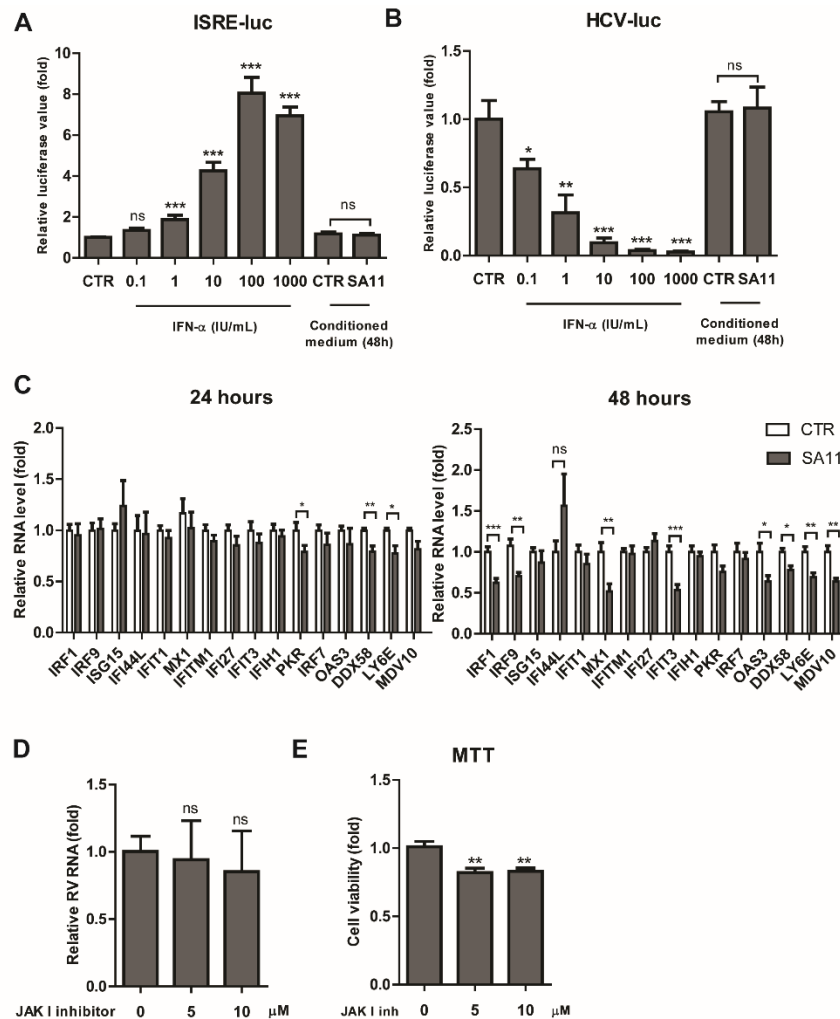


Figure 2. The increased expression of IFN genes does not result in production of detectable IFN protein and was not sufficient to limit RV SA11 replication. IFN production bioassay was performed in ISRE-luciferase (A) and HCV-luciferase (B) cell lines which are highly sensitive to IFN treatment. Conditioned medium derived from 48 hours post-RV infection on Caco2 cells was used ($n = 3$ independent experiments with each 2-3 replicates). (C) Caco2 cells were infected with RV SA11. Relative RNA levels of IFN-stimulated genes (ISGs) were examined at 24 and 48 hours post-infection as compared to uninfected cells ($n = 3$ independent experiments with each 2-3 replicates). (D) Pan-JAK I inhibitor had no effects on RV replication ($n = 3$ independent experiments with each 2-3 replicates). (E) Pan-JAK I inhibitor did not affect cell viability as determined by MTT assay (OD_{490} value) at 48 hours of treatment ($n = 3$ independent experiments with each 2 replicates). Data were presented as means \pm SEM., ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

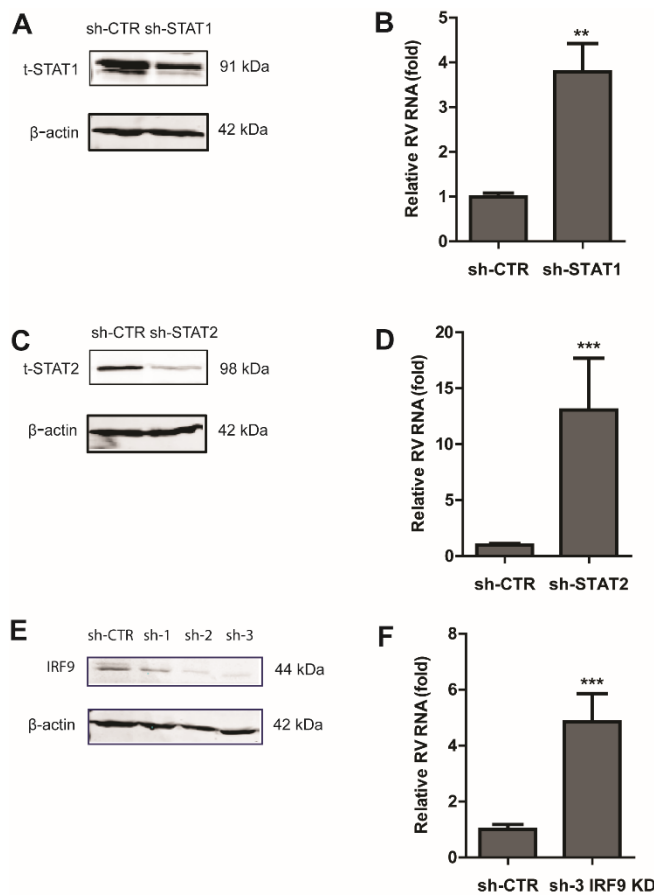


Figure 3. The key component of ISGF3 complex is necessary to restrict RV replication. (A) STAT1 knockdown by lentiviral shRNA vectors. Western blot analysis confirms a successful knockdown of total STAT1 protein. (B) Correspondingly, knockdown of STAT1 led to a significant increase of RV replication ($n = 3$ independent experiments with 3-4 replicates each). (C) Knockdown of STAT2 by lentiviral shRNA vectors. Western blot analysis shows a potent decrease of total STAT2 protein level. (D) Similarly, silencing of STAT2 resulted in a prominent increase of RV RNA level ($n = 3$ independent experiments with 3-4 replicates each). (E) IRF9 knockdown by lentiviral shRNA vectors. Western blot analysis shows a potent decrease of IRF9 protein level. (F) Knockdown of IRF9 led to a notable increase of RV replication ($n = 3$ independent experiments with 3-4 replicates each). Data were presented as means \pm SEM., ** $p < 0.01$; *** $p < 0.001$.

RV SA11 is sensitive to IFN treatment in human intestinal Caco2 cells and primary intestinal organoids

Since we did not find a significant role of endogenous IFN in restricting RV replication, we then investigated whether RV was sensitive to exogenous IFN treatment (Fig. 4A). Treatment of SA11-infected Caco2 cells with 100 and 1000 IU/mL of IFN α resulted in a potent inhibition of RV replication by $79 \pm 4\%$ ($P < 0.001$) and $98 \pm 0.7\%$ ($P < 0.001$) as measured in total RNA levels. Similarly, IFN β treatment at 100 and 1000 IU/mL dose-dependently inhibited total viral RNA levels by $60 \pm 9\%$ ($P < 0.01$) and $73 \pm 7\%$ ($P < 0.001$). Type II IFNs also strongly reduced RV replication, although the effects were not dose-dependent. At concentration of 100 and 1000 ng/mL, IFN γ restricted RV replication by $81 \pm 6\%$ ($P < 0.001$) and $68 \pm 7\%$ ($P < 0.001$).

0.01). Analysis of intra and extracellular RNA levels also demonstrated the inhibition of RV replication by type I and II IFNs in Caco2 cells (Supplementary Fig. S4).

Next, we investigated anti-RV effects of type III IFNs. Treatment of SA11-infected Caco2 cells with 100 and 1000 ng/mL of IFN λ 1 resulted in a notable restriction of RV replication by $76 \pm 11\%$ ($P < 0.01$) and $65 \pm 6\%$ ($P < 0.001$). Inhibition of RV replication was also observed with IFN λ 2 treatment. At a concentration of 100 and 1000 ng/mL, IFN λ 2 decreased RV replication by $61 \pm 17\%$ ($P < 0.01$) and $50 \pm 7\%$ ($P < 0.05$), respectively. At a similar concentration, IFN λ 3 significantly diminished RV replication by $52 \pm 7\%$ ($P < 0.05$) and $62 \pm 6\%$ ($P < 0.01$). Analysis of intra and extracellular RNA levels also demonstrated the effects of type III IFNs in limiting rotavirus replication in Caco2 cells (Supplementary Fig. S4).

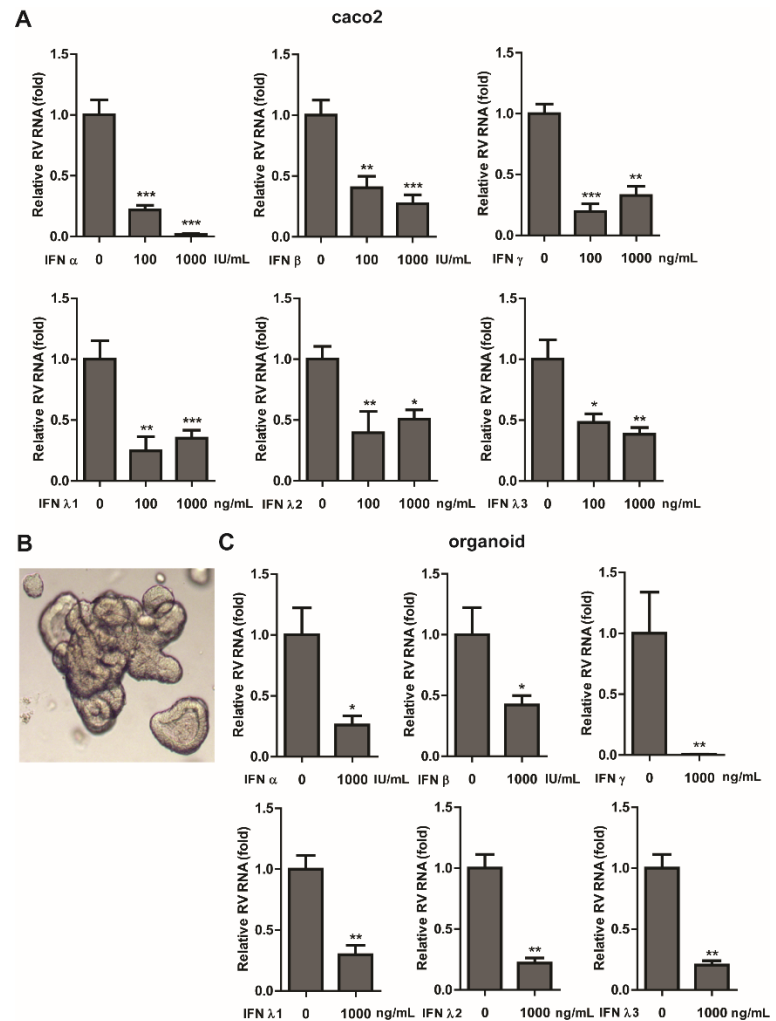
To further confirm the effects of various types of IFNs that we observed in the conventional 2D cell culture system, we employed a 3D culture model of primary intestinal organoids derived from one individual (P1) to more closely mimic the physiological situation *in vivo* (Fig. 4B). Treatment of SA11-infected organoid with 1000 IU/mL of IFN α and IFN β led to a significant reduction of intracellular viral RNA levels by $73.9 \pm 7.5\%$ ($P < 0.05$) and $57.8 \pm 7.7\%$ ($P < 0.05$), respectively. Surprisingly, the inhibition by IFN γ was more pronounced. Treatment of SA11-infected organoid with 1000 ng/mL of IFN γ significantly reduced intracellular viral RNA levels by $99.5 \pm 0.1\%$ ($P < 0.01$) (Fig. 4C). Similarly, treatment with 1000 ng/mL of IFN λ 1, IFN λ 2 or IFN λ 3 significantly diminished intracellular viral RNA levels by $70.2 \pm 7.6\%$ ($P < 0.01$), $78.2 \pm 4.2\%$ ($P < 0.01$) and $79.6 \pm 3.6\%$ ($P < 0.01$), respectively (Fig. 4C). Quantification of extracellular (secreted) RNA levels also showed a notable reduction of viral production (Supplementary Fig. S5). To confirm these findings, we obtained primary intestinal organoids from the second individual (P2). Similarly, treatment of SA11-infected organoid with IFN α (1000 IU/mL), IFN γ (1000 ng/mL) and IFN λ 1 (1000 ng/mL) significantly reduced total viral RNA levels by $74.6 \pm 5.1\%$ ($P < 0.01$), $85.5 \pm 4.4\%$ ($P < 0.01$) and $71.4 \pm 10.5\%$ ($P < 0.01$), respectively (Supplementary Fig. S6A). Collectively, these results suggest that all type of IFNs effectively inhibit RV replication, both in 2D and 3D culture model system.

Sensitivity of patient-derived RV strains to type I, II and III IFNs

Next, we evaluated the sensitivity of patient-derived RV strains against different types of IFNs. We treated human RV (G1P[8]) with IFN α 100 IU/mL (as representative of type I IFN),

IFN γ 100 ng/mL (type II IFN), IFN λ 1 and IFN λ 3 100 ng/mL (as representative of type III IFN) for 48 hours. Three out of four samples were sensitive to the inhibition by IFN α and all samples were inhibited by IFN γ (Fig. 5). Interestingly, only one sample which is sensitive to type III IFN treatment. Collectively, our data suggest that type I and II IFN more efficiently inhibit the replication of human RV strains as compared to type III IFN.

Figure 4. Exogenous treatment of type I, II and III IFNs inhibits RV SA11 infection. Antiviral activity of IFN α , IFN β , IFN γ , IFN λ 1, IFN λ 2 and IFN λ 3 treatment against RV SA11 infection on (A) Caco2 cells (n = 2-3 independent experiments with each of 3-4 replicates) and (C) organoids (n = 3 independent experiments with each of 2-3 replicates) at 48 hours after infection. Data were presented as means \pm SEM., * P < 0.05; ** P < 0.01; *** P < 0.001. (B) A representative picture of the morphology of human small intestinal organoid at day 4 post embedding in Matrigel. The organoid used in this experiment was derived from one individual (P1).



Induction of the known antiviral ISGs by all three types of IFNs

The observed anti-RV activity of type I, II and III IFNs prompted us to investigate whether all types of IFNs effectively induce the expression of known anti-viral ISGs in Caco2 cells and organoid. Although there are hundreds of ISGs, only a subset have broad or targeted antiviral effects²⁸. We have selectively investigated the expression of those known antiviral ISGs. Indeed, treatment of Caco2 cells with recombinant human IFN- α (1000 IU/mL), IFN- γ (1000 ng/mL) and IFN- λ 1 (1000 ng/mL) for 24 hours induced the expression a panel of ISGs

(Fig. 6A). Similarly, they also efficiently induced ISGs in organoids derived from both P1 (Fig. 6B) and P2 (Supplementary Fig. S6B). Interestingly, we observed a variation of the type and extent of ISG induction with different IFN treatment. For example, IRF1 and RTP4 were more induced by IFN- γ as compared to IFN- α in Caco2 cells. In contrast, DDX60 and IFI6 were more induced by IFN- α as compared to IFN- γ (Fig. 6A). In organoid (P1), several ISGs were more efficiently induced by IFN- λ 1 as compared to IFN- α and IFN- γ , including OASL, ISG15 and OAS1 (Fig. 6B).

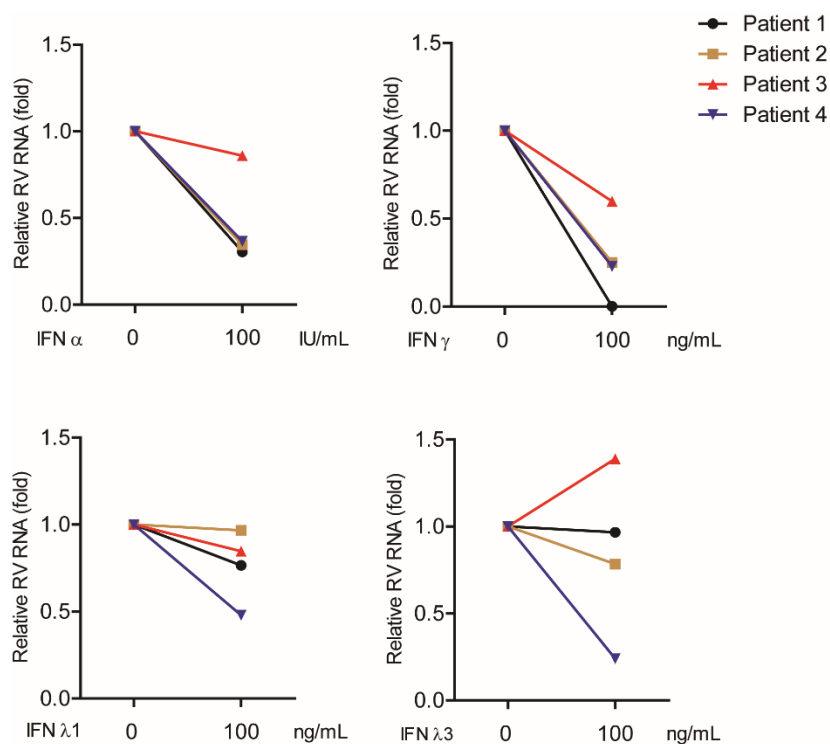


Figure 5. Sensitivity of patient-derived RV strains to type I, II and III IFNs. Caco2 cells were infected with four different patient-derived RV strains (G1P[8]) and treated with IFN α 100 IU/mL (as representative of type I IFN), IFN γ 100 ng/mL (type II IFN), IFN λ 1 and IFN λ 3 100 ng/mL (as representative of type III IFN). Distinct sensitivity was observed among these patient-derived RV samples. Human RV RNA levels were quantified by qRT-PCR at 48 hours post-infection and normalized to a reference gene GAPDH. The data are derived from an experiment for multiple patient-derived RV strains.

Inhibition of JAK-STAT signaling abrogates the anti-RV activity of IFN- α and IFN- γ

The ISG induction by type I and III IFNs is mediated via a similar pathway involving ISGF3 complex. For type II IFN, its signaling pathway involves phosphorylation and dimerization of STAT1 to form IFN γ activation factor (GAF)⁹. To investigate the role of JAK-STAT signaling pathway in the anti-RV effects of IFNs, we used JAK I inhibitor that predominantly inhibit JAK1 protein, the upstream element that is responsible for STAT1 and STAT2 phosphorylation. As expected, JAK I inhibitor (10 μ M) efficiently blocked IFN α - and IFN γ -induced ISG expression in Caco2 cells (Supplementary Fig. S7 and S8). Consistently, JAK I inhibitor treatment abolished the anti-RV effects of IFN- α and IFN γ in Caco2 cell lines (Fig. 7A and 7B, respectively). These data clearly indicate an important role of JAK-STAT pathway in mediating the anti-RV effects of IFNs.

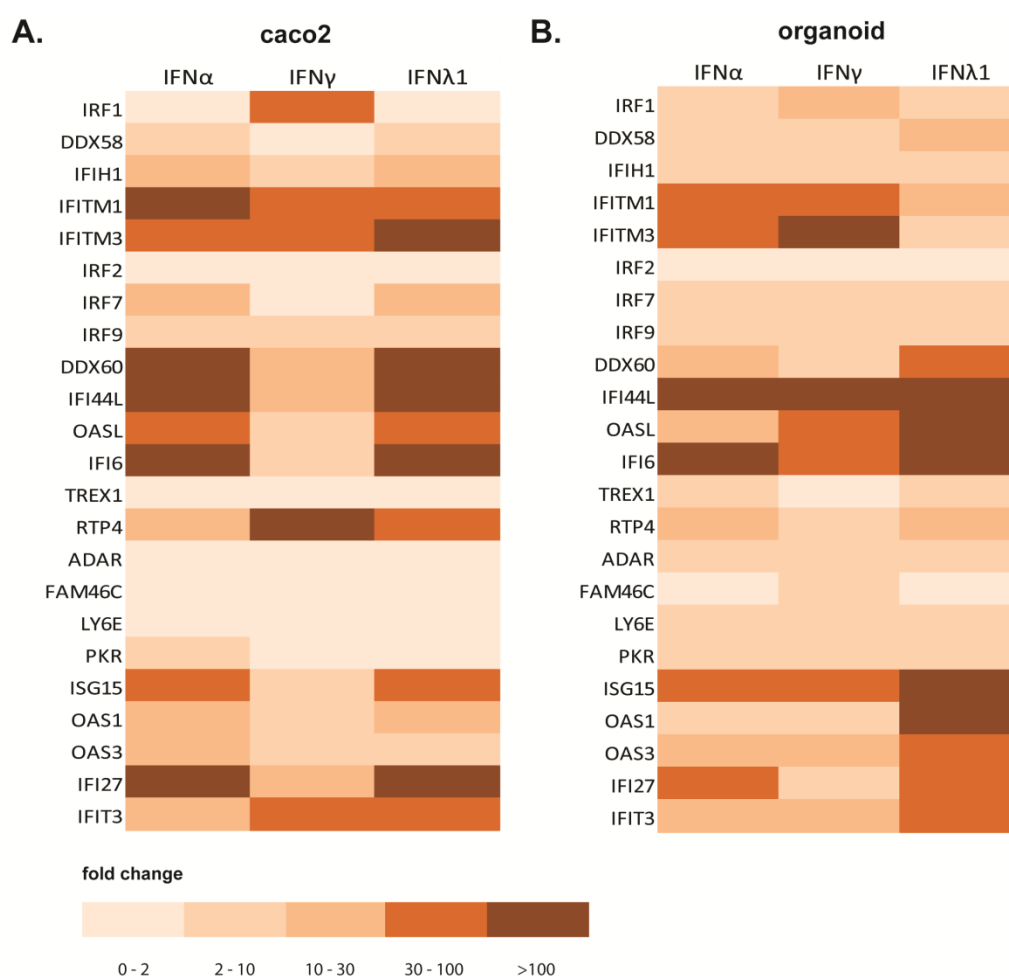


Figure 6. ISG induction by type I, II and III IFNs on Caco2 cells and organoids. Caco2 cells **(A)** and organoids **(B)** were stimulated with IFN α 1000 IU/mL (as representative of type I IFN), IFN γ 1000 ng/mL (type II IFN) and IFN λ 1 1000 ng/mL (as representative of type III IFN) for 24 hours. The expression levels of several ISGs were measured by qRT-PCR. The organoid used in this experiment was derived from one individual (P1).

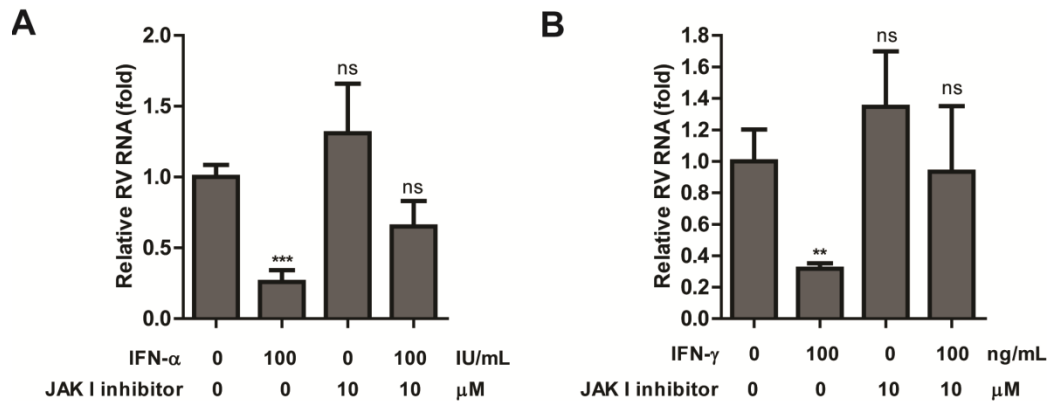


Figure 7. JAK I inhibitor block the anti-RV effects of IFN α and IFN γ . Caco2 cells were first infected with RV SA11 for 60 minutes. After four times washing, IFN α or IFN γ and *Pan*-JAK I inhibitor were added simultaneously to SA11-infected Caco2 cells and then cultured for 48 hours. *Pan*-JAK I inhibitor (10 μ M) can block the anti-RV effects of IFN α 100 IU/mL **(A)** and IFN γ 100 ng/mL **(B)** in Caco2 cells. (n = 3 independent experiments with 2-4 replicates each) Data were presented as means \pm SEM., *P < 0.05; **P < 0.01; ns, not significant.

Discussion

The *in vitro* study of RV biology had mainly been based on the conventional two-dimensional (2D) cell culture system of intestinal carcinoma-derived cell lines, including Caco2 and HT29 cell lines^{14,29-31}. They are homogenous immortalized cell lines that can functionally reflect the real biological processes in the humans intestinal epithelium. However, they are lacking three-dimensional (3D) higher order organisation present in the human intestine *in vivo*³². Recently, 3D models of primary intestinal organoids were developed for studying RV biology which better recapitulate the architecture and cellular composition of the human intestine³³⁻³⁵. Intestinal organoids contain heterogenous and non-transformed cell types, including enterocytes, enteroendocrine cells, goblet cells, Paneth cells and stem cells³⁵. Therefore, they enable us to investigate individual-specific response associated with histo-blood group antigen (HBGAs) profiles and microbiome diversity³⁵.

Transcriptional analysis of SA11-infected Caco2 cells revealed that RV predominantly induced type III (IFN λ 1) rather than type I (IFN α and IFN β) IFN responses. In other intestinal

epithelial cells, such as HT29, RV infection induces type I IFN response (IFN β) and subsequently regulates ISG expression^{29,30,36}. However, our findings are consistent with previous studies in human intestinal enteroids (organoids) infected with human RV strains in which predominant type III IFN responses (IFN λ 1 and IFN λ 2) were observed³⁴. The relatively low induction of type I IFNs in these human epithelial cells is also consistent with studies in murine RV³⁷. Following murine RV infection, type I IFN response was mainly produced by immune (hematopoietic) cells, not epithelial cells³⁷. A predominant type III IFN response was also observed in hepatocytes upon HCV³⁸ and HEV infection³⁹. However, both type I and III IFN were similarly induced following influenza virus infection in lung epithelial cells⁴⁰. Thus, all these findings suggest that preferential induction of type I and/or type III IFN response is virus- and cell type-specific and reflects the complex regulation of type I and III IFN induction following viral infections.

Despite a clear induction of type III IFN responses, our IFN production bioassay found undetectable levels of IFN proteins in the (conditioned) culture medium from SA11-infected Caco2 cells (Fig. 2A and 2B). Consistently, we did not observe ISG upregulation in SA11-infected Caco2 cells (Fig. 2C), indicating an absence of secreted IFN to stimulate ISG expression in an autocrine and/or paracrine manner. Further analysis by inhibiting downstream IFN signaling using JAK inhibitors demonstrated that RV replication levels were not altered. In human intestinal enteroids (organoids) infected with human RV strains, type III IFN induction was followed by stimulation of type III-dependent ISGs³⁴. These discrepancies may be due to different RV strains as well as the different cellular composition of both models. However, despite this ISG induction, blockade of type I and III IFN receptor had no effects on RV replication³⁴. Altogether, these findings indicate that endogenously produced IFNs (if any) were not sufficient to limit RV replication, even though they were able to induce ISGs in intestinal organoid models. These findings also suggest the ability of RV to subvert innate immune responses. It is known that RV have multiple ways to blunt innate IFN responses¹². It has been shown that RV nonstructural protein 1 (NSP1) interacts with IRF3 to promote its degradation, leading to attenuation of IFN induction⁴¹.

While previous studies mainly focused on the role of STAT1 in RV replication^{15,42}, here we highlighted the role of basal IFN signaling in constraining RV infection. Individual knockdown of ISGF3 component, i.e. STAT1, STAT2 and IRF9, led to an elevated level of RV replication.

Similarly, STAT1 knockout (KO) mice shed a significantly higher titer of RV than wild-type (WT) controls⁴². STAT1 is also shown to protect against lethal challenge of murine norovirus infection in mice⁴³. In dengue virus (DENV)-infected mice, STAT2 was essentially required to protect against DENV-mediated diseases independently of STAT1⁴⁴. Our findings are also consistent with our previous *in vitro* studies demonstrating that unphosphorylated ISGF3 complex is responsible for maintaining basal transcription of ISGs in the absence of IFN stimulation to provide a “combat-ready” antiviral state in the susceptible host²⁷. Thus, constitutive IFN signaling, ligand independent but maintained by background expression of its essential component, including STAT1, STAT2 and IRF9, are pivotal to restrict RV replication in the intestinal epithelium.

While endogenous IFN response was not able to reduce RV replication, exogenous IFN treatment was effective to limit RV replication. We demonstrated that all three types of IFNs have notable antiviral effects against simian RV SA11 both in Caco2 cells and human organoids. Patient-specific organoid lines have promising implications for personalized medicine. Noteworthy, our study employed organoids derived from only two individuals. In this aspect, using organoids derived from several numbers of patients would be much better in recapitulating inter-individual variations, including HBGAs profiles, microbiome diversity and genetic background^{32,35}.

We have also successfully cultivated four human-derived RV strains from acute diarrhea patients. Treatment with representative type I (IFN α), II (IFN γ) and III (IFN λ 1 and IFN λ 3) IFNs showed various sensitivity of human RV to IFNs, in which more pronounced inhibition was observed with type I and II rather than type III IFN treatment. In previous studies using human organoid models, type I IFN was more effective than type III IFN to suppress human RV replication³⁴. However, conflicting results were found from *in vivo* studies about the relative contributions of type I and III IFNs during RV infections^{25,26,45}. Type I IFN response plays a functional role to limit extra-intestinal spread in the mesenteric lymph node (MLN)⁴⁵. On the other hand, RV has the capacity to attenuate the antiviral actions of IFNs¹⁴. Our study therefore suggests that human RV may differentially adapt in homologous host.

While many previous studies mainly focused on the effects of type I and III IFNs, our study highlights the role of type II IFN (IFN γ) in limiting RV replication. Previous studies showed

that IFN γ level in the serum was significantly higher in children with RV diarrhea than those of control children¹⁷. In our study, transcriptional analysis showed that IFN γ mRNA level was not detectable from SA11-infected Caco2 cells (data not shown). However, it has been shown that IFN γ was produced from human peripheral blood mononuclear cells (PBMCs) stimulated with RV⁴⁶. Consistently, the level of IFN γ gene expression as well as secreted level in the supernatant of PBMCs were significantly elevated in children with RV diarrhea as compared with controls^{18,47}. These findings suggest that immune cells, and not epithelial Caco2 cells, were responsible for IFN γ production upon RV infection.

RV-specific CD4⁺ and CD8⁺ IFN γ ⁺ T cells were detected in the peripheral blood of RV-infected children and adults^{48,49}. IFN γ producing T cells were also observed following experimental vaccines in animals and associated with disease protection^{21,50,51}. It was previously shown that IFN γ inhibit RV entry into Caco2 cells²⁰. In our study, we found that human IFN γ significantly reduces RV replication, suggesting that IFN γ can inhibit RV infection at various steps of the life cycle in the infected cells. In apparent agreement, it was suggested that IFN γ responses critically determine the severity of RV diseases in children⁵².

ISGs are the ultimate effectors of IFN-mediated antiviral responses. Based on our findings, all three types of IFNs effectively induced a panel of well-known anti-viral ISGs both in Caco2 cells and in human organoids. However, further studies are needed to identify specific anti-RV ISGs to improve our understanding of immunity against RV infections. In conclusions, our study describes the role of both endogenous and exogenous IFN in RV infection, as well as the role of both basal and activated IFN signaling in limiting RV infection. These knowledge shall contribute to the better understanding of RV-host interactions and therapeutic development.

Material and Methods

Reagents

Type I human recombinant IFN alpha 2a (IFN α ; Thermo Scientific) and IFN beta 1a (IFN β ; Sigma-Aldrich; Catalog Number 14151); Type II IFN gamma (IFN γ ; BioLegend; Catalog# 570202), and Type III IFNs IL29 (IFN λ 1; Abnova), IL28A (IFN λ 2; Abnova) and IL28B (IFN λ 3; Abnova) were dissolved in culture medium. Stocks of Jak inhibitor I (Santa Cruz Biotech, CA) was dissolved in DMSO (Sigma-Aldrich, St Louis, MO, USA) with a final concentration of 5 mg/mL. Anti-STAT1 antibody (#9172) was purchased from Cell Signaling Technology. IRF9 antibody was obtained from LSBio (Life Span BioSciences, Inc). β -actin and STAT2 (sc-476) antibodies were purchased from Santa Cruz Biotechnology. Anti-rabbit or anti-mouse IRDye-conjugated antibodies were used as secondary antibodies for western blotting (Stressgen, Victoria, BC, Canada).

Viruses

Simian RV SA11, a broadly used laboratory strains, was employed. SA11 RV used in this study was prepared as described previously⁵³. RV genome copy numbers were determined by quantitative real-time polymerase chain reaction (qRT-PCR). A plasmid template was used to generate a standard curve by plotting the log copy number versus the cycle threshold (C_T) as previously described³³.

Human-derived RV strains (G1P[8]) were obtained from fecal samples of four RV patients and stored at -80 °C freezer (the Erasmus MC Biobank, Department of Viroscience, Erasmus Medical Center, Rotterdam). These samples were collected during diarrhea period and tested negative for enterovirus, parechovirus, norovirus genogroup I and II, adenovirus, astrovirus and sapovirus by qRT-PCR. The patient characteristics were shown in Supplementary Table S1.

Cell and human primary intestinal organoid culture

Caco2 cell line (human caucasian colon adenocarcinoma ECACC) was cultured in Dulbecco's modified Eagles's medium (DMEM; Lonza, Verviers, Belgium) containing 20% (vol/vol) heat-

inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis USA), 100 U/mL penicillin and streptomycin (Gibco, Grand Island, USA). The cells were maintained in 5% CO₂ at 37 °C in a humidified incubator.

Organoid culture was performed as described previously³³. Briefly, intestinal tissues taken from biopsy were vigorously shaken in 8 mM EDTA for 15 min at 4 °C. The EDTA solution was then discarded. Loosened crypts were collected by pipetting the solution up and down for 8-10 times through a 10 mL pipette and transferred into a 50 mL tube (Greiner Bioone, the Netherlands). The biopsies were repeatedly used for crypts collection (2-3 times). Crypt suspensions were pooled and centrifuged at 300 g for 5 min. The crypt pellets were resuspended in 2 mL complete medium containing growth factors CMGF-: advanced DMEM/F12 supplemented with 1% (vol/vol) GlutaMAX™ Supplement (Gibco, Grand island, USA), 10 mM HEPES. The crypts were collected by centrifugation at 130g for 5 min at 4 °C, suspended in matrigel (Corning, Bedford, USA) and placed in the center of a 24-well plate (40 µL per well). After the matrigel had solidified (15 min at 37 °C), organoids were maintained in culture medium at 37 °C, 5% CO₂. Culture medium was refreshed every 2-3 days, and organoids were passaged every 6-7 days.

Inoculation of SA11 and human-derived RV strains and treatment

Caco2 cells cultured in T75 flask were suspended and subsequently seeded into 48-well plate (5 * 10⁴ cells/well) in DMEM complemented with 20% (vol/vol) FCS and 100 IU/mL penicillin-streptomycin. After 2-3 days of culture, culture medium was removed when the cell confluence was about 80%. The cell layers were then washed twice with 500 µL PBS. Serum-free DMEM medium (100 µL) containing 5 µg/mL of trypsin (Gibco, Paisley, UK) and SA11 RV were added and incubated for 60 min at 37 °C with 5% CO₂ to allow efficient infection, followed by four times washing with PBS (500 µL each) to remove free virus particles. Subsequently, culture medium containing 5 µg/mL of trypsin (and indicated treatments) were added to the infected cells and incubated for 24 or 48 hours at 37 °C with 5% CO₂.

For organoid infection, SA11 RV (contain 5000 genome copies) was first activated with 5 µg/mL of trypsin at 37 °C with 5% CO₂ for 30 minutes. Subsequently, organoids were infected with the activated SA11 for 60 minutes at 37 °C with 5% CO₂, followed by four times washing

with PBS to discard the free viruses. Organoids were then aliquoted into 48-well plates that have been coated with 20% (vol/vol) Collagen R Solution (SERVA, Heidelberg, Germany) and maintained in culture medium containing indicated treatments at 37 °C with 5% CO₂.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total, intracellular or extracellular (secreted) RNA was isolated by using the Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified by a Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA). cDNA was made from total RNA using a cDNA Synthesis Kit (Takara Bio Inc, Shiga, Japan) with random hexamer primers. qRT-PCR of RV RNA and genes of interest were performed with a SYBRGreen-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to the manufacturer's instructions with the StepOnePlus System (Thermo Fisher Scientific Life Sciences). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a housekeeping gene to normalize (relative) gene expression using the $2^{-\Delta\Delta CT}$ formula. All primers used in this study are listed in Supplementary Table S2.

Lentiviral vector production and transfection assays

Lentiviral pLKO knockdown vectors (Sigma–Aldrich) targeting STAT1, STAT2 and IRF9 or scrambled control, were obtained from the Erasmus Center of Biomics. All shRNA sequences are listed in Supplementary Table S3. The lentiviral vectors were produced in human embryonic kidney epithelial cell line HEK 293T cells as previously described⁵⁴. The shRNA vectors exerting optimal gene knockdown were selected. To generate the gene knockdown cells, Caco2 cells were transduced with the lentiviral vectors and subsequently selected by adding puromycin (8 µg/mL; Sigma) to the culture medium. Knockdown and control Caco2 cells were infected with RV as previously described.

IFN production bioassay

The IFN production bioassay was performed to detect secreted IFN proteins in the culture medium as described previously⁵⁵. Briefly, the culture (conditioned) medium derived from control and SA11-infected Caco2 cells (48 hours) were collected and filtered through 0.45 µm pore size membrane. Two luciferase reporter models which are extremely sensitive to

IFN treatments were employed. Huh7.5-ET-Luc luciferase model is a hepatitis C virus (HCV) replicon (I389/NS3-3V/LucUbiNeo-ET) in which the HCV-related firefly luciferase activity (HCV-luc) can be potently inhibited by a low concentration of IFN- α treatments. Huh7-ISRE-luc is a luciferase reporter model in which the firefly luciferase gene was driven by multiple IFN-stimulated response elements (ISRE) promoter. In this model, the firefly luciferase activity can be potently stimulated by a low concentration of IFN- α treatment. Therefore, these two luciferase models can be employed to sensitively detect the presence of IFN proteins in the conditioned medium. Huh7 HCV-luc and ISRE-luc cells were cultured in DMEM supplemented with 10% FCS (vol/vol), 100 U/mL penicillin and streptomycin. For Huh7 HCV-luc, 250 μ g/mL G418 (Sigma-Aldrich) was added to the culture medium.

Western blot assay

Cultured cells were lysed in Laemmli sample buffer containing 0.1 M dithiothreitol (DTT) and heated for 5 min at 95°C. Cell lysates were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 100 min running at 110 V. The proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon-FL) for 1.5 hours with an electric current of 250 mA. Subsequently, the membrane was blocked with a mixture of 2.5 ml of blocking buffer (Odyssey) and 2.5 ml of PBS containing 0.05% Tween 20 for 1 hour at room temperature. This was followed by an overnight incubation with the indicated primary antibody (1:1000 dilution) at 4°C. The membrane was then washed three times, followed by incubation for 1 hour with IRDye-conjugated secondary antibody (1:5000 dilution) at room temperature. After washing three times, the protein bands were detected with the Odyssey 3.0 Infrared Imaging System. The intensity of the immunoreactive bands of blotted proteins was quantified by the Odyssey V3.0 software.

Immunofluorescence microscope assay

Caco2 cells were seeded on glass coverslips. After SA11 infection for 48 hours, cells were washed with PBS, fixed in 4% PBS-buffered formalin for 10 mins and blocked with tween-milk-glycine medium (PBS, 0.05% tween, 5g/L skim milk and 1.5g/L glycine). Samples were incubated with anti-rotavirus (ab181695) antibody (Abcam) overnight at 4 °C. Subsequently, samples were incubated with 1:1000 dilutions of Alexa FluorTM 594 goat anti-mouse

secondary antibodies (Invitrogen). Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Invitrogen). Images were detected using immunofluorescence microscope.

MTT assay

10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to Caco2 cells seeded in 96-well plates at indicated time points. The cells were incubated at 37 °C with 5% CO₂ for 3 hours. The culture medium was then removed and 100 µl of dimethyl sulfoxide (DMSO) was added to each well. The absorbance of each well was read on the microplate absorbance readers (BIO-RAD) at wavelength of 490 nm.

Study Approval

Human intestinal tissue were obtained during surgical resection. A written informed consent was signed by the volunteers or patients who agreed to participate. The study was approved by the Medical Ethical Committee of the Erasmus Medical Center (Medisch Ethische Toetsings Commissie Erasmus MC), and all experiments were performed in accordance with relevant guidelines and regulations.

Statistical Analysis

Statistical analysis was performed using the nonpaired, nonparametric test (Mann-Whitney test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). *P* values <0.05 were considered statistically significant.

Acknowledgements

This research is supported by the Dutch Digestive Foundation for a career development grant (No. CDG 1304 to Q. P.). It is also supported by the Indonesia Endowment Fund for Education (LPDP) for funding PhD fellowship to Mohamad S. Hakim and the China Scholarship Council for funding PhD fellowship to S. Chen (201606760056), Y. Yin (201307720045) and W. Wang (201303250056).

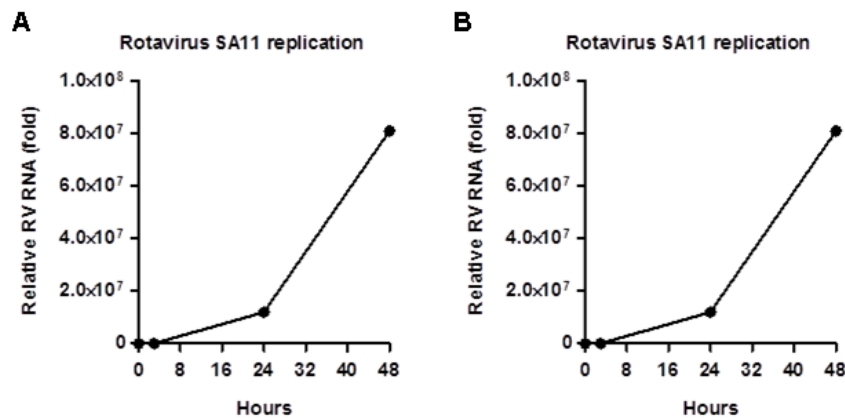
References

- 1 Greenberg, H. B. & Estes, M. K. Rotaviruses: from pathogenesis to vaccination. *Gastroenterology* **136**, 1939-1951, doi:10.1053/j.gastro.2009.02.076 (2009).
- 2 Lanata, C. F. *et al.* Global causes of diarrheal disease mortality in children <5 years of age: a systematic review. *PLoS One* **8**, e72788, doi:10.1371/journal.pone.0072788 (2013).
- 3 Walker, C. L. *et al.* Global burden of childhood pneumonia and diarrhoea. *Lancet* **381**, 1405-1416, doi:10.1016/S0140-6736(13)60222-6 (2013).
- 4 Desai, R. *et al.* Reduction in morbidity and mortality from childhood diarrhoeal disease after species A rotavirus vaccine introduction in Latin America - a review. *Mem Inst Oswaldo Cruz* **106**, 907-911, doi:S0074-02762011000800002 (2011).
- 5 Holloway, G. & Coulson, B. S. Innate cellular responses to rotavirus infection. *J Gen Virol* **94**, 1151-1160, doi:10.1099/vir.0.051276-0 (2013).
- 6 Deal, E. M., Jaimes, M. C., Crawford, S. E., Estes, M. K. & Greenberg, H. B. Rotavirus structural proteins and dsRNA are required for the human primary plasmacytoid dendritic cell IFN α response. *PLoS Pathog* **6**, e1000931, doi:10.1371/journal.ppat.1000931 (2010).
- 7 Randall, R. E. & Goodbourn, S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol* **89**, 1-47, doi:10.1099/vir.0.83391-0 (2008).
- 8 Donnelly, R. P. & Kotenko, S. V. Interferon-lambda: a new addition to an old family. *J Interferon Cytokine Res* **30**, 555-564, doi:10.1089/jir.2010.0078 (2010).
- 9 Plataniias, L. C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* **5**, 375-386, doi:10.1038/nri1604 (2005).
- 10 Schneider, W. M., Chevillotte, M. D. & Rice, C. M. Interferon-stimulated genes: a complex web of host defenses. *Annu Rev Immunol* **32**, 513-545, doi:10.1146/annurev-immunol-032713-120231 (2014).
- 11 Deal, E. M., Lahl, K., Narvaez, C. F., Butcher, E. C. & Greenberg, H. B. Plasmacytoid dendritic cells promote rotavirus-induced human and murine B cell responses. *J Clin Invest* **123**, 2464-2474, doi:10.1172/JCI60945 (2013).
- 12 Arnold, M. M., Sen, A., Greenberg, H. B. & Patton, J. T. The battle between rotavirus and its host for control of the interferon signaling pathway. *PLoS Pathog* **9**, e1003064, doi:10.1371/journal.ppat.1003064 (2013).
- 13 Graff, J. W., Ettayebi, K. & Hardy, M. E. Rotavirus NSP1 inhibits NF κ B activation by inducing proteasome-dependent degradation of beta-TrCP: a novel mechanism of IFN antagonism. *PLoS Pathog* **5**, e1000280, doi:10.1371/journal.ppat.1000280 (2009).
- 14 Holloway, G., Truong, T. T. & Coulson, B. S. Rotavirus antagonizes cellular antiviral responses by inhibiting the nuclear accumulation of STAT1, STAT2, and NF-kappaB. *J Virol* **83**, 4942-4951, doi:10.1128/JVI.01450-08 (2009).
- 15 Feng, N. *et al.* Variation in antagonism of the interferon response to rotavirus NSP1 results in differential infectivity in mouse embryonic fibroblasts. *J Virol* **83**, 6987-6994, doi:10.1128/JVI.00585-09 (2009).
- 16 De Boissieu, D., Lebon, P., Badoual, J., Bompard, Y. & Dupont, C. Rotavirus induces alpha-interferon release in children with gastroenteritis. *J Pediatr Gastroenterol Nutr* **16**, 29-32 (1993).
- 17 Jiang, B. *et al.* Cytokines as mediators for or effectors against rotavirus disease in children. *Clin Diagn Lab Immunol* **10**, 995-1001 (2003).
- 18 Azim, T. *et al.* Rotavirus-specific subclass antibody and cytokine responses in Bangladeshi children with rotavirus diarrhoea. *J Med Virol* **69**, 286-295, doi:10.1002/jmv.10280 (2003).
- 19 Dagenais, L., Pastoret, P. P., Van den Broecke, C. & Werenne, J. Susceptibility of bovine rotavirus to interferon. Brief report. *Arch Virol* **70**, 377-379 (1981).
- 20 Bass, D. M. Interferon gamma and interleukin 1, but not interferon alfa, inhibit rotavirus entry into human intestinal cell lines. *Gastroenterology* **113**, 81-89, doi:S0016508597003144 (1997).

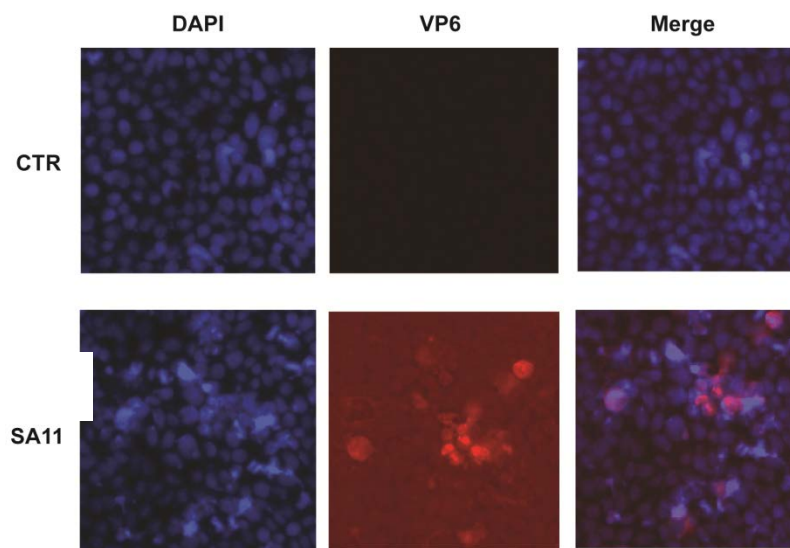
- 21 McNeal, M. M. *et al.* IFN-gamma is the only anti-rotavirus cytokine found after in vitro stimulation of memory CD4+ T cells from mice immunized with a chimeric VP6 protein. *Viral Immunol* **20**, 571-584, doi:10.1089/vim.2007.0055 (2007).
- 22 Schwers, A. *et al.* Experimental rotavirus diarrhoea in colostrum-deprived newborn calves: assay of treatment by administration of bacterially produced human interferon (Hu-IFN alpha 2). *Ann Rech Vet* **16**, 213-218 (1985).
- 23 Lecce, J. G., Cummins, J. M. & Richards, A. B. Treatment of rotavirus infection in neonate and weanling pigs using natural human interferon alpha. *Mol Biother* **2**, 211-216 (1990).
- 24 Angel, J., Franco, M. A., Greenberg, H. B. & Bass, D. Lack of a role for type I and type II interferons in the resolution of rotavirus-induced diarrhea and infection in mice. *J Interferon Cytokine Res* **19**, 655-659, doi:10.1089/107999099313802 (1999).
- 25 Pott, J. *et al.* IFN-lambda determines the intestinal epithelial antiviral host defense. *Proc Natl Acad Sci U S A* **108**, 7944-7949, doi:10.1073/pnas.1100552108 (2011).
- 26 Hernandez, P. P. *et al.* Interferon-lambda and interleukin 22 act synergistically for the induction of interferon-stimulated genes and control of rotavirus infection. *Nat Immunol* **16**, 698-707, doi:10.1038/ni.3180 (2015).
- 27 Wang, W. *et al.* Unphosphorylated ISGF3 drives constitutive expression of interferon-stimulated genes to protect against viral infections. *Sci Signal* **10**, doi:10.1126/scisignal.aah4248 (2017).
- 28 Schoggins, J. W. & Rice, C. M. Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol* **1**, 519-525, doi:10.1016/j.coviro.2011.10.008 (2011).
- 29 Frias, A. H. *et al.* Intestinal epithelia activate anti-viral signaling via intracellular sensing of rotavirus structural components. *Mucosal Immunol* **3**, 622-632, doi:10.1038/mi.2010.39 (2010).
- 30 Frias, A. H., Jones, R. M., Fifadara, N. H., Vijay-Kumar, M. & Gewirtz, A. T. Rotavirus-induced IFN-beta promotes anti-viral signaling and apoptosis that modulate viral replication in intestinal epithelial cells. *Innate Immun* **18**, 294-306, doi:10.1177/1753425911401930 (2012).
- 31 Cuadras, M. A., Feigelstock, D. A., An, S. & Greenberg, H. B. Gene expression pattern in Caco-2 cells following rotavirus infection. *J Virol* **76**, 4467-4482 (2002).
- 32 Dutta, D. & Clevers, H. Organoid culture systems to study host-pathogen interactions. *Curr Opin Immunol* **48**, 15-22, doi:10.1016/j.coi.2017.07.012 (2017).
- 33 Yin, Y. *et al.* Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. *Antiviral Res* **123**, 120-131, doi:10.1016/j.antiviral.2015.09.010 (2015).
- 34 Saxena, K. *et al.* A paradox of transcriptional and functional innate interferon responses of human intestinal enteroids to enteric virus infection. *Proc Natl Acad Sci U S A* **114**, E570-E579, doi:10.1073/pnas.1615422114 (2017).
- 35 Saxena, K. *et al.* Human Intestinal Enteroids: a New Model To Study Human Rotavirus Infection, Host Restriction, and Pathophysiology. *J Virol* **90**, 43-56, doi:10.1128/JVI.01930-15 (2015).
- 36 Hirata, Y., Broquet, A. H., Menchen, L. & Kagnoff, M. F. Activation of innate immune defense mechanisms by signaling through RIG-I/IPS-1 in intestinal epithelial cells. *J Immunol* **179**, 5425-5432, doi:10.179/8/5425 (2007).
- 37 Sen, A. *et al.* Innate immune response to homologous rotavirus infection in the small intestinal villous epithelium at single-cell resolution. *Proc Natl Acad Sci U S A* **109**, 20667-20672, doi:10.1073/pnas.1212188109 (2012).
- 38 Park, H. *et al.* IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection. *Hepatology* **56**, 2060-2070, doi:10.1002/hep.25897 (2012).
- 39 Yin, X. *et al.* Hepatitis E virus persists in the presence of a type III interferon response. *PLoS Pathog* **13**, e1006417, doi:10.1371/journal.ppat.1006417 (2017).
- 40 Crotta, S. *et al.* Type I and type III interferons drive redundant amplification loops to induce a transcriptional signature in influenza-infected airway epithelia. *PLoS Pathog* **9**, e1003773, doi:10.1371/journal.ppat.1003773 (2013).
- 41 Barro, M. & Patton, J. T. Rotavirus nonstructural protein 1 subverts innate immune response by inducing degradation of IFN regulatory factor 3. *Proc Natl Acad Sci U S A* **102**, 4114-4119, doi:10.1073/pnas.0408376102 (2005).

- 42 Vancott, J. L., McNeal, M. M., Choi, A. H. & Ward, R. L. The role of interferons in rotavirus infections and protection. *J Interferon Cytokine Res* **23**, 163-170, doi:10.1089/107999003321532501 (2003).
- 43 Karst, S. M., Wobus, C. E., Lay, M., Davidson, J. & Virgin, H. W. t. STAT1-dependent innate immunity to a Norwalk-like virus. *Science* **299**, 1575-1578, doi:10.1126/science.1077905 (2003).
- 44 Perry, S. T., Buck, M. D., Lada, S. M., Schindler, C. & Shresta, S. STAT2 mediates innate immunity to Dengue virus in the absence of STAT1 via the type I interferon receptor. *PLoS Pathog* **7**, e1001297, doi:10.1371/journal.ppat.1001297 (2011).
- 45 Lin, J. D. *et al.* Distinct Roles of Type I and Type III Interferons in Intestinal Immunity to Homologous and Heterologous Rotavirus Infections. *PLoS Pathog* **12**, e1005600, doi:10.1371/journal.ppat.1005600 (2016).
- 46 Yasukawa, M., Nakagomi, O. & Kobayashi, Y. Rotavirus induces proliferative response and augments non-specific cytotoxic activity of lymphocytes in humans. *Clin Exp Immunol* **80**, 49-55 (1990).
- 47 Xu, J. *et al.* Expression of Toll-like receptors and their association with cytokine responses in peripheral blood mononuclear cells of children with acute rotavirus diarrhoea. *Clin Exp Immunol* **144**, 376-381, doi:10.1111/j.1365-2249.2006.03079.x (2006).
- 48 Jaimes, M. C. *et al.* Frequencies of virus-specific CD4(+) and CD8(+) T lymphocytes secreting gamma interferon after acute natural rotavirus infection in children and adults. *J Virol* **76**, 4741-4749 (2002).
- 49 Rojas, O. L. *et al.* Human rotavirus specific T cells: quantification by ELISPOT and expression of homing receptors on CD4+ T cells. *Virology* **314**, 671-679, doi:S0042682203005075 (2003).
- 50 Yuan, L. *et al.* Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are correlated with protection against rotavirus diarrhea in gnotobiotic pigs. *Vaccine* **26**, 3322-3331, doi:10.1016/j.vaccine.2008.03.085 (2008).
- 51 Smiley, K. L. *et al.* Association of gamma interferon and interleukin-17 production in intestinal CD4+ T cells with protection against rotavirus shedding in mice intranasally immunized with VP6 and the adjuvant LT(R192G). *J Virol* **81**, 3740-3748, doi:10.1128/JVI.01877-06 (2007).
- 52 Mormile, R. Severe gastroenteritis and acute pancreatitis following rotavirus infection in children: The age-related failure of IFN-gamma? *Immunol Lett* **175**, 58-59, doi:10.1016/j.imlet.2016.04.017 (2016).
- 53 Knipping, K., Garssen, J. & van't Land, B. An evaluation of the inhibitory effects against rotavirus infection of edible plant extracts. *Virol J* **9**, 137, doi:10.1186/1743-422X-9-137 (2012).
- 54 Pan, Q. *et al.* Combined antiviral activity of interferon-alpha and RNA interference directed against hepatitis C without affecting vector delivery and gene silencing. *J Mol Med (Berl)* **87**, 713-722, doi:10.1007/s00109-009-0470-3 (2009).
- 55 Xu, L. *et al.* IFN regulatory factor 1 restricts hepatitis E virus replication by activating STAT1 to induce antiviral IFN-stimulated genes. *FASEB J* **30**, 3352-3367, doi:10.1096/fj.201600356R (2016).

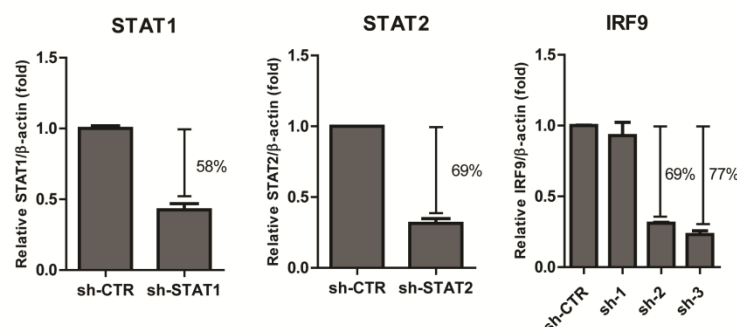
Supplementary Information



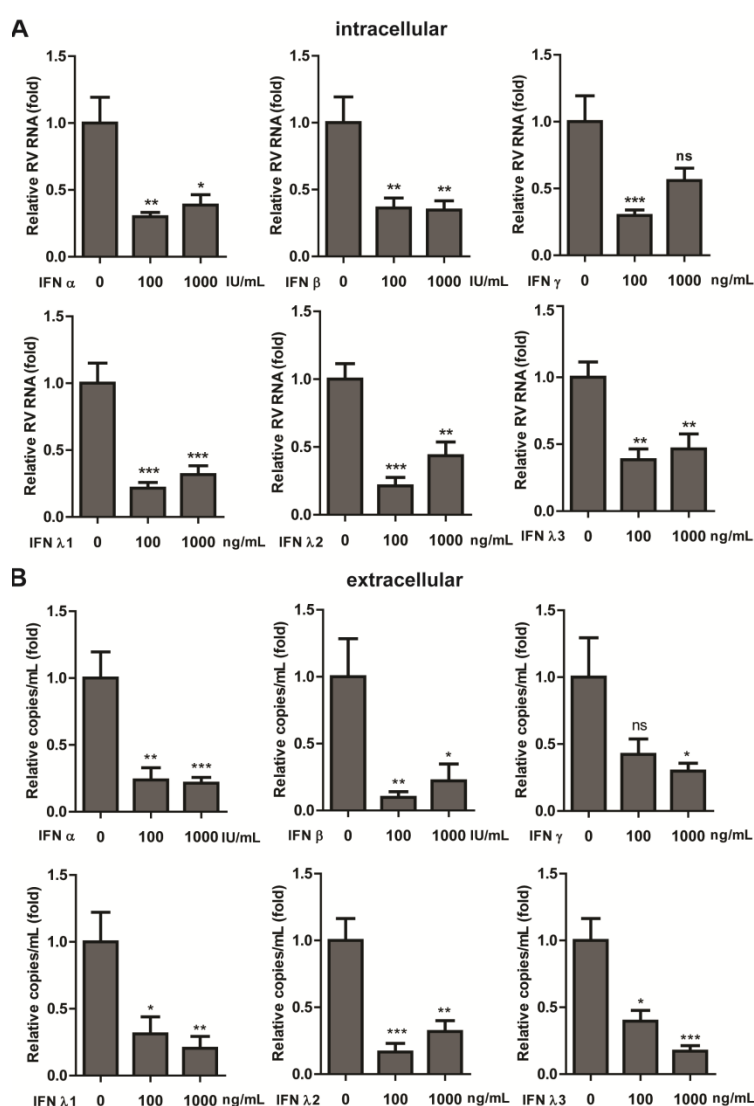
Supplementary Figure S1. Efficient replication of rotavirus SA11 in Caco2 cells as demonstrated by qRT-PCR. Intracellular (A) and secreted (extracellular) levels (B) of rotavirus SA11 were detected and quantified by qRT-PCR at 0 (before virus inoculation) as well as at 1, 24 and 48 hours after inoculation. For intracellular RNA quantification (A), RV RNA levels were shown as fold increases relative to 1 hour post infection. GAPDH was used as the housekeeping gene.



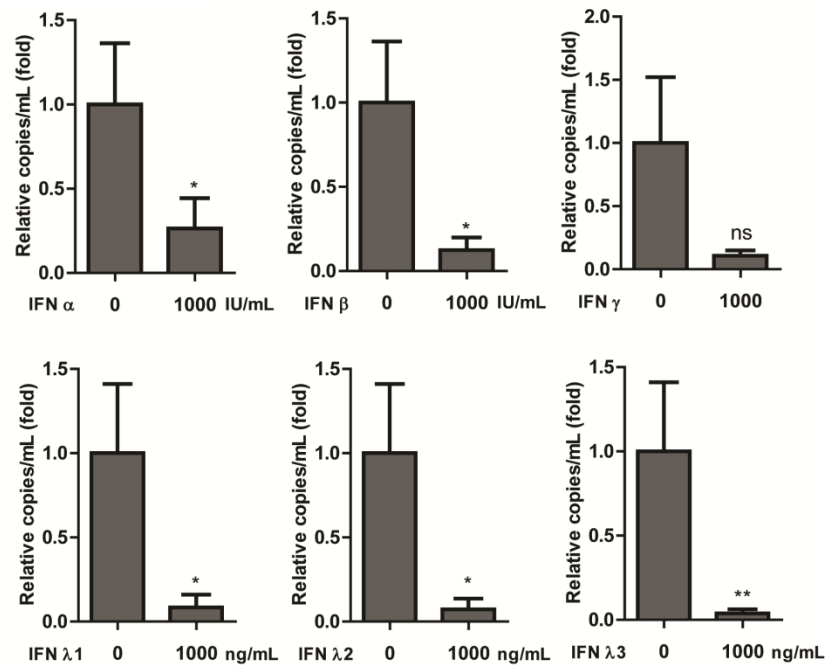
Supplementary Figure S2. Efficient replication of rotavirus SA11 in Caco2 cells as demonstrated by immunofluorescence stainings. Representative immunofluorescence stainings of ab181695 (targeting VP6 rotavirus protein) (red) after 48 hours infection of Caco2 cells with rotavirus SA11. Nuclei were visualized by DAPI (blue).



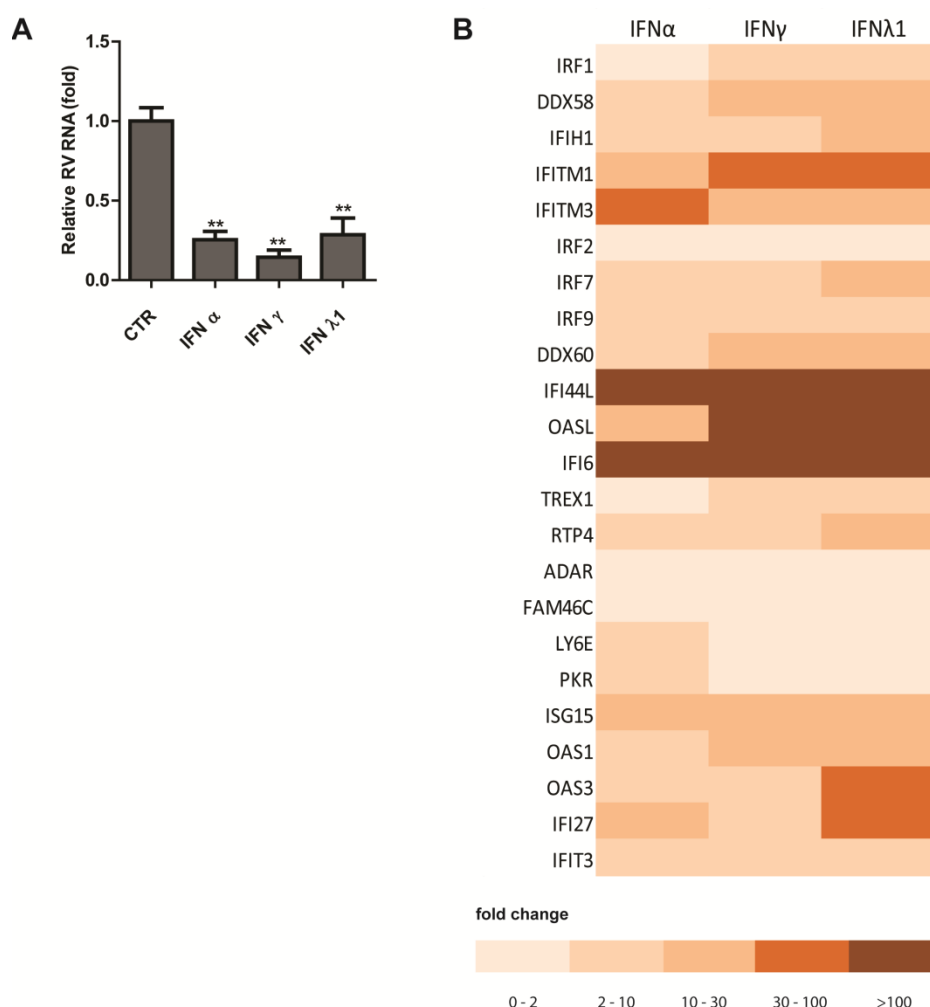
Supplementary Figure S3. Western blot assays showed a successful knockdown of STAT1 (58% reduction), STAT2 (69% reduction) and IRF9 proteins (69% and 77% reduction). β -actin served as an internal reference. (means \pm SEM, $n = 3$).



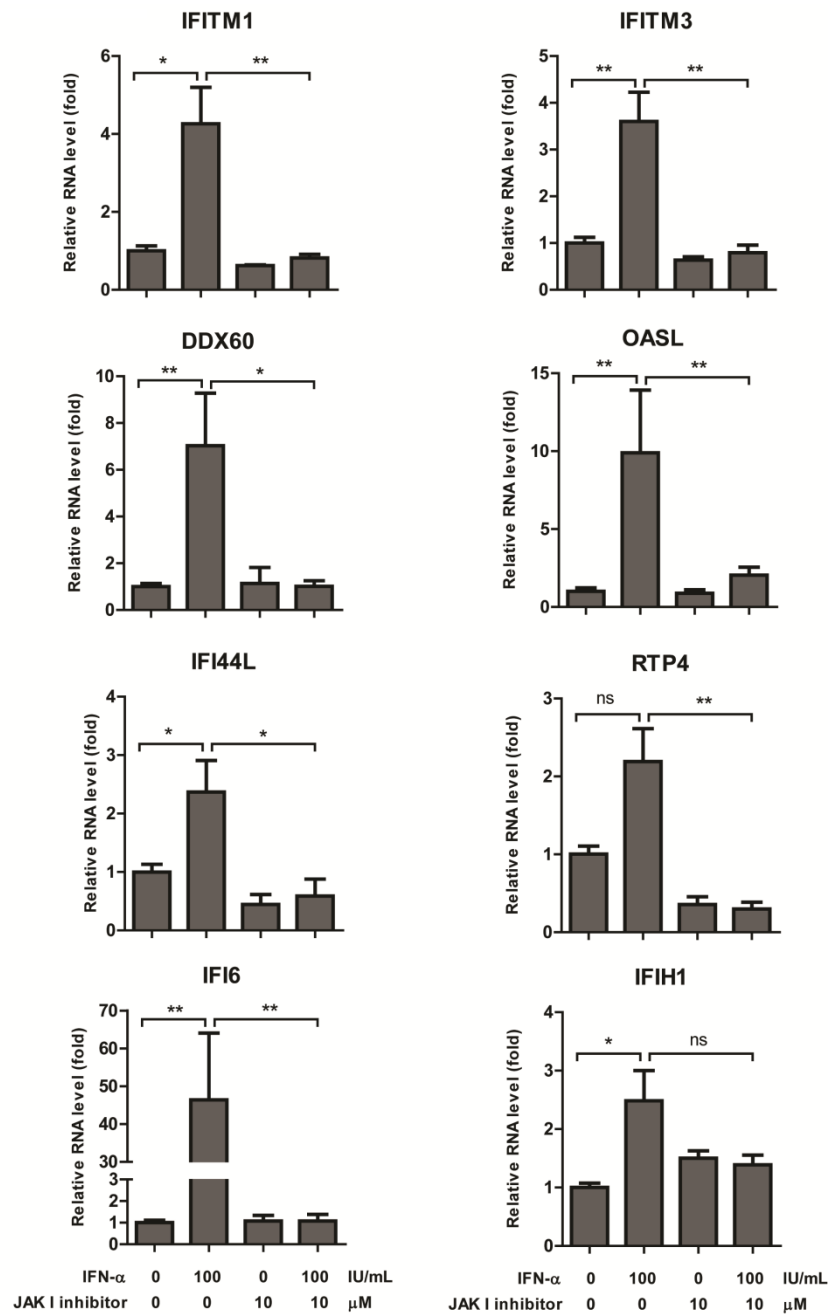
Supplementary Figure S4. The effects of exogenous treatment of type I, II and III IFNs on RV SA11 replication in Caco2 cells. Antiviral activities of IFN α , IFN β , IFN γ , IFN $\lambda 1$, IFN $\lambda 2$ and IFN $\lambda 3$ treatments against RV SA11 infection on Caco2 cells were determined by quantifying intracellular (A) and extracellular (secreted) (B) RNA levels at 48 hours post-infection. ($n = 2-3$ independent experiment with each of 3 replicates) Data were presented as means \pm SEM., * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns. not



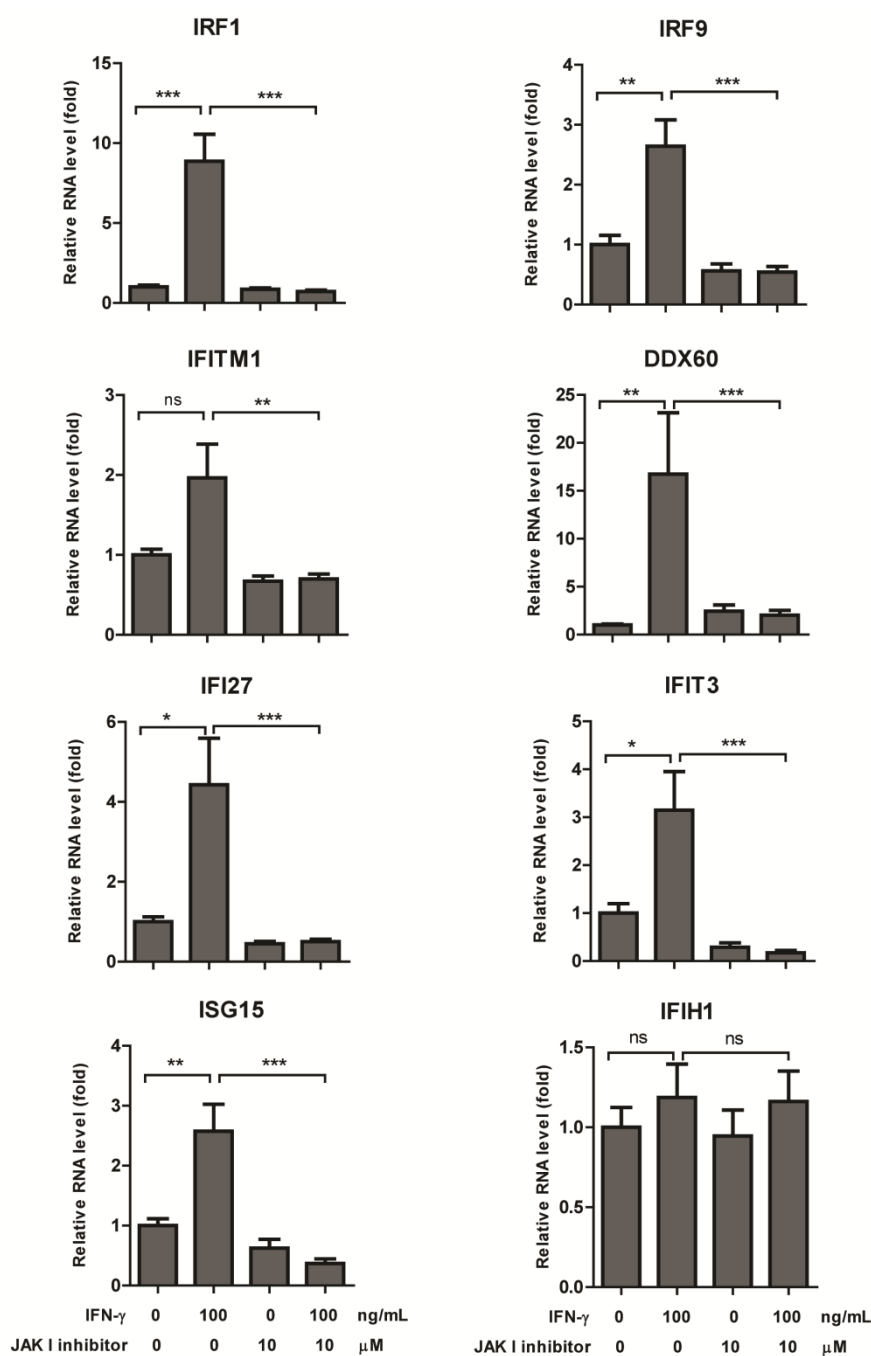
Supplementary Figure S5. The effects of type I, II and III IFNs on the extracellular level of RV SA11 in human organoids. Antiviral effects of IFN α , IFN β , IFN γ , IFN λ 1, IFN λ 2 or IFN λ 3 treatment against RV SA11 infection in organoids were determined by quantifying extracellular (secreted) RNA levels at 48 hours post-infection. The organoids were derived from one individual (P1). Data were presented as means \pm SEM., * P < 0.05; ** P < 0.01; ns, not significant. (n = 3 independent experiments with each of 2-3 replicates).



Supplementary Figure S6. (A) Antiviral effects of representative type I (IFN α , 1000 IU/mL), type II (IFN γ , 1000 ng/mL), and type III (IFN $\lambda 1$, 1000 ng/mL) treatment against RV SA11 infection in organoids were determined by quantifying total RV RNA levels at 48 hours post-infection. The organoid was derived from individual 2 (P2). Data were presented as means \pm SEM, ** $P < 0.01$. (n = 3 independent experiments with each of 1-2 replicates). **(B)** Organoids (P2) were stimulated with IFN α 1000 IU/mL, IFN γ 1000 ng/mL and IFN $\lambda 1$ 1000 ng/mL for 24 hours. The expression levels of several ISGs were measured by qRT-PCR.



Supplementary Figure S7. JAK I inhibitor blocks IFN α -induced ISGs in Caco2 cells. JAK I inhibitor (10 μ M) abrogated IFN α - (100 IU/mL) induced ISG expression as measured by qRT-PCR. Data were presented as means \pm SEM., * P < 0.05; ** P < 0.01; ns, not significant. (n = 3 independent experiments with each of 2-3 replicates)



Supplementary Figure S8. JAK I inhibitor abolishes IFN γ -induced expression of ISGs in Caco2 cells. JAK I inhibitor (10 μ M) abrogated IFN γ - (100 ng/mL) induced ISG expression as measured by qRT-PCR. Data were presented as means \pm SEM., * P < 0.05; ** P < 0.01; *** P < 0.001; ns, not significant. (n = 3 independent experiments with each of 2-3 replicates)

Supplementary Table S1. Patient characteristics.

Patient	Age (years)	Gender	Clinical symptoms	Virus Detection							
				Enterovirus	Parechovirus	Norovirus genogroups I	Norovirus genogroups II	Adenovirus	Astrovirus	Sapovirus	Rotavirus
1	74	Female	Congestive heart failure, myocarditis	No	No	No	No	No	No	No	Yes
2	27	Female	Fever, diarrhea, nausea, vomiting	No	No	No	No	No	No	No	Yes
3	67	Male	Fever, stomach ache, watery diarrhea (Kidney transplant)	No	No	No	No	No	No	No	Yes
4	1.5	Female	Vomiting, watery diarrhea	No	No	No	No	No	No	Yes	Yes

Supplementary Table S2. Primers used in this study.

Target Genes	Sequences (5' - 3')
Viruses	
RV SA11-F	TGGTTAAACGCAGGATCGGA
RV SA11-R	AACCTTTCCGCGTCTGGTAG
Human RV-F	ACCATCTACACATGACCCTC
Human RV-R	CACATAACGCCCCTATAGCC
Human Genes	
IRF1-F	GAGGAGGTGAAAGACCAGAGCA
IRF1-R	TAGCATCTCGGCTGGACTTCGA
DDX58-F	CACCTCAGTTGCTGATGAAGGC
DDX58-R	GTCAGAAGGAAGCACTTGCTACC
IFIH1(MDA5)-F	GCTGAAGTAGGAGTCAAAGCCC
IFIH1(MDA5)-R	CCACTGTGGTAGCGATAAGCAG
IFITM1-F	GGCTTCATAGCATTGCGCTACTC
IFITM1-R	AGATGTTTCAGGCACTTGCGGT
IFITM3-F	CTGGGCTTCATAGCATTGCGCT
IFITM3-R	AGATGTTTCAGGCACTTGCGGT
IRF2-F	TAGAGGTGACCACTGAGAGCGA
IRF2-R	CTCTTCATCGCTGGGCACACTA
IRF7-F	CCACGCTATACCATCTACCTGG
IRF7-R	GCTGCTATCCAGGGAAGACACA
IRF9-F	CCACCGAAGTTCAGGTAACAC
IRF9-R	AGTCTGCTCCAGCAAGTATCGG
DDX60-F	GGTGTTTTACCAGGGAGTATCG
DDX60-R	CCAGTTTTGGCGATGAGGAGCA
IFI44L-F	TGCACTGAGGCAGATGCTGCG
IFI44L-R	TCATTGCGGCACACCACTACAG
OASL-F	GTGCCTGAAACAGGACTGTTGC
OASL-R	CCTCTGCTCCACTGTCAAGTGG
IFI6-F	TGATGAGCTGGTCTGCGATCCT
IFI6-R	GTAGCCCATCAGGGCACCAATA
TREX-F	GCATCTGTCACTGGAGACCACA
TREX-R	CAGTGGTTGTGACAGCAGATGG
RTP4-F	GACGCTGAAGTTGGATGGCAAC
RTP4-R	GTGGCACAGAATCTGCACTTGG
ADAR-F	TCCGTCTCCTGTCCAAAGAAGG
ADAR-R	TTCTTGCTGGGAGCACTCACAC
FAM46C-F	CCTTGAACAGCAGAGGAAGTTGG
FAM46C-R	GGAGATGAGGTTGAGAGTCTGC
LY6E-F	GACCAGGACAACACTGCGTGA
LY6E-R	AAGCCACACCAACATTGACGCC
PKR-F	GAAGTGGACCTCTACGCTTTGG
PKR-R	TGATGCCATCCCGTAGGTCTGT
ISG15-F	CTCTGAGCATCCTGGTGAGGAA
ISG15-R	AAGGTCAGCCAGAACAGGTCGT
OAS1-F	AGGAAAGGTGCTTCCGAGGTAG

OAS1-R	GGACTGAGGAAGACAACCAGGT
OAS3-F	CCTGATTCTGCTGGTGAAGCAC
OAS3-R	TCCCAGGCAAAGATGGTGAGGA
IFI27-F	CGTCCTCCATAGCAGCCAAGAT
IFI27-R	ACCCAATGGAGCCCAGGATGAA
IFIT3-F	CCTGGAATGCTTACGGCAAGCT
IFIT3-R	GAGCATCTGAGAGTCTGCCAA
IFNα1-F	CGCCTGTGATCCAGGATTATCC
IFNα1-R	TGGTGTGTGCTCTGGCTTTTAC
IFNλ1-F	CAGCAAGTTCTCTAAGCCCACC
IFNλ1-R	GTCATTACGGACTCTGGTCTG
IFNα-F	GACTCCATCTTGGCTGTGA
IFNα-R	TGATTTCTGCTCTGACAACCT
IFNβ1-F	CTTGGATTCTACAAAGAAGCAGC
IFNβ1-R	TCCTCCTTCTGGAAGTCTGCA
IFN-γ-F	GAGTGTGGAGACCATCAAGGAAG
IFN-γ-R	TGCTTTGCGTTGGACATTCAAGTC
IFNλ1 (IL-29)-F	GGAAGACAGGAGAGCTGCAACT
IFNλ1 (IL-29)-R	AACTGGGAAGGGCTGCCACATT
IFNλ2/3 (IL-28)-F	TCGCTTCTGCTGAAGGACTGCA
IFNλ2/3 (IL-28)-R	CCTCCAGAACCTTCAGCGTCAG
GAPDH-F	TGTCCCCACCCCAATGTATC
GAPDH-R	CTCCGATGCCTGCTTCACTACCT

Supplementary Table S3. Lentiviral sh-RNA sequences used in this study.

Name	Oligo Sequences (5' - 3')
shSTAT1	CCGGCGACAGTATGATGAACACAGTCTCGAGACTGTGTTTCATCATACTGTCGTTTTT
shSTAT2	CCGGGCTGAGCCATAGGTCTAAATACTCGAGTATTTAGACCTATGGCTCAGCTTTTT
shIRF9-1	CCGGTTCAAGGCCTGGGCAATATTTCTCGAGAAATATTGCCAGGCCTGAATTTTTG
shIRF9-2	CCGGGAGACTTGGTCAGGTACTTTCTCGAGGAAAGTACCTGACCAAGTCTCTTTTTG
shIRF9-3	CCGGCTCAGTAGTTGTCCGTGATAACTCGAGTTATCACGGACAACCTACTGAGTTTTTG

Chapter 7

TNF- α Exerts Potent Anti-rotavirus Effects via the Activation of Classical NF- κ B Pathway

Mohamad S. Hakim^{1,2}, Shihao Ding¹, Sunrui Chen¹, Yuebang Yin¹, Junhong Su^{1,3}, C. Janneke van der Woude¹, Gwenny M. Fuhler¹, Maikel P. Peppelenbosch¹, Qiuwei Pan¹, Wenshi Wang¹

- 1 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, the Netherlands
- 2 Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
- 3 Medical Faculty, Kunming University of Science and Technology, Kunming, PR China.

Virus Research 2018; 253: 28-37.

Abstract

Active virus-host interactions determine the outcome of pathogen invasions. It has been shown that in isolated dendritic cells (DCs), rotavirus can induce the expression of tumor necrosis factor α (TNF- α), a vital cytokine mediating host immune responses. However, the role of TNF- α in rotavirus infection is unknown. In this study, we demonstrated that TNF- α has potent anti-rotavirus effects, independent of type I interferon production. Blocking of TNF- α by infliximab, a clinically available TNF α antibody, totally abrogated this effect. Mechanistic studies revealed that the anti-rotavirus effect of TNF- α was achieved by NF κ B-regulated genes via the activation of classical nuclear factor κ B (NF- κ B) signaling. Our study reveals the pivotal role and the mechanism-of-actions of TNF- α in the host defense against rotavirus. Thus, this knowledge may contribute to the better understanding of the complexity of rotavirus-host interactions.

Keywords: antiviral; cytokines; NF- κ B signaling; rotavirus; TNF- α

1. Introduction

Rotavirus is a viral pathogen that causes severe gastroenteritis worldwide, especially in children under five years of age (Tate et al., 2012). It is also prevalent in immunocompromised patients, such as pediatric and adult organ transplant recipients (Yin et al., 2015b). Notably, gastrointestinal infections including rotavirus have been recognized as potential cause of exacerbation and induction of flares in inflammatory bowel disease (IBD) patients (Masclee et al., 2013). Rotavirus primarily infects mature enterocytes at the tips of the intestinal villus, leading to acute diarrhea (Greenberg and Estes, 2009). However, systemic infections can occur which involve multiple extra-intestinal organs, including liver, kidney and the central nervous system (Chiappini et al., 2005).

The innate and adaptive immune systems play an essential role in the limitation of rotavirus infection in infected hosts (Angel et al., 2012; Holloway and Coulson, 2013). Rotavirus can induce the production of interferons (IFNs) and cytokines, including interferon α (IFN- α), IFN- β , tumor necrosis factor α (TNF- α), interleukin 6 (IL-6) and also IL-8 in dendritic cells (DCs) (Deal et al., 2010; Mesa et al., 2007; Rosales-Martinez et al., 2016). Previous studies have demonstrated a role for the different types of IFNs in constraining rotavirus infection (Hernandez et al., 2015; Lin et al., 2016; Saxena et al., 2017). In addition, we have previously shown that rotavirus modulates the expression of interferon-stimulated genes (ISGs) that cooperatively mediate an anti-viral state in the infected cells (Yin et al., 2015a). On the other hand, rotavirus efficiently develops strategies to counteract these anti-viral responses (Arnold et al., 2013; Ding et al., 2016), indicating an active and dynamic virus-host interplay following infection.

TNF- α was first described as a serum factor that mediates killing of tumors *in vitro*, from which it derives its name (Carswell et al., 1975). Further studies discovered that TNF- α is a potent and essential mediator of inflammatory responses. Aberrant regulations of TNF- α have been associated with many immune-mediated inflammatory diseases, such as rheumatoid arthritis (RA) and IBD. This has led to the development of therapeutic agents targeting TNF- α that are now successfully used in the clinic (Kalliolias and Ivashkiv, 2016; Sedger and McDermott, 2014). However, these TNF- α antagonists are well known to increase the risk of severe viral (including rotavirus) and bacterial infections, thus limiting

their use in groups of patients (Kim and Solomon, 2010; Sedger and McDermott, 2014). This phenomenon highlights the fact that TNF- α has either direct or indirect effects against bacterial and viral infections. Indeed, several studies have demonstrated the anti-viral and anti-bacterial properties of TNF- α against influenza virus (Seo and Webster, 2002), hepatitis C (HCV) and E (HEV) viruses (Wang et al., 2016), poxviruses (Bartee et al., 2009), and *Mycobacterium tuberculosis* (Flynn et al., 1995), either alone or in combination with IFNs.

However, the potential activity of TNF- α on rotavirus has not yet been described. Here we report that TNF- α has potent anti-viral effects against rotavirus. However, its anti-rotavirus effect is totally independent of interferon production and the IFN signaling pathway. Importantly, these effects are achieved by the induction of NF κ B-regulated genes through the activation of classical nuclear factor κ B (NF- κ B) signaling. This study therefore strengthens the role of TNF- α production as a host immune response in defending against viral infections.

2. Materials and Methods

2.1. Reagents

Recombinant human TNF- α (Peprotech, USA) was dissolved in phosphate-buffered saline (PBS) to a final concentration of 100 μ g/mL. Human recombinant IL32A (GeneTex), IL8 (Abnova), CXCL10 (BioLegend), CXCL11 (BioLegend), CCL20 (BioLegend) and CCL2 (BioLegend) was dissolved to a final concentration of 10 μ g/mL. Stock of JAK I inhibitor (Santa Cruz Biotech, CA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA) with a final concentration of 5 mM. RelA (p65) antibody (C22B4, #4764) was purchased from Cell Signaling Technology (Leiden, The Netherlands). β -actin and TNFR1 antibody (sc-8436) were purchased from Santa Cruz Biotechnology. β -tubulin antibody (ab6046) was purchased from Abcam. Anti-VP4 mouse monoclonal antibody (HS-2) was generously provided by Professor Harry Greenberg (Stanford University School of Medicine, USA). Anti-rabbit or anti-mouse IRDye-conjugated antibodies were used as secondary antibodies for western blotting (Stressgen, Victoria, BC, Canada).

2.2. Rotavirus SA11 and human-derived strain

A well-characterized and broadly used laboratory strain, simian rotavirus SA11, was employed. SA11 rotavirus strain used in this study was prepared as previously described (Knipping et al., 2012). Rotavirus genome copy numbers were determined by quantitative real-time polymerase chain reaction (qRT-PCR) referring to a plasmid template using a standard curve calculation method as described previously (Yin et al., 2015a). A standard curve was generated by plotting the log copy number versus the cycle threshold (C_T) value (Supplementary Figure 1). Human rotavirus (huRV) strains were isolated from rotavirus diarrhea patients as described previously (Yin et al., 2015a).

2.3. Cell culture

Caco2 cell line (human caucasian colon adenocarcinoma ECACC) was cultured in Dulbecco's modified Eagles's medium (DMEM; Lonza, Verviers, Belgium) supplemented with 20% (vol/vol) heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis USA), 100 U/mL penicillin and 100 U/mL streptomycin (Gibco, Grand Island, USA). The cells were maintained in 5% CO₂ at 37 °C in a humidified incubator.

2.4. Inoculation of SA11 and huRV rotavirus and treatment

Caco2 cell lines (not differentiated) maintained in T75 flasks were suspended and subsequently seeded into 48-well plates (5×10^4 cells/well) in DMEM containing 20% (vol/vol) FCS and 100 U/mL penicillin-streptomycin. When the cell confluence was approximately 80% after 2-3 days of culture, culture medium was discarded. The cell monolayer was then washed twice with PBS. 100 µL of serum-free DMEM medium containing 5 µg/mL of trypsin (Gibco, Paisley, UK) and SA11 rotavirus (MOI 0.7) were added and incubated at 37 °C with 5% CO₂ for 60 min for infection, followed by three times washing with PBS to remove free, uninfected virus particles. Subsequently, the cells were added with serum-free culture medium containing 5 µg/mL of trypsin (and indicated treatments) and incubated at 37 °C with 5% CO₂ for 48 hours. Inoculation protocol of huRV strains is similar to to inoculation of SA11 rotavirus.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (Takara Bio Inc) with random hexamer primers. qRT-PCR was performed with a SYBRGreen-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was considered as a reference gene to normalize gene expression. Relative gene expressions were normalized to GAPDH using the formula $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT_{\text{sample}} - \Delta\Delta CT_{\text{control}}$). The SA11 rotavirus sense and anti-sense primers target 564-585 and 719-699 of the VP6 segment of SA11 genome, respectively. All primers used in this study are listed in Supplementary Table 1.

2.6. IFN production bioassay

The IFN production bioassay was performed to detect secreted IFN proteins in the culture medium as described previously (Xu et al., 2016). Caco2 cells were seeded into 48-well plates and stimulated with TNF- α 10 ng/mL. After 24 hours, medium was removed and the cell monolayer was washed three times with PBS. Subsequently, the medium was refreshed and cultured for another 24 hours to let the produced IFNs secreted into the medium. The culture (conditioned) medium were then collected and filtered through 0.45 μm pore size membrane. To detect the presence of IFNs, Huh7-ISRE-luc reporter cell lines was used. Huh7-ISRE-luc is a luciferase reporter model in which the firefly luciferase gene was driven by multiple IFN-stimulated response elements (ISRE) promoter. In this model, the firefly luciferase activity can be potently stimulated by a low concentration of IFN- α treatment. Huh7-ISRE-luc cells were cultured in DMEM supplemented with 10% FCS (vol/vol), 100 U/mL penicillin and streptomycin.

2.7. Gene knockdown using lentiviral vectors

Lentiviral pLKO knockdown vectors (Sigma-Aldrich) targeting TNFR1 and RelA (p65) or scrambled control, were obtained from the Erasmus Center of Biomics and produced in human embryonic kidney epithelial cell line HEK 293T cells as described previously (Pan et al.,

2009). After a pilot study, the shRNA vectors exerting optimal gene knockdown were selected. All shRNA sequences are listed in Supplementary Table 2. As the lentiviral vectors also encode a puromycin resistance gene, transduced cells were subsequently selected by adding puromycin (8 µg/mL; Sigma) to the cell culture medium. Knockdown and control Caco2 cells were infected with rotavirus SA11 as previously described.

2.8. Caco2-based NF-κB and AP-1 luciferase reporter cell lines and measurement of luciferase activity

NF-κB and AP-1 luciferase reporter cells were generated by transducing Caco2 cells with lentiviral vectors expressing the firefly luciferase gene under the control of the promoters containing the NF-κB and AP-1 motifs, respectively (System Biosciences).

To measure the luciferase activity, luciferin potassium salt (100 mM; Sigma) was added to the cells and incubated for 20 minutes at 37 °C. The luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG Lab Tech, Offenburg, Germany).

2.9. Western blot assay

After discarding the culture medium (supernatants), cultured cells were lysed in Laemmli sample buffer containing 0.1 M DTT and heated for 5 minutes at 95 °C, followed by loading onto a 10% sodium dodecyl sulfate polyacrylamide gel and separation by electrophoresis (SDS-PAGE). After 90 mins running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-FL) for 1.5 hours with an electric current of 250 mA. Subsequently, the membrane was blocked with a mixture of 2.5 ml blocking buffer (Odyssey) and 2.5 ml PBS containing 0.05% Tween 20, followed by overnight incubation with primary antibodies (1:1000) at 4 °C. The membrane was then washed 3 times, followed by incubation for 1 hour with IRDye-conjugated secondary antibody (1:5000). After washing 3 times, protein bands were detected with the Odyssey 3.0 Infrared Imaging System.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Serum samples were collected from Crohn's disease patients and stored at -80 °C. Serum TNF- α levels were measured by an ELISA kit (eBioscience, USA) according to the manufacturer's instructions. The absorbance value was measured at 450 nm in an automatic microplate reader. The results were calculated based on a standard curve.

2.11. MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) (10 mM) was added to Caco2 cells cultured in 96-well plates at indicated time points (24 and 48 hours) following TNF- α and cytokine treatments. The cells were then incubated at 37 °C with 5% CO₂ for 3 hours. Subsequently, the culture medium was removed and 100 μ l of dimethyl sulfoxide (DMSO) was added to each well. The absorbance of each well was measured on the microplate absorbance readers (BIO-RAD) at the wavelength of 490 nm.

2.12. Immunofluorescence and confocal microscope assay

Caco2 cells were seeded on glass coverslips in 6-well plates. Then, Caco2 cells were infected with SA11 for 48 hours. Subsequently, cells were washed with PBS, fixed in 4% PBS-buffered formalin for 10 mins and blocked with tween-milk-glycine medium (PBS, 0.05% tween, 5g/L skim milk and 1.5g/L glycine). Samples were incubated with 1:500 dilution of anti-rotavirus (ab181695) antibody (Abcam) overnight at 4 °C. Subsequently, samples were incubated with 1:1000 dilution of Alexa FluorTM 594 goat anti-mouse secondary antibodies (Invitrogen). Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Invitrogen). Finally, the images were detected using immunofluorescence microscope.

For analysis of RelA protein, Caco2 cells on glass coverslips in 6-well plates were infected with either mock, SA11, huRV 1 and huRV 2 rotavirus for 8 hours. Subsequently, cells were washed twice with PBS. The mock- or rotavirus-infected cells were then stimulated with TNF- α (100 ng/mL) for 1 hour. Fixed and permeabilized cells were stained with antibodies to RelA (C22B4, #4764, Cell Signaling Technology, Leiden, The Netherlands), followed by anti-rabbit IgG(H+L),F(ab')₂ Fragment (Alexa Fluor 488 conjugate) secondary antibodies. The RelA proteins were visualized with confocal microscopy. Nuclei were stained by DAPI (blue).

2.13. Statistical Analysis

Statistical analysis was performed using the nonpaired, nonparametric test (Mann-Whitney test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). *P* values <0.05 were considered statistically significant.

2.14. Ethical Statement

The use of serum samples from IBD patients was approved by the Medical Ethical Committee of the Erasmus Medical Center (Medisch Ethische Toetsings Commissie Erasmus MC). Informed consents were signed by the volunteers or patients who agreed to participate.

3. Results

3.1. *TNF- α* has potent anti-viral activity against rotavirus

TNF- α serves as a host immune response against a variety of pathogen invasions. It has been previously reported that virus infection, such as influenza virus, induces TNF- α expression in lung epithelial cells, which subsequently inhibits influenza virus replication (Seo and Webster, 2002). To investigate whether rotavirus infection can also induce *tnfa* gene transcription, we employed the widely used Caco2 intestinal epithelium cell line. Infection of these cells with SA11 rotavirus did not trigger transcription of *tnfa* gene at 6, 24, 36 and 48 hours post-infection (Supplementary Figure 2A). In addition, analysis of *IL1B* and *IL8* gene expression levels showed that there were no changes of these genes upon rotavirus infection in Caco2 cells (Supplementary Figure 2B and 2C). This finding, which is in agreement with previous studies (Cuadras et al., 2002), suggests that in our experimental system, Caco2 cells do not produce TNF- α upon rotavirus infection.

Since rotavirus does not stimulate *tnfa* gene transcription in Caco2 cells, we next investigated the effects of exogenous TNF- α treatment on rotavirus replication. Treatment of SA11 rotavirus-infected Caco2 cells with human recombinant TNF- α for 24 and 48 hours significantly inhibited viral RNA levels. At concentrations of 10 and 100 ng/mL for 24 hours, TNF- α decreased total viral RNA levels by $41 \pm 8\%$ ($P < 0.05$) and $61 \pm 4\%$ ($P < 0.01$), respectively (Figure 1A). The inhibitions were more pronounced at 48 hours after infections,

where TNF- α decreased total viral RNA levels by $67 \pm 5\%$ ($P < 0.01$) and $71 \pm 4\%$ ($P < 0.01$) at concentrations of 10 and 100 ng/mL, respectively (Figure 1A). In addition, intracellular viral RNA levels quantified at 48 hours post-infections were significantly reduced by $92 \pm 0.3\%$ ($P < 0.001$) and $92 \pm 2\%$ ($P < 0.001$) at 10 and 100 ng/mL, respectively (Figure 1B). These inhibitions were also more potent than those at 24 hours, where TNF- α inhibited $55 \pm 7\%$ ($P < 0.01$) and $70 \pm 4\%$ ($P < 0.001$) of intracellular viral RNA levels at concentrations of 10 and 100 ng/mL, respectively (Figure 1B). Consistently, human TNF- α significantly reduced rotavirus secretion by the infected Caco2 cells as shown in viral titer quantification (Figure 1C). A notable reduction was also shown in the western blot analysis of VP4 protein (Figure 1D) and immunofluorescence analysis of VP6 protein of SA11-infected Caco2 cells (Figure 1E). Importantly, MTT assay demonstrated that TNF- α did not exert cytotoxicity to Caco2 cells (Supplementary Figure 3A).

It is well known that TNF- α is involved in IBD pathogenesis and is associated with the disease's activity. TNF- α may influence the expression of pro-inflammatory genes in IBD patients (Slebioda and Kmiec, 2014). To get further insight about the role of TNF- α in rotavirus infection, we collected serum samples from anti-TNF- α naïve Crohn's disease patients. The serum TNF- α levels were measured by ELISA and 4 serum samples with relatively high TNF- α levels were selected (Supplementary Figure 4A). Notably, all selected serum samples exerted anti-rotavirus effect (as determined by total viral RNA levels) as compared to the control serum (Supplementary Figure 4B). These results suggest that TNF- α may indirectly influence rotavirus infection.

3.2. Anti-rotavirus effect of TNF- α is independent of interferon production and JAK-STAT pathway activity

Since TNF- α can induce type I IFNs in several cell lines, we first investigated the straightforward possibility that TNF- α merely induces IFN- α and IFN- β expression to mediate its anti-rotavirus effect in Caco2 cells. As demonstrated by qRT-PCR, basal constitutive expression of *IFN- α* and *IFN- β* is low, compared to the reference genes *GAPDH* (Figure 2A). In addition, TNF- α treatment for 48 hours did not significantly increase *IFN- α* and *IFN- β* (Figure 2B) gene expression. To further confirm the absence of IFN production in our experimental systems, the conditioned medium from the TNF α -stimulated cells were

collected and were used to perform an IFN production bioassay. We used Huh7-ISRE-luciferase reporter cell lines, in which the firefly luciferase gene expression is driven by multiple IFN-stimulated response elements (ISRE) promoter. As shown in Figure 2C, the conditioned medium was not able to stimulate IFN response and confirmed the absence of IFN proteins secreted in the culture medium.

Type I IFNs signal through activation of the Janus Kinases (JAKs) protein which leads to phosphorylation of Signal Transducers and Activators of Transcription (STAT) family of proteins. This JAK-STAT signaling ultimately promotes expression of IFN-stimulated genes (ISGs) as the ultimate anti-viral effector molecules (Wang et al., 2017). To further rule out the possibility of JAK-STAT pathway involvement, we combined TNF- α with *pan*-JAK I inhibitors which potently block JAK-STAT signaling (Wang et al., 2016). Consistently, addition of JAK I inhibitor (5 μ M) did not abolish the anti-rotavirus effects of TNF- α (Figure 2D).

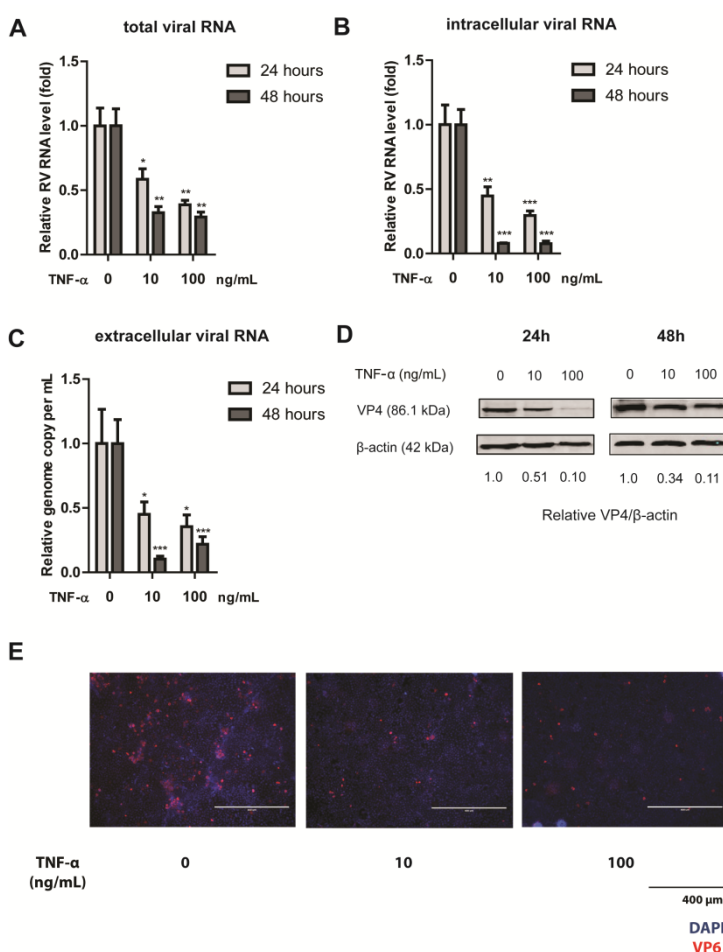


Figure 1. Anti-rotavirus effects of TNF- α . TNF- α treatment (10 and 100 ng/mL) significantly inhibits rotavirus replication as measured by total **(A)**, intracellular **(B)** and extracellular (secreted) **(C)** rotavirus RNA levels in Caco2 cells both at 24 and 48 hours post-infections ($n = 6-8$, means \pm SEM; (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Mann-Whitney test). **(D)** Western blot analysis of VP4 protein indicates inhibitory effect of 10 and 100 ng/mL TNF- α against rotavirus at 24 and 48 hours post-infections. **(E)** Immunofluorescence staining of VP6 rotavirus protein (red) after 48 hours infection of Caco2 cells with rotavirus SA11. Nuclei were visualized by DAPI (blue).

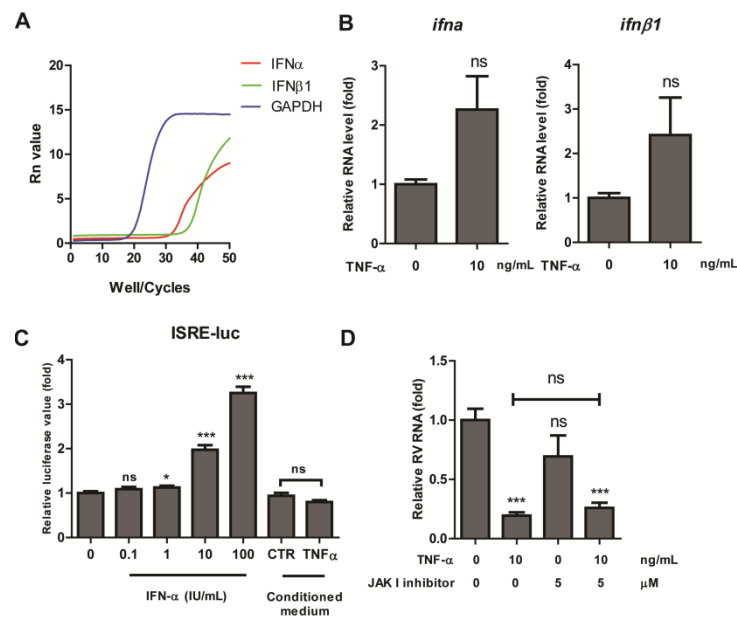


Figure 2. Anti-rotavirus effect of TNF- α is independent of interferon and the JAK-STAT signaling. (A) The relative basal *IFN- α* and *IFN- β 1* gene expression levels of Caco2 cell lines were determined by qRT-PCR. GAPDH served as a reference gene. **(B)** *IFN- α* and *IFN- β 1* gene expression levels in Caco2 cells were not induced following 10 ng/mL TNF- α stimulation for 48 hours as quantified by qRT-PCR (n = 12). **(C)** ISRE *firefly* luciferase activity in Huh7-ISRE-luc model treated with the conditioned medium for 48 hours (n = 11). **(D)** JAK I inhibitor (5 μ M) did not abolish the anti-rotavirus effects of TNF- α at 48 hours post-infections (n = 6). Data are presented as means \pm SEM (* P < 0.05; *** P < 0.001; ns, not significant; Mann-Whitney test).

3.3. TNF receptor 1 is essential for TNF- α induced anti-rotavirus effect

TNF- α exerts its biological effect through interaction with two different receptors expressed in the target cells, TNF receptor 1 (TNFR1) and TNFR2. TNFR1 is ubiquitously expressed by almost all human tissues and can be activated by both soluble and transmembrane TNF- α . In contrast, TNFR2 expression is limited to certain cell types, such as immune cells, mesenchymal stem cells and endothelial cells (Kalliolias and Ivashkiv, 2016). In addition, it is suggested that TNFR2 can only be activated by transmembrane TNF- α . Thus, TNFR1 serves as the major signaling component for soluble TNF- α *in vivo* (Kalliolias and Ivashkiv, 2016). Therefore, this encouraged us to investigate whether anti-rotavirus effects of TNF- α was mediated via TNFR1.

First, Caco2 cells were transduced with integrating lentiviral vectors expressing shRNA that specifically target TNFR1. Among three tested shRNA vectors, sh-96 vector showed the most potent gene silencing capacity, as determined by qRT-PCR (Figure 3A) and western blot analysis (Figure 3B). As expected, TNF α -induced *CXCL10* expression was abolished in TNFR1

knockdown cells which was examined at 6 hours following TNF- α stimulation (Figure 3C). Caco2 cells with sh-96 TNFR1 knockdown and scrambled sh-RNA (sh-CTR) as control were subsequently infected with SA11 rotavirus. In sh-CTR transfected cells, treatment with 10 ng/mL TNF- α significantly reduced viral RNA levels ($80 \pm 2.4\%$ inhibition; $P < 0.001$). However, the anti-rotavirus effect of TNF- α was abolished upon TNFR1 silencing (Figure 3D). To validate these results, the clinically used anti-TNF α antibody, infliximab (Remicade[®]), was used. Infliximab binds specifically to TNF- α and blocks its interaction with TNF receptors (Sedger and McDermott, 2014). As expected, infliximab efficiently abrogated TNF α -induced *CXCL10* expression at 6 hours after stimulation (Figure 3E). Consistently, combining TNF- α with infliximab completely blocked the anti-rotavirus effects of TNF- α at 48 hours after infections (Figure 3F).

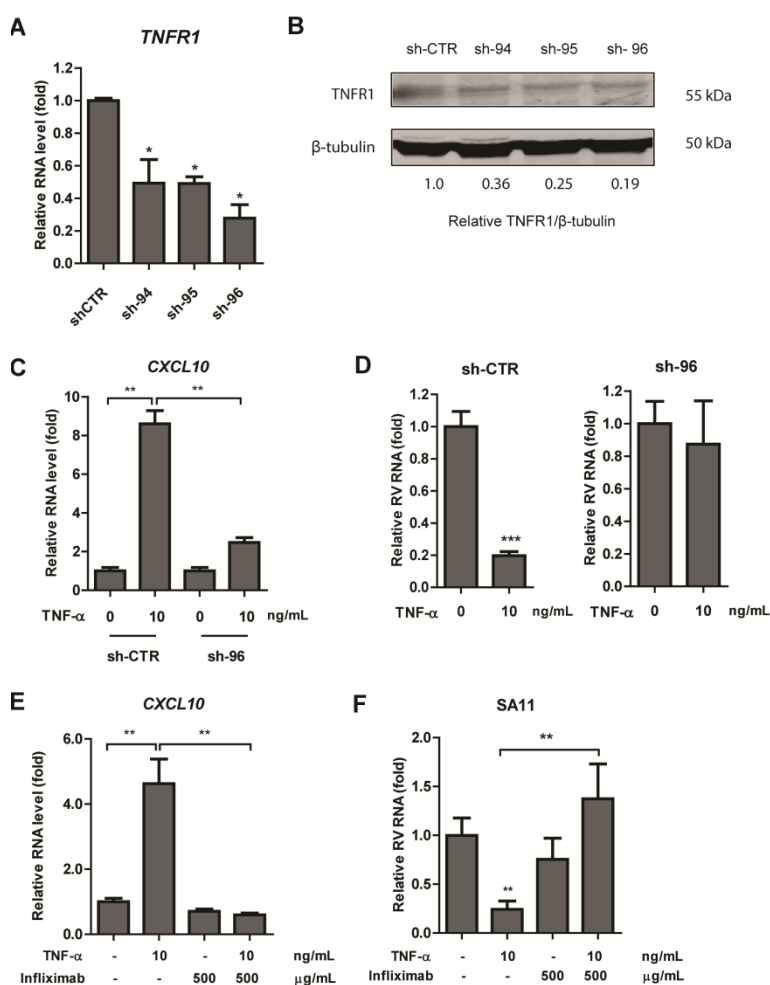


Figure 3. Anti-rotavirus effect of TNF- α is mediated via TNF receptor 1 (TNFR1). (A) qRT-PCR ($n = 4$) and (B) western blot analysis of TNFR1 knockdown by lentiviral sh-RNA vectors in Caco2 cells. (C) TNFR1 knockdown (sh-96) abolished TNF α -induced *CXCL10* expression at 6 hours following stimulation ($n = 6$). (D) TNFR1 knockdown (sh-96) led to the blockade of TNF α -induced anti-rotavirus activity at 48 hours post-infections ($n = 6$). (E) The TNF- α inhibitor (Infliximab) (500 μ g/mL) abrogated the induction of *CXCL10* gene following TNF- α stimulation (10 ng/mL) for 6 hours ($n = 5$). (F) Infliximab (500 μ g/mL) blocked anti-rotavirus effects of 10 ng/mL TNF- α as measured by qRT-PCR at 48 hours post infection ($n = 6$). Data are presented as means \pm SEM (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Mann-Whitney test).

3.4. *TNF- α mediates anti-rotavirus activity through NF- κ B signaling*

At the cellular level, binding of TNF- α to TNFR1 activates downstream c-JUN N-terminal kinase (JNK), leading to the transcription of AP-1 target genes. Additionally, TNFR1 activates NF- κ B, resulting in induced expression of NF- κ B target genes (Cabal-Hierro and Lazo, 2012). To dissect whether the anti-rotavirus effects of TNF- α are mediated via the AP-1 or NF- κ B pathway, we first constructed Caco2-based stable NF- κ B and AP-1 driven luciferase reporter cell lines. As shown in figure 4A, TNF- α stimulation resulted in profound induction NF- κ B driven luciferase activity in a dose-dependent manner at 12, 24 and 48 hours after stimulation, yet no effect on AP-1 driven luciferase activity was observed. Noteworthy, the maximum induction of NF- κ B driven luciferase activity was observed at 12 hours after stimulation (Figure 4A). Based on these results, we hypothesized that the anti-rotavirus effects of TNF- α on Caco2 cells are mediated via the classical NF- κ B signaling pathway, rather than the AP-1 pathway.

The endpoint of TNF- α signaling is activation of the heterodimeric NF- κ B complex, consisting of the RelA (p65) and p50 (Cabal-Hierro and Lazo, 2012). To examine the role of this RelA (p65)-p50 complex in mediating anti-rotavirus effects of TNF- α , Caco2 cells were transduced with integrating lentiviral shRNA vectors to silence RelA. Two of the three tested shRNA vectors targeting RelA profoundly downregulate *RelA* both at mRNA and protein level (Figure 4B and 4C). Importantly, RelA knockdown promoted SA11 rotavirus replication by 2.2 ± 0.3 fold ($P < 0.01$) (Figure 4D), supporting its importance in the control of rotavirus infection. In RelA knockdown cells, induction of *CXCL10* expression was efficiently blocked following TNF- α (10 ng/mL) stimulation for 6 hours (Figure 4E). Next, Caco2 cells with sh-10 RelA knockdown and scrambled sh-RNA as control (sh-CTR) were infected with SA11 rotavirus with and without treatment of 10 ng/mL of TNF- α . As before, treatment of sh-CTR transfected cells with 10 ng/mL TNF- α significantly decreased viral RNA levels ($60 \pm 6.5\%$ inhibition; $P < 0.001$). However, this anti-rotavirus effect was abrogated in RelA knockdown cells (Figure 4F), demonstrating that NF- κ B signaling pathway is essential to mediate anti-rotavirus effects of TNF- α .

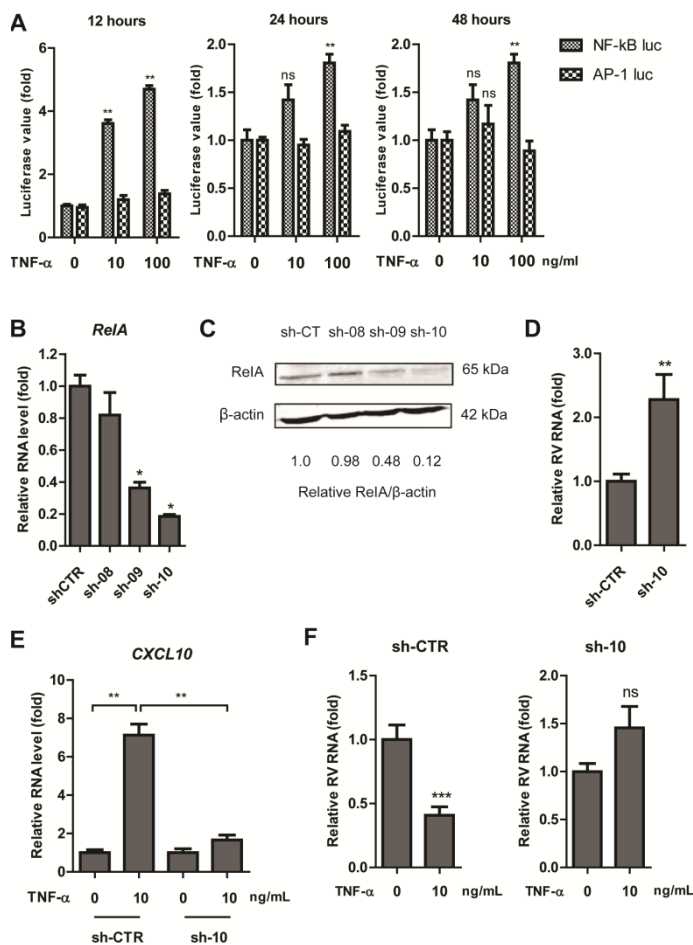


Figure 4. Anti-rotavirus effect of TNF- α is mediated via NF- κ B signaling. (A) In Caco2-based NF- κ B reporter cell lines, TNF- α induced activation of NF- κ B-related luciferase signals in a dose-dependent manner as measured at 12, 24 and 48 hours, while AP1-related luciferase signals were not affected ($n = 4-6$). qRT-PCR ($n = 4$) (B) and western blot (C) analysis of RelA (p65) knockdown by lentiviral sh-RNA vectors in Caco2 cells. (D) RelA (p65) knockdown (sh-10) promoted rotavirus replication as quantified by total RNA levels in Caco2 cells at 48 hours post-infections ($n = 8$). (E) RelA (p65) knockdown (sh-10) led to the blockade of TNF α -induced CXCL10 expression at 6 hours post-stimulations ($n = 6$). (F) RelA knockdown (sh-10) resulted in the blockade of TNF α -induced anti-rotavirus activity at 48 hours post-infections ($n = 6-8$). Data are presented as means \pm SEM (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant; Mann-Whitney test).

3.5. TNF α -induced cytokines are the downstream effectors to exert anti-rotavirus activity

Following TNF- α stimulation, the activated p65:p50 NF- κ B complex translocates to the nucleus where it binds to specific DNA motifs located in the promoter region of its target genes. This event will regulate, either induce or suppress, the expression of TNF α -modulated genes in a cell-type-specific manner (Kalliolias and Ivashkiv, 2016). These NF κ B-regulated genes play an essential role in the host immune response such as pro-inflammatory cytokine and chemokine regulation. Therefore, we examined a panel of cytokines and chemokines which may be induced by human TNF- α in Caco2 cell lines.

As shown in figure 5A, 10 ng/mL TNF- α stimulation for 6 and 24 hours led to increased transcription of the genes encoding IL-8, IL-32, CCL2, CCL20, CXCL10 and CXCL11 genes in Caco2 cells. To dissect whether these TNF α -induced cytokines could inhibit rotavirus infection, we treated SA11-infected Caco2 cells with those cytokines at a concentration of 10 ng/mL. As shown in figure 5B, treatment with IL32, IL8, CXCL11 and CCL20 potentially inhibited rotavirus replication as measured by qRT-PCR. Western blot analysis of VP4 protein confirmed these results (Figure 5C). Importantly, MTT assay revealed that these cytokines did not affect cell growth and cytotoxicity to Caco2 cells (Supplementary Figure 5). These results collectively show that TNF α -induced cytokines exert a powerful anti-rotavirus activity.

3.6. Sensitivity of human rotavirus (huRV) derived from clinical strains to TNF- α treatment.

Previous studies have indicated that several rotavirus strains, including Wa (human), RRV (simian), and OSU (porcine), have different mechanisms of antagonizing NF- κ B activation. Those mechanisms include degradation of β -TrCP and inhibition the nuclear translocation of NF- κ B (Graff et al., 2009; Holloway et al., 2009). To extend our results, we employed human rotavirus (huRV)-derived clinical strains isolated from two diarrhea patients (Yin et al., 2015a). Caco2 cells infected with two huRV-derived clinical strains were treated with TNF- α 100 ng/mL for 48 hours. As shown in Figure 6, huRV strain 1 replication was notably inhibited by TNF- α treatment ($70 \pm 6\%$ inhibition; $P < 0.01$), in contrast to huRV strain 2 which was resistant to TNF- α treatment. These results suggest that huRV strains have different sensitivity to TNF- α treatment.

To further investigate the different sensitivity of rotavirus strains against TNF- α treatment observed in our study, we analyzed the ability of different rotavirus strains to block translocation of RelA to the nucleus (Supplementary Figure 5). In mock-infected Caco2 cells, the RelA protein level in the nucleus was increased following TNF- α stimulation (100 ng/mL) for 1 hour, compared with mock-infected unstimulated cells. Unexpectedly, in SA11-, huRV 1- and huRV2-infected Caco2 cells, the nuclear accumulation of RelA was also elevated following TNF- α stimulation (Supplementary Figure 5).

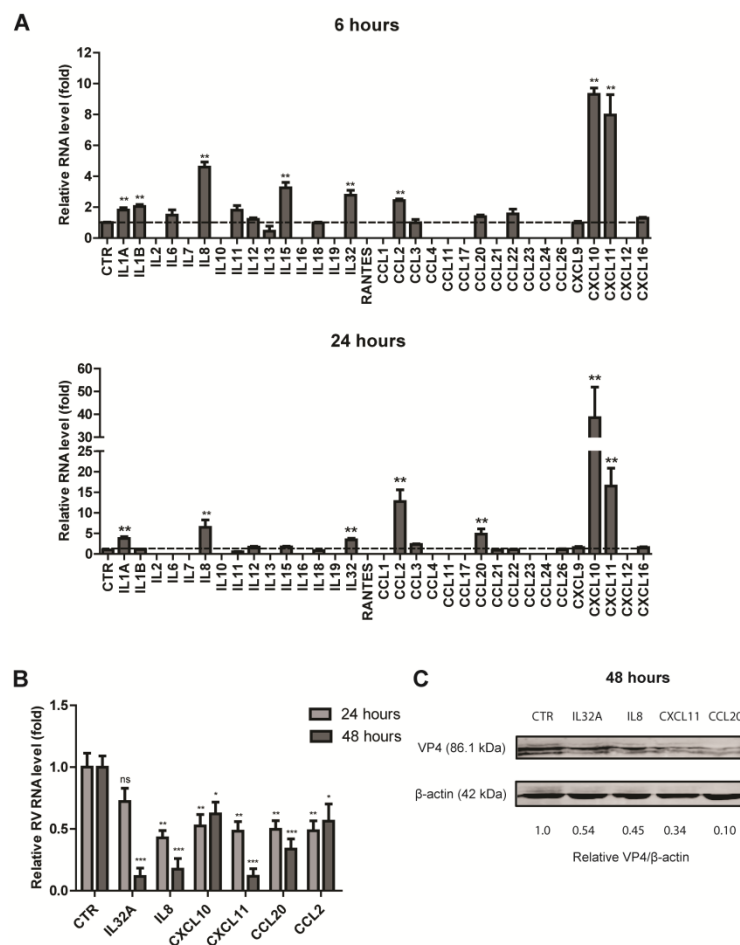
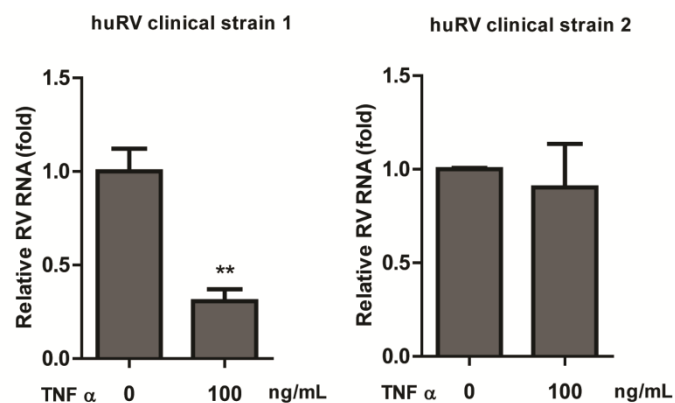


Figure 5. TNF α -induced cytokines exert anti-rotavirus activity. (A) Expression profile of 33 cytokines in Caco2 cells as quantified by qRT-PCR analysis. Some cytokines were up-regulated with 10 ng/mL TNF- α stimulation of Caco2 cells for 6 and 24 hours ($n = 6$). (B) IL32A, IL8, CXCL10, CXCL11, CCL20 and CCL2 significantly inhibited rotavirus replication as quantified by qRT-PCR of total RNA levels, both at 24 and 48 hours ($n = 6-8$). (C) Western blot analysis of VP4 protein indicates the inhibitory effect of 10 ng/mL of IL32, IL8, CXCL11 and CCL20 against rotavirus at 48 hours post-infections. Data are presented as means \pm SEM (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant; Mann-Whitney test).

Figure 6. Sensitivity of human rotavirus (huRV) clinical strains to TNF- α treatment. TNF- α treatment for 48 hours (100 ng/mL) significantly inhibits replication of huRV clinical strain 1 (huRV 1) as measured by total rotavirus RNA levels in Caco2 cells; while replication of huRV clinical strain 2 (huRV 2) was not affected. ($n = 3$, means \pm SEM, ** $P < 0.01$, Mann-Whitney test).



4. Discussion

TNF- α was first known for its anti-cancer activity *in vitro* (Carswell et al., 1975). The subsequent development of recombinant technology allowed the dissection of the multiple roles of TNF- α in the human body, which includes inflammatory responses, immune

regulatory functions, and anti-microbial immunity (Sedger and McDermott, 2014). Inappropriate regulation of TNF- α has been implicated in various human diseases, leading to the use of TNF- α inhibitors in the clinic to treat these TNF α -related diseases. The subsequent increased of viral and bacterial infections in these patients suggests that TNF- α plays an essential role in anti-viral and anti-microbial immunity (Kim and Solomon, 2010).

Timely and rapid immune responses against invading viral pathogens are critical for the host to clear such infections. These early responses mainly depend on anti-viral immunity mediated by innate immune cells that produce various cytokines, such as IFNs, interleukins and TNF- α that act directly to eliminate infections and indirectly by promoting subsequent development of a more specific adaptive immunity (Holloway and Coulson, 2013). In infected children, increased levels of IFN- α were detected in the blood, and related to the clinical outcome (De Boissieu et al., 1993). Thus, investigating innate responses is crucial for understanding virus-host pathogenesis during rotavirus infections (Arnold et al., 2013).

Previous studies have shown the increased production of TNF- α by DCs (Deal et al., 2010) and macrophages (Mohanty et al., 2010) upon rotavirus stimulation. Elevated levels of TNF- α were found in children with rotavirus diarrhea as compared to healthy controls (Azim et al., 1999). Furthermore, in children with rotavirus diarrhea, a significantly higher level of TNF- α was found in serum of those who developed fever and had more frequent episodes of diarrhea (Jiang et al., 2003). It is probably associated with the effect of TNF- α on ion secretion in human intestinal epithelial cells (Oprins et al., 2000). This suggests that TNF- α may contribute to the immunity and the disease pathogenesis. However, the exact role of TNF- α in rotavirus infection has not yet been extensively studied. Here we demonstrated that TNF- α has a strong anti-rotavirus effect both at intracellular and extracellular (excreted) levels. We also showed that the clinically used TNF- α inhibitors (infliximab) can completely block this effect, supporting caution of the use of these treatments in patients with latent or active viral infections.

In several cell lines, TNF- α induces the production of type I IFNs (IFN- β) (Ahrens et al., 1990; Hughes et al., 1988; Jacobsen et al., 1989; Yarilina et al., 2008). Thus, we first hypothesized a straightforward explanation that anti-rotavirus effect of TNF- α is merely mediated via type I IFN and consequently, classical IFN-signaling (JAK-STAT pathway activation). However,

treatment of Caco2 cells with TNF- α did not significantly increase *ifna* and *ifnb1* gene expression levels, and consequently did not result in IFN production (Fig. 2B and 2C). In addition, combination treatment with TNF- α and the *pan*-JAK I inhibitor did not abolish the anti-rotavirus effect of TNF- α . Thus, we conclude that the anti-rotavirus effect of TNF- α is independent of IFN production and the JAK-STAT pathway.

TNF- α signals mainly through TNFR1 receptor and stimulates downstream signaling pathways that ultimately activates two major transcription factors, NF- κ B (classical NF- κ B pathway) and c-Jun (AP-1 pathway) (Cabal-Hierro and Lazo, 2012; Kalliolias and Ivashkiv, 2016). Our experiments using Caco2-based NF- κ B and AP-1 reporter cell lines showed that TNF- α signals via NF- κ B and not via AP-1. NF- κ B is activated following rotavirus infection and is involved in the regulation of cytokine productions (Rollo et al., 1999), indicating its importance in the host defense against rotavirus. Indeed, the following loss-of-function assays by knocking down RelA (p65), one of the main components of the NF- κ B protein complex, led to an increase of rotavirus replication. Furthermore, knockdown of RelA abrogates anti-rotavirus effect of TNF- α . This indicates the importance of NF- κ B in restricting rotavirus infection and also in mediating anti-rotavirus effects of TNF- α .

It is important to note that several studies have reported that other rotavirus strains can inhibit NF- κ B activation (Graff et al., 2009) or its TNF α -induced translocation to the nucleus (Holloway et al., 2009). Noteworthy, the NSP1 protein of rotavirus contains a conserved C-terminal phosphodegron-like (PDL) motif which disrupts NF- κ B activation by interfering with I κ B degradation (Morelli et al., 2015). However, our data suggested that translocation of RelA to the nucleus was not blocked by SA11 as well as both human rotavirus strains 1 and 2, as demonstrated by confocal microscopy analysis (Fig. S5). Other mechanisms, including blocking of RelA binding to its promoter, may be involved. Collectively, our results showed that anti-rotavirus effect of TNF- α is mediated via TNFR1 receptor and classical NF- κ B signaling.

Several cytokines are induced following exposure of dendritic cells to rotavirus, such as IL-6, IL-8, CXCL-10 and CCL5 (Deal et al., 2010; Rosales-Martinez et al., 2016). Several cytokines have been associated with clinical symptoms in rotavirus diarrheal children (Jiang et al., 2003). Surprisingly, we found that TNF α -induced cytokines, such as IL-8, CXCL10, CXCL11,

have potent anti-rotavirus effects. Further experiments are therefore required to clarify their mechanism of action in inhibiting rotavirus infection. Noteworthy, anti-TNF α treatment in Crohn's disease patients is associated with modulation of pro-inflammatory genes, including IL1B and CXCL11 (Leal et al., 2015). Accordingly, our results from patients' samples suggest that a high level of TNF- α in serum may modulate rotavirus infection indirectly by affecting cytokine levels in these patients' sera.

It is important to note that under inflammatory conditions, infected cells are continuously exposed to various cytokines. The effect of these cytokines could be influenced by the presence of other cytokines (Bartee et al., 2008). Several studies have shown a synergistic anti-viral effect of TNF- α and type I IFNs (IFN- α and IFN- β) in the setting of HCV, HEV (Wang et al., 2016), respiratory virus (Fink et al., 2013) and poxvirus infections (Bartee et al., 2009). TNF- α can also synergize with type II IFNs (IFN- γ) in the setting of cytomegalovirus (CMV) infection (Lucin et al., 1994). Therefore, it is interesting to further investigate whether these combined effects can be observed in rotavirus infections.

5. Conclusion

We demonstrate a novel role of TNF- α in inhibiting rotavirus replication. This anti-rotavirus effect is mediated via the classical NF- κ B signaling pathway, independent of IFN production and JAK-STAT pathway activity. Furthermore, this finding elucidates the increased risk of viral infection upon TNF- α inhibitor treatments used in the clinic. Thus, this knowledge may contribute to a better clinical management of these patients.

Acknowledgements

The authors thank to Professor Harry Greenberg (Stanford University School of Medicine, USA) for generously providing the mouse monoclonal antibody against rotavirus VP4 protein. This work is supported by the Dutch Digestive Foundation for a career development grant (No. CDG 1304 to Q. P.). This research is also supported by the Indonesia Endowment Fund for Education (LPDP) for funding PhD fellowship to Mohamad S. Hakim and the China Scholarship Council for funding PhD fellowship to W. Wang (201303250056), S. Chen (201606760056) and Y. Yin (201307720045).

Potential conflicts of interest

The authors declare no conflict of interest.

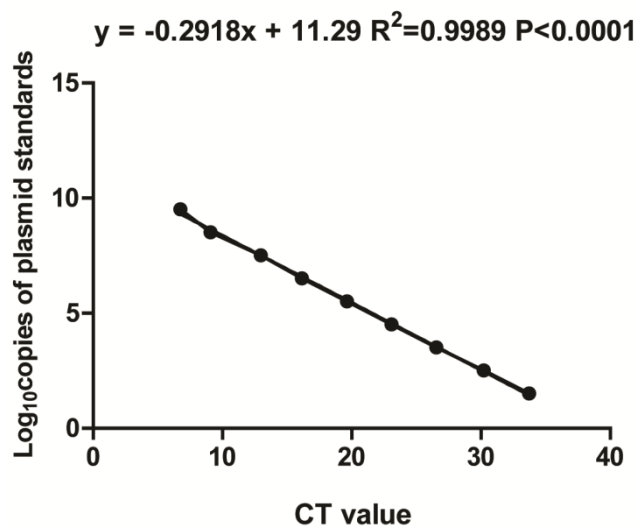
References

- Ahrens, P.B., Besancon, F., Memet, S., Ankel, H., 1990. Tumour necrosis factor enhances induction by beta-interferon of a ubiquitin cross-reactive protein. *J Gen Virol* 71 (Pt 8), 1675-1682.
- Angel, J., Franco, M.A., Greenberg, H.B., 2012. Rotavirus immune responses and correlates of protection. *Curr Opin Virol* 2, 419-425.
- Arnold, M.M., Sen, A., Greenberg, H.B., Patton, J.T., 2013. The battle between rotavirus and its host for control of the interferon signaling pathway. *PLoS Pathog* 9, e1003064.
- Azim, T., Ahmad, S.M., Sefat, E.K., Sarker, M.S., Unicomb, L.E., De, S., Hamadani, J.D., Salam, M.A., Wahed, M.A., Albert, M.J., 1999. Immune response of children who develop persistent diarrhea following rotavirus infection. *Clin Diagn Lab Immunol* 6, 690-695.
- Bartee, E., Mohamed, M.R., Lopez, M.C., Baker, H.V., McFadden, G., 2009. The addition of tumor necrosis factor plus beta interferon induces a novel synergistic antiviral state against poxviruses in primary human fibroblasts. *J Virol* 83, 498-511.
- Bartee, E., Mohamed, M.R., McFadden, G., 2008. Tumor necrosis factor and interferon: cytokines in harmony. *Curr Opin Microbiol* 11, 378-383.
- Cabal-Hierro, L., Lazo, P.S., 2012. Signal transduction by tumor necrosis factor receptors. *Cell Signal* 24, 1297-1305.
- Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., Williamson, B., 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A* 72, 3666-3670.
- Chiappini, E., Azzari, C., Moriondo, M., Galli, L., de Martino, M., 2005. Viraemia is a common finding in immunocompetent children with rotavirus infection. *J Med Virol* 76, 265-267.
- Cuadras, M.A., Feigelstock, D.A., An, S., Greenberg, H.B., 2002. Gene expression pattern in Caco-2 cells following rotavirus infection. *J Virol* 76, 4467-4482.
- De Boissieu, D., Lebon, P., Badoual, J., Bompard, Y., Dupont, C., 1993. Rotavirus induces alpha-interferon release in children with gastroenteritis. *J Pediatr Gastroenterol Nutr* 16, 29-32.
- Deal, E.M., Jaimes, M.C., Crawford, S.E., Estes, M.K., Greenberg, H.B., 2010. Rotavirus structural proteins and dsRNA are required for the human primary plasmacytoid dendritic cell IFNalpha response. *PLoS Pathog* 6, e1000931.
- Ding, S., Mooney, N., Li, B., Kelly, M.R., Feng, N., Loktev, A.V., Sen, A., Patton, J.T., Jackson, P.K., Greenberg, H.B., 2016. Comparative proteomics reveals strain-specific beta-TrCP degradation via rotavirus NSP1 hijacking a host cullin-3-Rbx1 complex. *PLoS Pathog* 12, e1005929.
- Fink, K., Martin, L., Mukawera, E., Chartier, S., De Deken, X., Brochiero, E., Miot, F., Grandvaux, N., 2013. IFNbeta/TNFalpha synergism induces a non-canonical STAT2/IRF9-dependent pathway triggering a novel DUOX2 NADPH oxidase-mediated airway antiviral response. *Cell Res* 23, 673-690.
- Flynn, J.L., Goldstein, M.M., Chan, J., Triebold, K.J., Pfeffer, K., Lowenstein, C.J., Schreiber, R., Mak, T.W., Bloom, B.R., 1995. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity* 2, 561-572.
- Graff, J.W., Ettayebi, K., Hardy, M.E., 2009. Rotavirus NSP1 inhibits NFkappaB activation by inducing proteasome-dependent degradation of beta-TrCP: a novel mechanism of IFN antagonism. *PLoS Pathog* 5, e1000280.
- Greenberg, H.B., Estes, M.K., 2009. Rotaviruses: from pathogenesis to vaccination. *Gastroenterology* 136, 1939-1951.

- Hernandez, P.P., Mahlakoiv, T., Yang, I., Schwierzeck, V., Nguyen, N., Guendel, F., Gronke, K., Ryffel, B., Holscher, C., Dumoutier, L., Renauld, J.C., Suerbaum, S., Staeheli, P., Diefenbach, A., 2015. Interferon-lambda and interleukin 22 act synergistically for the induction of interferon-stimulated genes and control of rotavirus infection. *Nat Immunol* 16, 698-707.
- Holloway, G., Coulson, B.S., 2013. Innate cellular responses to rotavirus infection. *J Gen Virol* 94, 1151-1160.
- Holloway, G., Truong, T.T., Coulson, B.S., 2009. Rotavirus antagonizes cellular antiviral responses by inhibiting the nuclear accumulation of STAT1, STAT2, and NF-kappaB. *J Virol* 83, 4942-4951.
- Hughes, T.K., Kaspar, T.A., Coppenhaver, D.H., 1988. Synergy of antiviral actions of TNF and IFN-gamma: evidence for a major role of TNF-induced IFN-beta. *Antiviral Res* 10, 1-9.
- Jacobsen, H., Mestan, J., Mitnacht, S., Dieffenbach, C.W., 1989. Beta interferon subtype 1 induction by tumor necrosis factor. *Mol Cell Biol* 9, 3037-3042.
- Jiang, B., Snipes-Magaldi, L., Dennehy, P., Keyserling, H., Holman, R.C., Bresee, J., Gentsch, J., Glass, R.I., 2003. Cytokines as mediators for or effectors against rotavirus disease in children. *Clin Diagn Lab Immunol* 10, 995-1001.
- Kallioli, G.D., Ivashkiv, L.B., 2016. TNF biology, pathogenic mechanisms and emerging therapeutic strategies. *Nat Rev Rheumatol* 12, 49-62.
- Kim, S.Y., Solomon, D.H., 2010. Tumor necrosis factor blockade and the risk of viral infection. *Nat Rev Rheumatol* 6, 165-174.
- Knipping, K., Garssen, J., van't Land, B., 2012. An evaluation of the inhibitory effects against rotavirus infection of edible plant extracts. *Virol J* 9, 137.
- Leal, R.F., Planell, N., Kajekar, R., Lozano, J.J., Ordas, I., Dotti, I., Esteller, M., Masamunt, M.C., Parmar, H., Ricart, E., Panes, J., Salas, A., 2015. Identification of inflammatory mediators in patients with Crohn's disease unresponsive to anti-TNFalpha therapy. *Gut* 64, 233-242.
- Lin, J.D., Feng, N., Sen, A., Balan, M., Tseng, H.C., McElrath, C., Smirnov, S.V., Peng, J., Yasukawa, L.L., Durbin, R.K., Durbin, J.E., Greenberg, H.B., Kotenko, S.V., 2016. Distinct roles of type I and type III interferons in intestinal immunity to homologous and heterologous rotavirus infections. *PLoS Pathog* 12, e1005600.
- Lucin, P., Jonjic, S., Messerle, M., Polic, B., Hengel, H., Koszinowski, U.H., 1994. Late phase inhibition of murine cytomegalovirus replication by synergistic action of interferon-gamma and tumour necrosis factor. *J Gen Virol* 75 (Pt 1), 101-110.
- Masclee, G.M., Penders, J., Pierik, M., Wolffs, P., Jonkers, D., 2013. Enteropathogenic viruses: triggers for exacerbation in IBD? A prospective cohort study using real-time quantitative polymerase chain reaction. *Inflamm Bowel Dis* 19, 124-131.
- Mesa, M.C., Rodriguez, L.S., Franco, M.A., Angel, J., 2007. Interaction of rotavirus with human peripheral blood mononuclear cells: plasmacytoid dendritic cells play a role in stimulating memory rotavirus specific T cells in vitro. *Virology* 366, 174-184.
- Mohanty, S.K., Ivantes, C.A., Mourya, R., Pacheco, C., Bezerra, J.A., 2010. Macrophages are targeted by rotavirus in experimental biliary atresia and induce neutrophil chemotaxis by Mip2/Cxcl2. *Pediatr Res* 67, 345-351.
- Morelli, M., Dennis, A.F., Patton, J.T., 2015. Putative E3 ubiquitin ligase of human rotavirus inhibits NF-kappaB activation by using molecular mimicry to target beta-TrCP. *MBio* 6, e02490-14.
- Oprins, J.C., Meijer, H.P., Groot, J.A., 2000. TNF-alpha potentiates the ion secretion induced by muscarinic receptor activation in HT29cl.19A cells. *Am J Physiol Cell Physiol* 278, C463-472.
- Pan, Q., Henry, S.D., Metselaar, H.J., Scholte, B., Kwekkeboom, J., Tilanus, H.W., Janssen, H.L., van der Laan, L.J., 2009. Combined antiviral activity of interferon-alpha and RNA interference directed against hepatitis C without affecting vector delivery and gene silencing. *J Mol Med (Berl)* 87, 713-722.
- Rollo, E.E., Kumar, K.P., Reich, N.C., Cohen, J., Angel, J., Greenberg, H.B., Sheth, R., Anderson, J., Oh, B., Hempson, S.J., Mackow, E.R., Shaw, R.D., 1999. The epithelial cell response to rotavirus infection. *J Immunol* 163, 4442-4452.

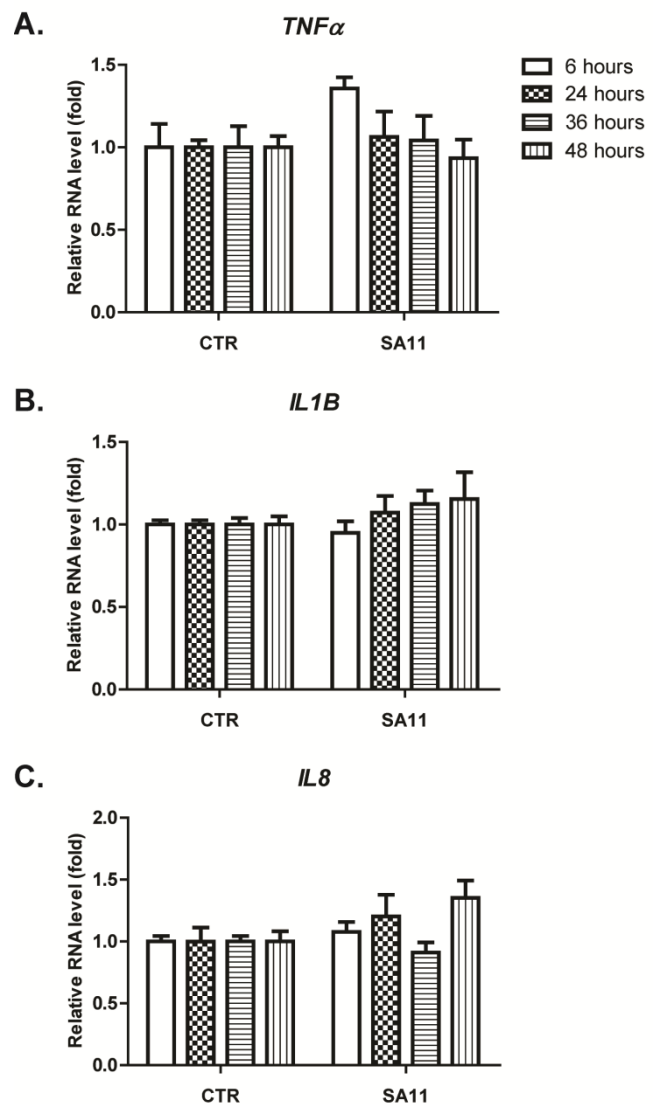
- Rosales-Martinez, D., Gutierrez-Xicotencatl, L., Badillo-Godinez, O., Lopez-Guerrero, D., Santana-Calderon, A., Cortez-Gomez, R., Ramirez-Pliego, O., Esquivel-Guadarrama, F., 2016. Rotavirus activates dendritic cells derived from umbilical cord blood monocytes. *Microb Pathog* 99, 162-172.
- Saxena, K., Simon, L.M., Zeng, X.L., Blutt, S.E., Crawford, S.E., Sastri, N.P., Karandikar, U.C., Ajami, N.J., Zachos, N.C., Kovbasnjuk, O., Donowitz, M., Conner, M.E., Shaw, C.A., Estes, M.K., 2017. A paradox of transcriptional and functional innate interferon responses of human intestinal enteroids to enteric virus infection. *Proc Natl Acad Sci U S A* 114, E570-E579.
- Sedger, L.M., McDermott, M.F., 2014. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. *Cytokine Growth Factor Rev* 25, 453-472.
- Seo, S.H., Webster, R.G., 2002. Tumor necrosis factor alpha exerts powerful anti-influenza virus effects in lung epithelial cells. *J Virol* 76, 1071-1076.
- Slebiada, T.J., Kmiec, Z., 2014. Tumour necrosis factor superfamily members in the pathogenesis of inflammatory bowel disease. *Mediators Inflamm* 2014, 325129.
- Tate, J.E., Burton, A.H., Boschi-Pinto, C., Steele, A.D., Duque, J., Parashar, U.D., Network, W.H.-c.G.R.S., 2012. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. *Lancet Infect Dis* 12, 136-141.
- Wang, W., Xu, L., Brandsma, J.H., Wang, Y., Hakim, M.S., Zhou, X., Yin, Y., Fuhler, G.M., van der Laan, L.J., van der Woude, C.J., Sprengers, D., Metselaar, H.J., Smits, R., Poot, R.A., Peppelenbosch, M.P., Pan, Q., 2016. Convergent transcription of interferon-stimulated genes by TNF-alpha and IFN-alpha augments antiviral activity against HCV and HEV. *Sci Rep* 6, 25482.
- Wang, W., Xu, L., Su, J., Peppelenbosch, M.P., Pan, Q., 2017. Transcriptional regulation of antiviral interferon-stimulated genes. *Trends Microbiol* 25, 573-584.
- Xu, L., Zhou, X., Wang, W., Wang, Y., Yin, Y., Laan, L.J., Sprengers, D., Metselaar, H.J., Peppelenbosch, M.P., Pan, Q., 2016. IFN regulatory factor 1 restricts hepatitis E virus replication by activating STAT1 to induce antiviral IFN-stimulated genes. *FASEB J* 30, 3352-3367.
- Yarilina, A., Park-Min, K.H., Antoniv, T., Hu, X., Ivashkiv, L.B., 2008. TNF activates an IRF1-dependent autocrine loop leading to sustained expression of chemokines and STAT1-dependent type I interferon-response genes. *Nat Immunol* 9, 378-387.
- Yin, Y., Bijvelds, M., Dang, W., Xu, L., van der Eijk, A.A., Knipping, K., Tuysuz, N., Dekkers, J.F., Wang, Y., de Jonge, J., Sprengers, D., van der Laan, L.J., Beekman, J.M., Ten Berge, D., Metselaar, H.J., de Jonge, H., Koopmans, M.P., Peppelenbosch, M.P., Pan, Q., 2015a. Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. *Antiviral Res* 123, 120-131.
- Yin, Y., Metselaar, H.J., Sprengers, D., Peppelenbosch, M.P., Pan, Q., 2015b. Rotavirus in organ transplantation: drug-virus-host interactions. *Am J Transplant* 15, 585-593.

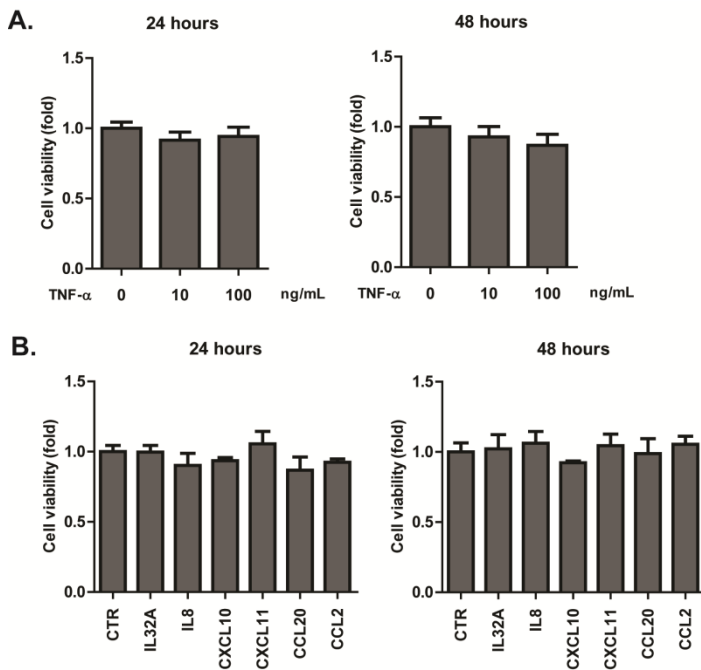
Supplementary Figures



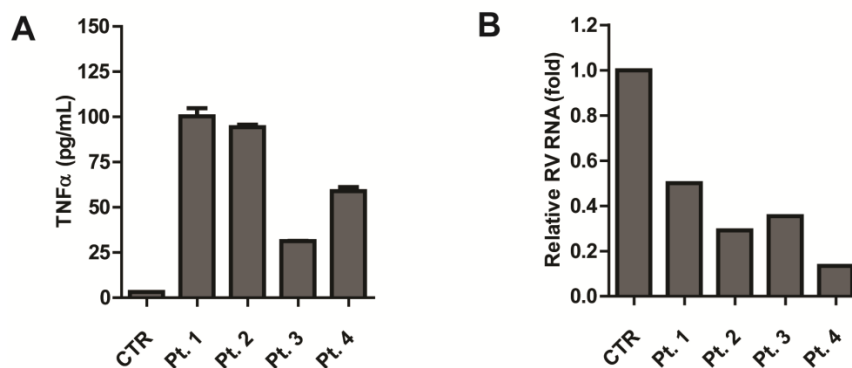
Supplementary Figure 1. Standard curve for quantifying rotavirus genome copy numbers. An SA11 amplicon was first cloned into the pCR2.1-TOPO vector. The isolated plasmid was diluted from 10^{-2} to 10^{-10} dilution and then amplified and quantified by qRT-PCR. A standard curve was generated by plotting the cycle threshold (CT) value with regard to the log copy number value.

Supplementary Figure 2. A. SA11 rotavirus infection did not modulate *tnfa*, *IL1B* and *IL8* gene expression levels in Caco2 cells at 6, 24, 36 and 48 hours post-infection. Caco2 cells were infected with rotavirus SA11. Relative RNA levels of *tnfa* (A), *IL1B* (B) and *IL8* (C) genes were examined at 6, 24, 36 and 48 hours post infection and compared to uninfected cells. Data were normalized to GAPDH and presented as means \pm SEM (n = 9).

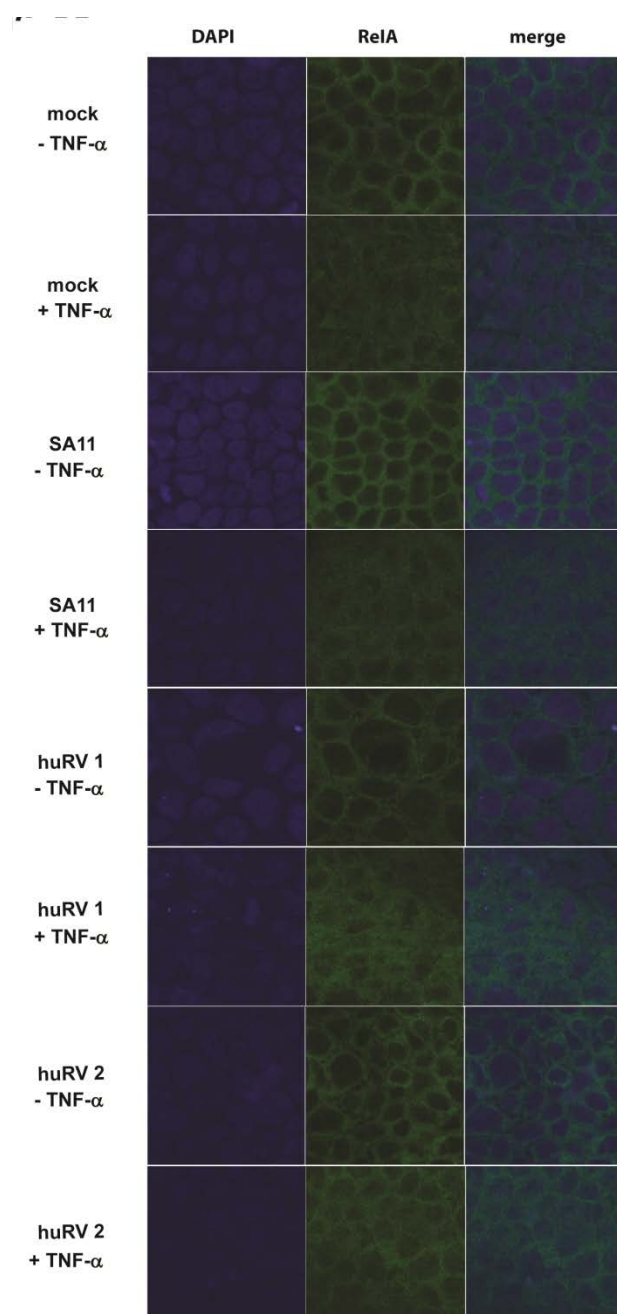




Supplementary Figure 3. The effects of TNF- α and cytokines on cell viability. TNF- α (10 and 100 ng/mL) **(A)** and several cytokines (10 ng/mL of IL32A, IL8, CXCL10, CXCL11, CCL20, CCL2) **(B)** did not affect cell viability as determined by MTT assay (OD₄₉₀ value) at 24 and 48 hours of treatment (n = 6-8).



Supplementary Figure 4. (A) TNF- α levels in serum samples from anti-TNF- α treatment naïve Crohn's disease patients were quantified by ELISA. **(B)** All patient serum samples showed anti-rotavirus activity as compared to the control serum sample as quantified by qRT-PCR at 48 hours post-infections.



Supplementary Figure 5. Rotavirus infection did not inhibit TNF α -induced nuclear accumulation of RelA. Caco2 cells were infected with either mock, SA11, huRV 1 and huRV 2 rotavirus for 8 hours. Subsequently, the mock- or rotavirus-infected cells were stimulated with TNF- α (100 ng/mL) for 1 hour. Fixed and permeabilized cells were stained with antibodies to RelA (green), and visualized with confocal microscopy. Nuclei were visualized by DAPI (blue).

Supplementary Tables

Supplementary Table 1. Primers used in the study

Sequence of SA11 rotavirus primers (targeting VP6 gene segment)

Primers	Sequences
Sense	TGGTTAAACGCAGGATCGGA
Anti-sense	AACCTTTCCGCGTCTGGTAG

Sequence of human-derived rotavirus primers (targeting NSP3 gene segment)

Primers	Sequences
Sense	ACCATCTACACATGACCCTC
Anti-sense	CACATAACGCCCTATAGCC

Primer sequences of human genes

Primers	Sequences
GAPDH-F	GTCTCCTCTGACTTCAACAGCG
GAPDH-R	ACCACCCTGTTGCTGTAGTAGCCAA
TNFα-F	CTCTTCTGCCTGCTGCACTTTG
TNFα-R	ATGGGCTACAGGCTTGTCCTC
IL8-F	CACTGCGCCAACACAGAAATTA
IL8-R	ACTTCTCCACAACCCTCTGCAC
IL1B-F	CCCTAAACAGATGAAGTGCTCCTT
IL1B-R	GTAGCTGGATGCCGCCAT
IFNα-F	GACTCCATCTTGGCTGTGA
IFNα-R	TGATTTCTGCTCTGACAACT
IFNβ1-F	CTTGGATTCTACAAAGAAGCAGC
IFNβ1-R	TCCTCCTTCTGGAAGTCTGCA
TNFR1-F	CCGCTTCAGAAAACCACTCAG
TNFR1-R	ATGCCGGTACTGGTTCTTCCTG
RelA-F	TGAACCGAAACTCTGGCAGCTG
RelA-R	CATCAGCTTGCGAAAAGGAGCC

Supplementary Table 2. shRNA sequences

Name	Oligo Sequences
shTNFR1-94	CCGGCATTGGTTTAATGTATCGCTACTCGAGTAGCGATACATTAAACCAATGTT TTTG
shTNFR1-95	CCGGACCGGCATTATTGGAGTGAAACTCGAGTTTCACTCCAATAATGCCGGTT TTTTG
shTNFR1-96	CCGGTCCTGTAGTAACTGTAAGAAACTCGAGTTTCTTACAGTTACTACAGGATT TTTG
shRelA-08	CCGGGCAGGCTATCAGTCAGCGCATCTCGAGATGCGCTGACTGATAGCCTGCT TTTT
shRelA-09	CCGGCACCATCAACTATGATGAGTTCTCGAGAACTCATCATAGTTGATGGTGT TTTT
shRelA-10	CCGGCCTGAGGCTATAACTCGCCTACTCGAGTAGGCGAGTTATAGCCTCAGGT TTTT

Chapter 8

6-thioguanine inhibits rotavirus replication through suppression of Rac1 GDP/GTP cycling

Yuebang Yin,^{1,2,3‡} Sunrui Chen,^{2‡} **Mohamad S. Hakim,**^{2,4‡} Wenshi Wang,² Lei Xu,² Wen Dang,²
Changbo Qu,² Auke P. Verhaar,² Junhong Su,^{1,2,5} Gwenny M. Fuhler,² Maikel P.
Peppelenbosch,² and Qiuwei Pan²

1 Center for Biomedical Research, Northwest Minzu University, Lanzhou, China

2 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands.

3 Institute of Subtropical Agriculture, Chinese Academic of Sciences, Changsha 410125, China

4 Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

5 Medical Faculty, Kunming University of Science and Technology, Kunming, PR China

‡ These authors contributed equally to this work.

Antiviral Research 2018; 156: 92-101.

Abstract

Rotavirus infection has emerged as an important cause of complications in organ transplantation recipients and might play a role in the pathogenesis of inflammatory bowel disease (IBD). 6-thioguanine (6-TG) has been widely used as an immunosuppressive drug for organ recipients and treatment of IBD in the clinic. This study aims to investigate the effects and mode-of-action of 6-TG on rotavirus replication. Human intestinal Caco2 cell line, 3D model of human primary intestinal organoids, laboratory rotavirus strain (SA11) and patient-derived rotavirus isolates were used. We have demonstrated that 6-TG significantly inhibits rotavirus replication in these intestinal epithelium models. Importantly, gene knockdown or knockout of Rac1, the cellular target of 6-TG, significantly inhibited rotavirus replication, indicating the supportive role of Rac1 for rotavirus infection. We have further demonstrated that 6-TG can effectively inhibit the active form of Rac1 (GTP-Rac1), which essentially mediates the anti-rotavirus effect of 6-TG. Consistently, ectopic over-expression of GTP-Rac1 facilitates, but an inactive Rac1 (N17) or a specific Rac1 inhibitor (NSC23766) inhibits rotavirus replication. In conclusion, we have identified 6-TG as an effective inhibitor of rotavirus replication via the inhibition of Rac1 activation. Thus, for transplantation patients or IBD patients infected with rotavirus or at risk of rotavirus infection, the choice of 6-TG as a treatment appears rational.

Keywords: intestinal organoids; Rac1; rotavirus; 6-thioguanine

Introduction

Rotavirus, a double stranded RNA (dsRNA) virus in the *Reoviridae* family, is a major cause of gastroenteritis, particularly in children younger than 5 years of age. As a major global health problem, this virus causes 114 million diarrhea episodes, 2.4 million hospitalizations, and an estimated 215,000 deaths worldwide annually (Grimwood and Buttery, 2007; Tate et al., 2016). Although rotavirus infection mainly occurs in developing countries, it also results in over 200 deaths and more than 87,000 hospital admissions in infants in European Union (Vesikari et al., 2007). Besides young children, organ transplantation patients are also susceptible to rotavirus infection irrespective of their age, causing long-term diarrhea and even death due to graft failure (Yin et al., 2015b). Although two global licensed rotavirus vaccines have been launched, no specific antiviral treatment is available.

A thio analogue of the naturally occurring purine base guanine, 6-thioguanine (6-TG), has been used in the clinic since the early 1950s (Munshi et al., 2014). 6-TG was initially developed to treat cancer; whereas currently it is widely used as an immunosuppressive agent in organ transplantation. It is also used as treatment for acute lymphoblastic leukemia in children and for autoimmune diseases (Bourguine et al., 2011). In particular, 6-TG is often used to treat inflammatory bowel disease (IBD) (de Boer et al., 2006). IBD including Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis (IC) represent a heavy burden in Western countries (Kolho et al., 2012). Although the causes of exacerbations of IBD remain poorly characterized, gastrointestinal infections including rotavirus might induce flares in IBD (Masclee et al., 2013). Thus, preventing or treating rotavirus infection in these patients is of importance.

Upon ingestion, 6-TG is first metabolized into 6-thioguanosine monophosphate (6-TGMP), and subsequently into 6-thioguanosine diphosphate (6-TGDP), and finally into 6-thioguanosine triphosphate (6-TGTP) (Chouchana et al., 2012). Among these metabolites, 6-TGDP and 6-TGTP are able to compete with endogenous guanosine phosphates for Rac1 binding and to form 6-TGNP•Rac1 complexes. These complexes are in turn incapable to support the formation of the active configuration of Rac1, a process that Rac1 interacts with GTP. Thus, 6-TG indirectly provokes inhibition of Rac1-dependent signaling (Shin et al., 2016), which has substantial consequences for cellular physiology. As a member of the Ras

superfamily of Rho GTPases, GTP-bound Rac1 mediates a myriad of cellular processes including actin reorganization and gene transcription. Intriguingly, IBD is characterized by hyperactivation of Rac1 in the phagocyte compartment. This is associated with reduced effector function of Rac1, which is sensitive to 6-TG treatment (Parikh et al., 2014). The inhibition of Rac1 resulting from 6-TG treatment restores innate immune functionality of phagocytes in IBD patients, contributing to disease remission (Parikh et al., 2014). Thus, Rac1 hyperactivation appears an important immunosuppressive effector in human pathophysiology, at least in the phagocyte compartment.

Given the clinical relevance and the potential in revealing mechanistic insight, we have investigated the effects and mechanism-of-action of 6-TG on rotavirus replication. To this end, we have demonstrated that 6-TG effectively combats rotavirus replication through inhibition of Rac1 activation.

Materials and Methods

Viruses and reagents

Simian rotavirus SA11 strain and patient-derived rotavirus isolates (G1P[8]) were prepared as previously described (Yin et al., 2015a; Yin et al., 2016).

Stocks (0.1 mg/mL) of 6-TG (Sigma-Aldrich) were dissolved in alkali solution (1 M NaOH, 50 mg/ml), and NSC23766 (Merck Millipore) was dissolved in H₂O (2 mM). All chemicals were stored in 25 µL aliquots and frozen at -80 °C.

Conventional enterocyte culture and human primary intestinal organoid culture

Human colon cancer cell line Caco2 and human embryonic kidney cell line 293T (HEK 293T) cells were grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco, Breda, The Netherlands) supplemented with 20% (vol/vol) heat-inactivated fetal calve serum (FCS) (Hyclone, Lonan, Utah) and penicillin (100 IU/mL)/streptomycin (100 mg/mL) (Invitrogen-Gibco) at 37°C in a humidified 5% CO₂ incubator. Rac1 knockout mouse embryonic fibroblast (MEF) cells were cultured in DMEM supplemented with 10% FCS, Penicillin (100

IU/mL)/streptomycin (100 mg/mL), L-Glutamine (Gibco[®] by Life Technologies), non-essential amino acids (Gibco[®] by Life Technologies) and sodium-pyruvate (Gibco[®] by Life Technologies). Caco2 cells with stable knockdown of Rac1 were generated by transduction of lentiviral shRNA (produced in HEK293T cells) targeting Rac1 and selected with puromycin (6 µg/mL) as described previously (Yin et al., 2016). The shRNA targeting sequences used in this study were listed in Table S1.

3D culture of human intestinal organoids was performed as previously described (Yin et al., 2015a).

Rotavirus inoculation and drug treatment

Inoculation and treatment of Caco2 cells and human intestinal organoids with SA11 and patient-derived rotavirus were performed as previously described (Yin et al., 2016).

Viability assay of cells or organoids

The viability of cells or organoids was determined by 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, Caco2 cells (1×10^4 cells/well) or organoids were seeded into a 96-well culture plate and incubated with various concentrations of 6-TG or NSC23766 for 48 h, followed by adding 500 µg/mL of MTT solution to each well and incubation at 37 °C for 3 hrs. Subsequently, the medium was removed, and replaced with 100 µL DMSO and incubated at 37 °C for 50 min. Then, the absorbance was measured at 490 nm in an enzyme-linked immunosorbent assay reader (BIO-RAD). The effects of 6-TG, NSC294002, IFN α and ribavirin on host cell viability were determined by MTT assay (Fig. S1).

Transfection of plasmids

A constitutively active Rac1V12 and dominant inactive Rac1N17 plasmids were prepared as previously described (Apolloni et al., 2013). HEK 293 cells were seeded into 6-well plates for Rac activation assay or 48-well plates for infection assay with ~70% confluence. The cells were washed with PBS, followed by adding 500 µL of Opti-MEM[®] reduced serum medium with 2 µg Rac1V12 or Rac1N17 plasmids and 10 µg polyethylenimine (PEI) per well of a 6-well plate, or 100 µL of Opti-MEM[®] reduced serum medium (Thermo Fisher Scientific) with

0.25 µg Rac1V12 or Rac1N17 plasmids and 1.25 µg polyethylenimine (PEI) (Sigma-Aldrich) per well of a 48-well plate. After 4 to 5 hrs of incubation, 2 mL or 0.5 mL of DMEM containing 10% FCS was added to each well of 6-well plate or 48-well plate, respectively. Transfected cells were infected with rotavirus for 24 hrs.

Quantitative real-time PCR (qRT-PCR)

Total cellular RNA was isolated using a NucleoSpin[®] RNA kit (MACHEREY-NAGEL, Düren, Germany) following the manufacturer's protocol, and quantified with a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was synthesized using the reverse transcription system from TAKARA according to manufacturer's instructions (TAKARA BIO INC). The resulting cDNA was diluted 1:10, and 2 µL of the diluted cDNA was used for qRT-PCR with primers listed in Table S1. All qRT-PCR experiments were performed by SYBR-Green-based (Applied Biosystems SYBR Green PCR Master Mix; Thermo Fisher Scientific Life Science) real-time PCR with the StepOnePlus System (Thermo Fisher Scientific Life Sciences). The expression of target mRNA was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Gene expression analysis was performed by the $\Delta\Delta C_T$ method (Yin et al., 2015a; Yin et al., 2016).

Rac activation assay

The levels of Rac1-bound GTP were detected using Rac interactive binding (CRIB) domain of PAK (aa56-272) as described previously (Fuhler et al., 2008). In brief, GST-PAKcrib protein was pre-coupled to glutathione-sepharose beads (Sigma-Aldrich) for 45 min at 4 °C. Caco2 cells treated with various concentrations of 6-TG or NSC23766 (48 h, seeded in 6-well-plate) were lysed for 10 min in lysis buffer (50 mM Tris, pH 7.4, 10% glycerol, 200mM NaCl, 1% NP-40, 2mM MgCl₂, 2 mM sodium orthovanadate, and protease inhibitors). Cell lysates were incubated with pre-coupled beads for 45 min. Then, agarose beads were washed 3 times with 1x lysis buffer, followed by boiling in Laemmli buffer, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15%) analysis.

Western blotting

Cell lysates were subjected to SDS-PAGE, and proteins were transferred to PVDF membrane (Immobilon-FL). Rac1 monoclonal antibody (1:1000, rabbit; cell signaling) and SA11 rotavirus

VP4 [1:1000, HS-2, a mouse monoclonal antibody (provided by Professor Harry Greenberg, Stanford University School of Medicine, USA)] were detected by Western blotting analysis. Detection of β -actin was served as loading control (1:1000, mouse monoclonal; Santa Cruz) as previously described (Versteeg et al., 2002).

Serial passaging of rotavirus with 6-TG treatment

To investigate whether rotavirus can develop resistance to 6-TG treatment, viruses were passaged in both MA104 and Caco2 cells in the absence of drug (vehicle control) or in the presence of gradually increasing concentrations of the drug (1000 ng/mL of 6-TG for passage 1-10 and 2000 ng/mL of 6-TG for passage 11-20). In brief, MA104 or Caco2 cells in 24-well plate were inoculated with 200 μ L virus (MOI=0.7) at 37 °C for 1 hr, followed by adding 6-TG or without drug (as control). After 48 hrs, both cells and supernatant were harvested, subsequently frozen, thawed once, and centrifuged. The supernatant containing passaged viruses was stored at -80 °C until used for the next passage. Viruses were serially passaged by using 1 aliquot of viral stock from the preceding passage to infect fresh MA104 or Caco2 cells. The effect of each passage of virus (same titer) was quantified by qRT-PCR.

IC50 and CC50 calculation

IC50 and CC50 calculation were described previously (Yin et al., 2016).

Statistics

Data are presented as means, and statistical comparison between different groups was performed by Mann-Whitney test (two-tailed) using GraphPad Prism 5. Error bars represent the SEM, and *P* value <0.05 was considered statistically significant.

Results

6-TG remarkably inhibits rotavirus replication

To assess the effects of 6-TG on rotavirus replication, we treated SA11 rotavirus-infected Caco2 cells with various concentrations (0.001 – 10000 ng/mL) of 6-TG for 48 hrs. This resulted in dose-dependent inhibition of rotavirus RNA replication (Fig. 1A, *n* = 3-17, **P*<0.05,

*** $P < 0.001$). The IC_{50} value of 6-TG against SA11 rotavirus was 3.0×10^{-13} M, which is substantially below the concentrations reached in patients undergoing 6-TG or azathioprine therapy. CC_{50} of 6-TG to Caco2 cells was 9.8×10^{-6} M and selectivity index (SI, CC_{50}/IC_{50}) was 3.3×10^7 (Table S2). This was further confirmed at the infectious virus production levels and protein levels of the rotavirus VP4 protein (Fig. 1B and 1C). To further verify the effect of 6-TG on rotavirus replication, SA11 rotavirus-infected Caco2 cells were treated with 6-TG at different time points (6, 12, 24 and 48 hrs), demonstrating that 6-TG significantly inhibited rotavirus RNA replication in time-dependent Manner (Fig. 1D, $n = 4-8$, * $P < 0.05$, ** $P < 0.01$). These results were substantiated by experiments in human primary intestinal organoids, a model system that recapitulates many aspects of the intestinal epithelium, including the presence of a villus domain and a crypt domain (Fig. 1E) (Sato and Clevers, 2013). 6-TG significantly inhibited SA11 rotavirus replication and virus production in these organoids (Fig. 1F and G, $n = 6$, * $P < 0.05$, ** $P < 0.01$). Our results were essentially repeated using five patient-derived rotavirus isolates in Caco2 cells but also in human intestinal organoids (Table S3). Treatment with 100 ng/mL 6-TG inhibited patient-derived isolates in both Caco2 cells (Fig. 1H) and human intestinal organoids (Fig. 1I). Hence, 6-TG significantly counteracts rotavirus replication at clinically relevant concentrations.

Development of resistance to 6-TG is uncommon for rotavirus

Rotavirus was serially passaged in the presence of escalating concentrations of 6-TG to assess the potential of drug resistance development. As a control, wild-type SA11 rotavirus was passaged in the absence of the drug. During the initial ten passages, infected cultures were exposed to 1000 ng/mL of 6-TG; whereas the drug concentration was increased to 2000 ng/mL at the subsequent later passages. After 20 passages, the effects of 6-TG (100 ng/mL) on each representative passage (both drug treated and untreated) of rotavirus was quantified by qRT-PCR. Our results indicate that rotavirus remains sensitive to 6-TG treatment in MA104 cells (Fig. 2A) and Caco2 cells (Fig. 2B). Therefore, rotavirus does not easily develop 6-TG resistance, suggesting that the antiviral effects of 6-TG likely depend on a cellular target but not a direct interaction with the virus itself.

Rac1, the cellular target of 6-TG, sustains rotavirus replication

Although initially characterized as a nucleotide synthesis inhibitor, in the last decade it has become clear that many of the effects of 6-TG, at least in gastrointestinal pathophysiology, depend on its potency to inhibit Rac1 (Shin et al., 2016). Thus, we investigated the potential role of Rac1 on rotavirus replication. To this end, we performed a lentiviral RNAi-mediated loss-of-function assay to silence the Rac1 gene. Two (no. 9689 and 9691) out of five RNAi vectors showed potent knockdown of the target gene (Fig. 3A). Importantly, these two RNAi vectors resulted in $65.0 \pm 0.1\%$ ($n = 10$, $P < 0.001$) and $61.8 \pm 0.2\%$ ($n = 6$, $P < 0.05$) reduction of SA11 rotavirus viral RNA, respectively (Fig. 3B). In apparent agreement, we obtained Rac1 knockout mouse embryonic fibroblasts cells (MEFs), and their status as a *bona fide* knockout was confirmed by western blot assay (Fig. 3C). Rotavirus replication in Rac1 knockout MEFs (-/-) was significantly less efficient ($62.4 \pm 0.2\%$; $n = 4$; $P < 0.05$), compared with the replication in wild type MEF (If/If) (Fig. 3D). Thus, decreased Rac1 levels correlate with increased resistance towards rotavirus replication.

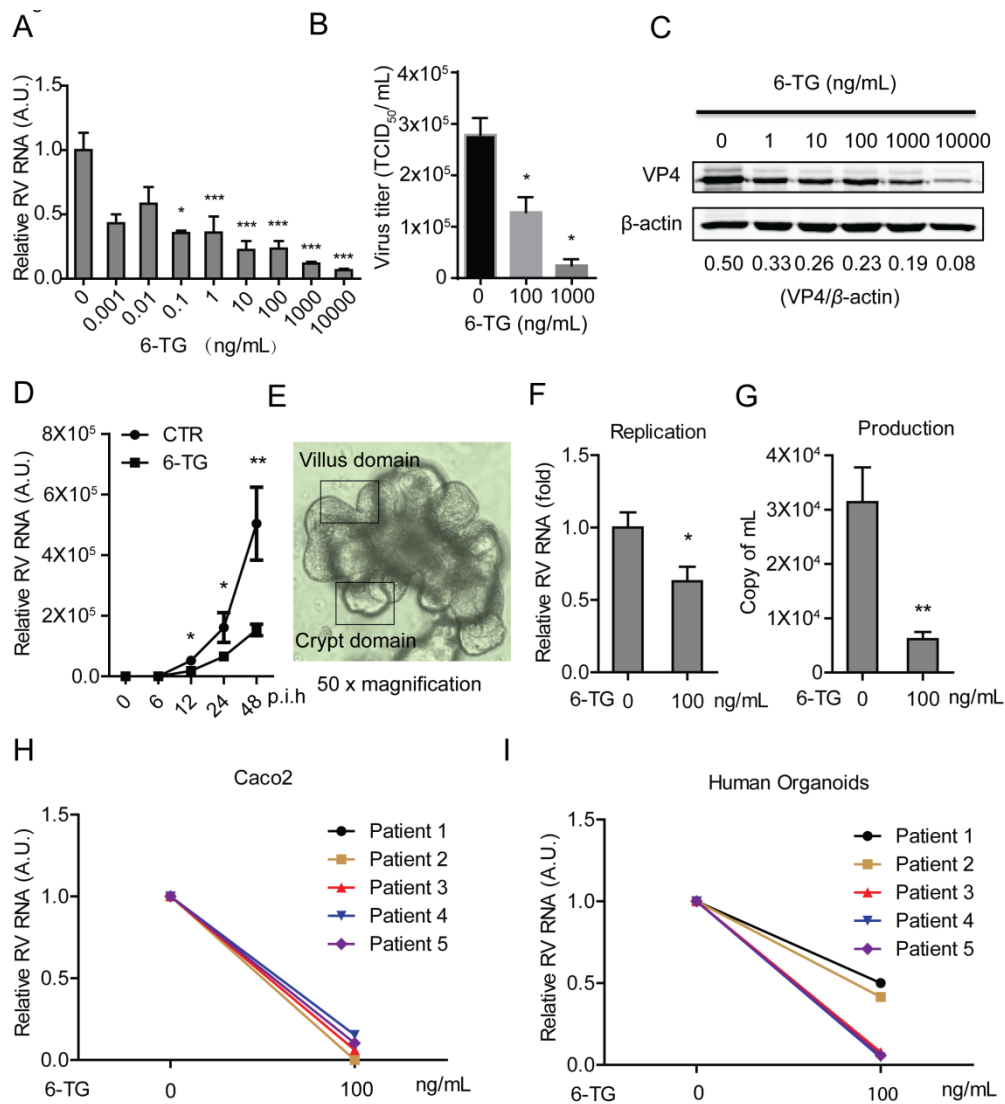


Fig. 1. 6-TG effectively inhibits rotavirus replication. (A) Treatment with 6-TG (48 hrs) significantly inhibited viral genomic RNA in SA11 rotavirus infected Caco2 cells (MOI = 0.7) in a dose-dependent manner ($n = 6-17$, means \pm SEM, $*P < 0.05$, $***P < 0.001$, Mann-Whitney test, A.U. denotes artificial unit). (B) Effects of 6-TG on the production of infectious viral particles determined by TCID₅₀ method. Each bar represents the TCID₅₀/mL (mean \pm SEM) ($n = 3$, $P < 0.05$, Mann-Whitney test). (C) Treatment with 6-TG (48 h) significantly inhibited viral VP4 protein in SA11 rotavirus infected Caco2 cells in a dose-dependent manner (the densitometric analysis of immunoreactive bands in western blots was quantified by ImageJ software, and the ratio of VP4/β-actin was expressed in arbitrary units). (D) Treatment with 6-TG (6 - 48 hrs) significantly inhibited viral genomic RNA in SA11 rotavirus infected Caco2 cells (MOI = 0.7) in a time-dependent manner ($n = 4-8$, means \pm SEM, $*P < 0.05$, $**P < 0.01$, Mann-Whitney test, p.i.h denotes hour of post infection). (E) The morphology of human primary small intestinal organoids with clear villus domain and crypt domain. (F) Treatment with 6-TG (48 hrs) significantly inhibited genomic RNA in SA11 rotavirus (MOI = 7) infected human intestinal organoids ($n = 6$, means \pm SEM, $*P < 0.05$, Mann-Whitney test). (G) Treatment with 6-TG (48 hrs) significantly inhibited viral production in SA11 rotavirus infected human intestinal organoids ($n = 6$, means \pm SEM, $**P < 0.01$, Mann-Whitney test). (H) Treatment with 100 ng/mL of 6-TG (48 hrs) inhibited viral RNA of patient-derived rotavirus isolates in Caco2 cells. (I) Treatment with 100 ng/mL of 6-TG (48 hrs) inhibited viral RNA of patient-derived rotavirus isolates in human intestinal organoids.

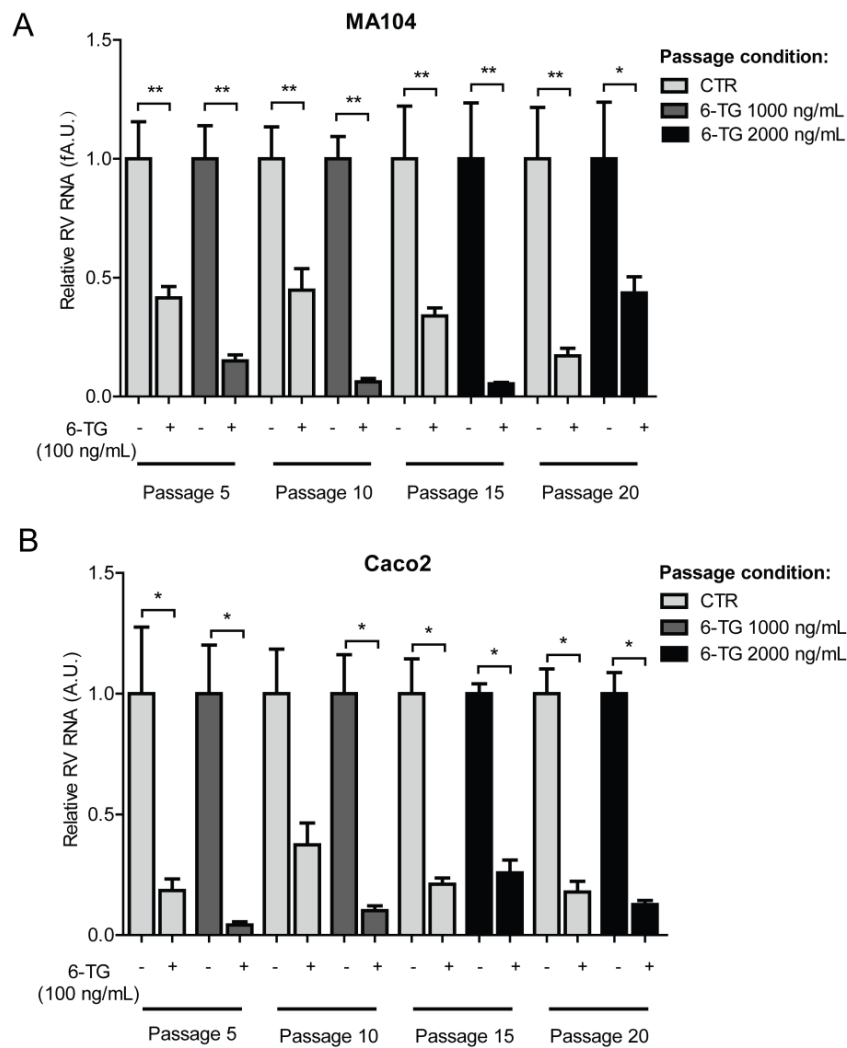


Fig. 2. 6-TG has a high barrier to drug resistance development. Rotavirus was serially passaged in MA104 or Caco2 cells exposed to no 6-TG (as control) and increasing concentrations of 6-TG until 20 passages (infected cultures were exposed to 1000 ng/mL of 6-TG in passage 1-10; whereas the drug concentration was increased to 2000 ng/mL at the subsequent later passages). The effect of 6-TG (100 ng/mL) on passage 5, 10, 15 and 20 (the drug treated and untreated) of rotavirus was quantified using qRT-PCR. 6-TG retained its anti-rotavirus activity even with continuous exposure to 6-TG for 20 passages in MA104 cells (A) and Caco2 cells (B).

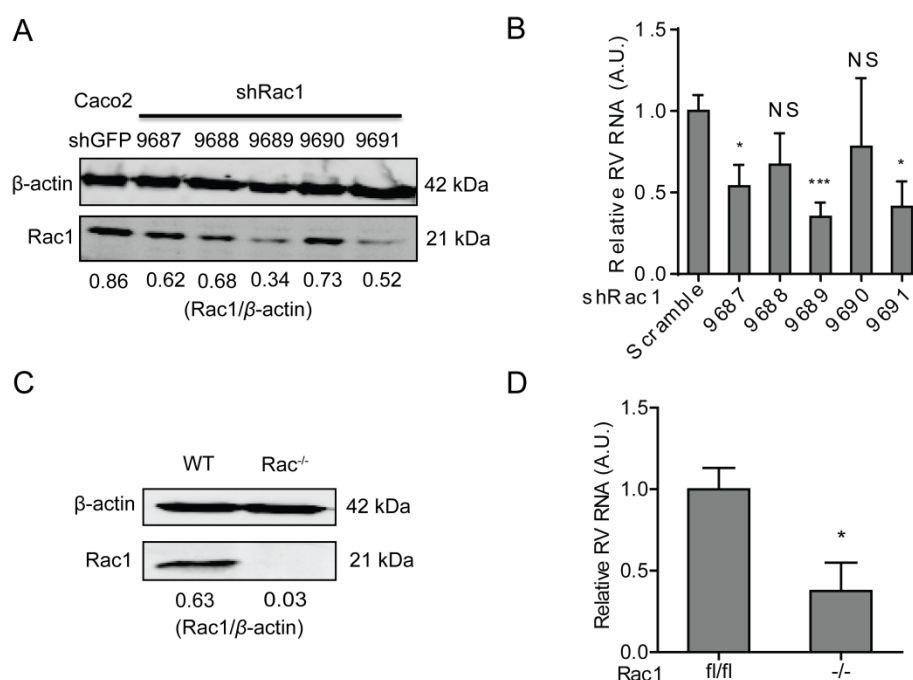


Fig. 3. Rac1, the drug target of 6-TG, sustains rotavirus replication. (A) Western blot assay detected Rac1 in transduced Caco2 cells transduced lentiviral RNAi vectors against Rac1 (The ratio of Rac1/β-actin was expressed in arbitrary units). (B) Three (No. 9687, 9689 and 9691) out of five lentiviral shRNA vectors inhibited rotavirus genomic RNA ($n = 6-10$, means \pm SEM, $*P < 0.05$, $***P < 0.001$, Mann-Whitney test). (C) Western blot assay confirmed knockout of Rac1 in Rac1 knockout (-/-) MEF cells. (D) SA11 rotavirus replication was significantly attenuated in Rac1 knockout (-/-) MEF cells ($n = 4$, means \pm SEM, $*P < 0.05$, Mann-Whitney test).

Efficient rotavirus replication requires Rac1 activation

Rac1 acts as binary switch in cellular biochemistry, and it is only capable of provoking signaling in the active GTP-bound form (Shin et al., 2016). NSC23766, a specific GTP-Rac1 inhibitor was able to effectively inhibit GTP-Rac1 accumulation in Caco2 cells, as evident when tested in GTP-Rac1 specific pull-down assay (Fig. 4A and B). Importantly, treatment with either 5 or 25 μ M NSC23766 for 48 hrs resulted in $58.8 \pm 0.1\%$ ($n = 8$; $P < 0.05$) and $77.48 \pm 0.1\%$ ($n = 10$; $P < 0.001$) reduction on viral RNA levels, respectively (Fig. 4C). The IC_{50} value of NSC23766 against SA11 rotavirus was 1.1×10^{-8} M, the CC_{50} of NSC23766 to Caco2 cells was 3.1×10^{-4} M, and the SI was 2.8×10^4 (Table. S2). The inhibitory effect of NSC23766 on the rotavirus infectious was further verified by a western blot assay, revealing that treatment with either 1, 5 or 25 μ M NSC23766 significantly inhibits rotavirus VP4 protein synthesis in Caco2 cells (Fig. 4D). The effect of this drug was further confirmed by experiments in human primary intestinal organoids, which indicated $91.1 \pm 0.1\%$ ($n = 6$; $P < 0.05$) reduction of viral RNA in the organoids (Fig. 4E) following treatment with NSC23766.

Next, we tested the effects of constitutively active and dominant negative forms of Rac1 on rotavirus replication. This was done by transfection of the active Rac1V12 plasmid or the dominant negative Rac1N17 plasmid. Expression of both plasmids was successful when tested by flow cytometry (Fig. 4F). Accordingly, Rac1V12-transfected cells displayed abundant GTP-Rac1, which in contrast is low in Rac1N17-transfected cells, as determined in a Rac pull-down assay (Fig. 4G). These results were confirmed by analyzing abundance of phospho-PAK2 status (Fig. 4H). Importantly, Rac1V12 promoted, but Rac1N17 inhibited rotavirus replication (Fig. 4I). Taken together, activation of Rac1 supports rotavirus replication.

6-TG inhibits rotavirus via suppression of Rac1 GDP/GTP cycling

The inhibition of the activation of Rac1 by 6-TG was reported in many cell types (Fuhler et al., 2008; Shin et al., 2016; Tiede et al., 2003). Employing the Rac1 pull-down assay (Fig. 4A), we observed that 6-TG potently inhibited GTP-Rac1 accumulation; whereas corresponding western blots did not show reduced overall Rac1 levels in Caco2 cells following 6-TG treatment (Fig. 5A). Functional studies using Rac1 knockdown Caco2 cells (Fig. 5B and 5C) were performed to demonstrate that both 6-TG and NSC23766 require Rac1 to combat rotavirus replication. In agreement, pharmacological Rac1 inhibitors did not inhibit rotavirus replication in Rac1 knockout MEF cells (Fig. 5D). Thus 6-TG inhibits rotavirus via suppression of Rac1 activation.

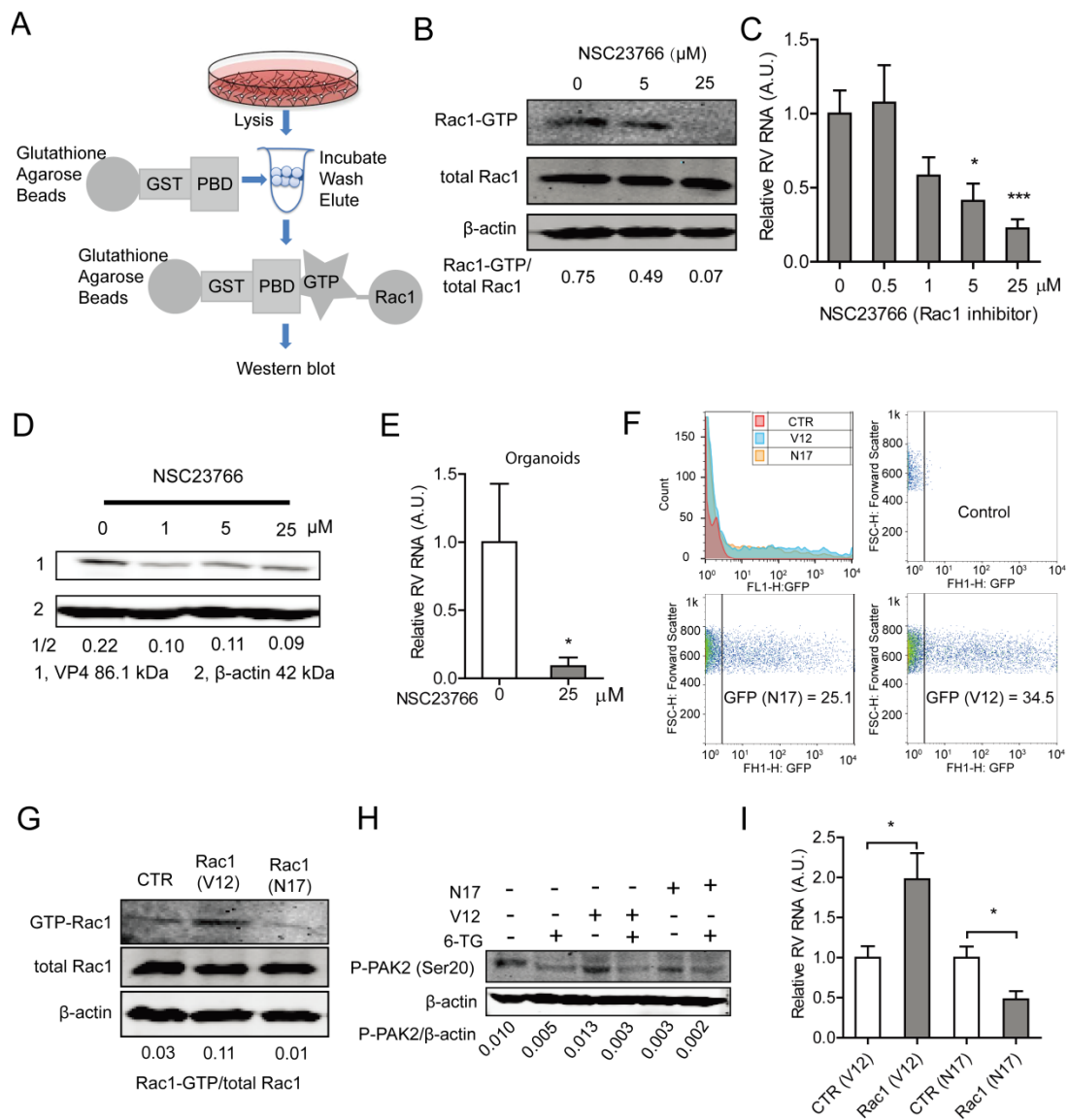


Fig. 4. The activated form of Rac1 is required for supporting rotavirus replication. (A) Schematic depicting the pull-down assay. (B) NSC23766 inhibited GTP-Rac1 as detected by pull-down assay (The ratio of GTP-Rac1/Rac was expressed in arbitrary units). (C) Treatment with NSC23766 (48 hrs) significantly inhibited viral genomic RNA in SA11 rotavirus infected Caco2 cells in a dose-dependent manner ($n = 8-10$, means \pm SEM, $*P < 0.05$, $***P < 0.001$, Mann-Whitney test). (D) Treatment with NSC23766 (48 hrs) significantly inhibited viral VP4 protein in SA11 rotavirus infected Caco2 cells in a dose-dependent manner (The ratio of VP4/ β -actin was expressed in arbitrary units). (E) Treatment with NSC23766 (48 hrs) significantly inhibited viral RNA in SA11 rotavirus infected human intestinal organoids ($n = 6$, means \pm SEM, $*P < 0.05$, Mann-Whitney test). (F) Flow cytometric analysis of green fluorescence indicated the percentages of transduced cells with Rac1V12 and Rac1N17 plasmids. Median fluorescence identity (MFIs) of control, N17 and V12 are 3.43, 35.5 and 84.3, respectively. (G) Pull-down and western blot assays showed higher level of GTP-Rac1 with transduction of Rac1V12 but lower level of GTP-Rac1 with Rac1N17 plasmids (The ratio of GTP-Rac1/Rac was expressed in arbitrary units). (H) Detection of phospho-PAK2 indicated successful transduction of Rac1V12 and Rac1N17 plasmids. (I) Rac1V12 transduction facilitates but Rac1N17 inhibits rotavirus replication ($n = 10$, means \pm SEM, $*P < 0.05$, Mann-Whitney test).

6-TG has no combination effect with IFN α , but moderately antagonistic effect with ribavirin on rotavirus replication

Interferon-alpha (IFN α) and ribavirin are widely used as general antivirals, being confirmed to significantly inhibit rotavirus replication *in vitro* (Yin et al., 2015a). Consistently, we again demonstrated the inhibitory effects of IFN α and ribavirin on rotavirus RNA (Fig. 6A, and 6E) and viral protein (Fig. 6B and 6F) in a dose-dependent manner. The IC₅₀ value of IFN α against SA11 rotavirus was 3.1×10^{-5} IU, CC₅₀ of IFN α to Caco2 cells was 18706 IU, and SI was 6.0×10^8 (Table S2). The IC₅₀ value of ribavirin against SA11 rotavirus was 1.6×10^{-7} M, CC₅₀ of ribavirin to Caco2 cells was 3.02×10^{-2} M and SI was 1.9×10^5 (Table S2). Next, we assessed the combinatory antiviral effects of 6-TG with IFN α or ribavirin. The combination of 6-TG and IFN α resulted in no combination (no synergy or antagonism) antiviral effect, with a synergy volume of $-2.8 \mu\text{M}^2\%$ (Fig. 6C and 6D). However, the combination of 6-TG and ribavirin resulted in moderately antagonistic antiviral effect, with a synergy volume of $-26.02 \mu\text{M}^2\%$ (Fig. 6G and 6H).

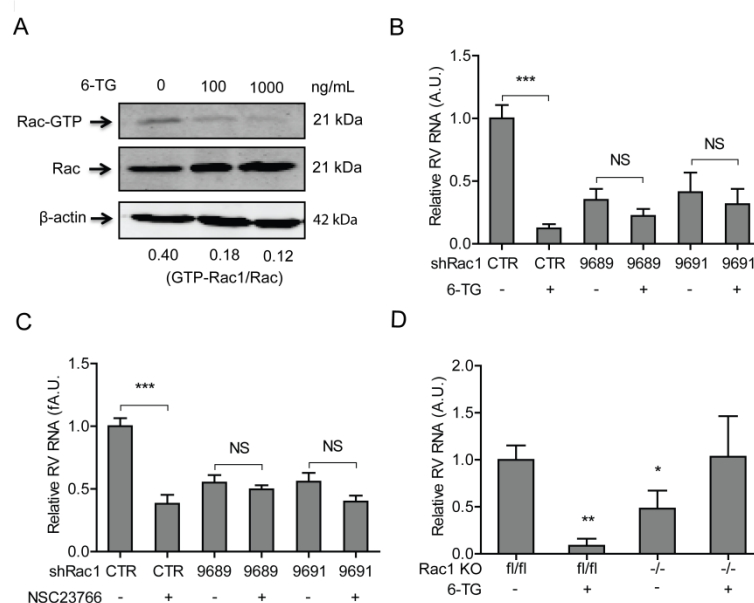


Fig. 5. 6-TG inhibits rotavirus via suppression of Rac1 activation. (A) 6-TG inhibited GTP-Rac1 as detected by pull-down assay. (B) Anti-rotavirus effect of 6-TG (100 ng/mL) was attenuated in Rac1 knockdown Caco2 cells. (C) Anti-rotavirus effect of NSC23766 (25 μg ng/mL) was attenuated in Rac1 knockdown Caco2 cells. (D) The anti-rotavirus effect of 6-TG (100 ng/mL) was attenuated in Rac1 knockout (-/-) MEF cells (n = 6-8, means \pm SEM, * P < 0.05, ** P < 0.01, Mann-Whitney test).

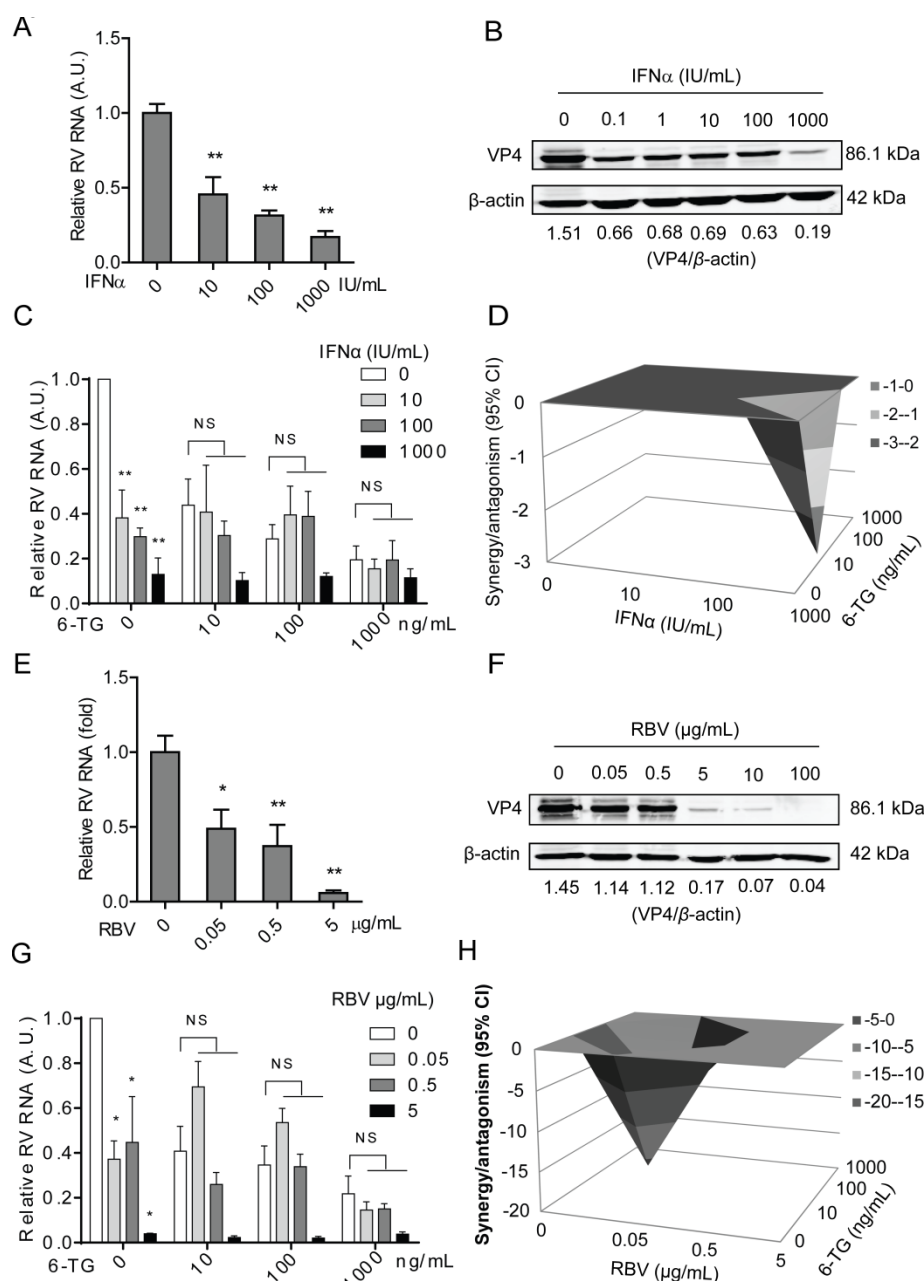


Fig. 6. The effects of the combination of 6-TG with IFN α , or ribavirin on rotavirus replication. (A) Treatment with IFN α (48 hrs) significantly inhibited viral genomic RNA in SA11 rotavirus infected Caco2 cells in a dose-dependent manner ($n = 4$, means \pm SEM, $**P < 0.01$, Mann-Whitney test). (B) Treatment with IFN α (48 hrs) remarkably inhibited viral VP4 protein in SA11 rotavirus infected Caco2 cells in a dose-dependent manner (The ratio of VP4/ β -actin was expressed in arbitrary units). (C) Effect of the combination of various concentrations of 6-TG and IFN α on rotavirus replication in Caco2 cells. (D) Synergy plot representing the percentage of antiviral activity above/below the expected activity for the 6-TG-IFN α combination based on the data shown in C. (E) Treatment with ribavirin (48 hrs) significantly inhibited viral genomic RNA in SA11 rotavirus infected Caco2 cells in a dose-dependent manner ($n = 4-7$, means \pm SEM, $*P < 0.05$, $**P < 0.01$, Mann-Whitney test). (F) Treatment with ribavirin (48 hrs) remarkably inhibited viral VP4 protein in SA11 rotavirus infected Caco2 cells in a dose-dependent manner (The ratio of VP4/ β -actin was expressed in arbitrary units). (G) Effect of the combination of various concentrations of 6-TG and ribavirin on rotavirus replication

in Caco2 cells. (H) Synergy plot representing the percentage of antiviral activity above/below the expected activity for the 6-TG-ribavirin combination based on the data shown in G.

Discussion

In this study, we have demonstrated that 6-TG effectively inhibits rotavirus replication via inhibition of the Rac1 activity. This is particularly interesting in view that rotavirus replication is especially an issue in organ transplantation recipients and in patients with IBD. 6-TG is a therapeutic option for both groups of patients. Based on the findings presented in the current study, the choice of 6-TG for these patients appears rational, in particular when they are at risk of rotavirus infection (Yin et al., 2015b).

Our results fit well with the momentum of studies that document antiviral activity of 6-TG. For instance, it has been reported that 6-TG can combat Middle East respiratory syndrome coronavirus infection by augmenting interferon responses (Cheng et al., 2015) and it has also been reported that Simian virus 40 DNA replication is antagonized by 6-TG (Maybaum et al., 1987). Interestingly, it was reported that vaccinated IBD patients had lower titers of hepatitis B surface antibody (HBsAb), which might be influenced by the use of immunosuppressants including 6-TG (Watts et al., 2017).

As a 6-thiopurine (6-TP) prodrug, 6-TG is converted into pharmacologically active deoxy-6-thioguanosine phosphate (also called 6-thioguanine nucleotide) and 6-thioguanosine phosphate (6-TGNP). 6-TGNP can bind to Rac1 to form the 6-TGNP•Rac1 complex inactivating Rac1 (Shin et al., 2016). As a major player of the Rho family of small GTPases, Rac1 plays a vital role in various cellular signaling pathways to regulate a wide variety of cell functions including gene transcription, cell proliferation, apoptosis, motility, and redox signaling (D'Ambrosi et al., 2014). The expression of Rac1 is ubiquitous, but it has two conformational states including an inactive GDP-bound form and an active GTP-bound form (Bosco et al., 2009). It exerts biological functions mainly through activation of Rac1 (i.e. GTP-bound form) (Bosco et al., 2009). Many viruses interfere with or employ the conformational states of Rac1 to regulate their infection. At early stages of African swine fever virus (ASFV) infection, Rac1 is activated, and inhibition of Rac1 is able to suppress production of this virus (Quetglas et al., 2012). Rac1 is found to be activated during intracellular mature virus (MV) of Vaccinia virus entry (Mercer and Helenius, 2008).

Although rotavirus replication *per se* does not affect the activation of Rac1 (Fig. S2), we have demonstrated that the loss-of-function of Rac1 by gene knockdown or knockout significantly impairs rotavirus replication, which is in line with the previous finding that knockdown Rac1 could significantly inhibit Enterovirus 1 (EV1) infection (Karjalainen et al., 2008). More specifically, the activation of Rac1 is required as shown by the opposing effects of ectopic over-expression of the active or inactive forms of Rac1 on rotavirus replication. This mechanistically explains the potent anti-rotavirus effects of the GTP-Rac1 inhibitors, 6-TG and NSC23766. Of note, NSC23766 has been shown to inhibit the replication of several influenza viruses including a human virus strain from the 2009 pandemic and highly pathogenic avian virus strains (Dierkes et al., 2014).

Despite the absence of approved medications for treating rotavirus, the widely used general antivirals including ribavirin and IFN α have been studied on rotavirus in experimental models (Yin et al., 2015a). Here, we have evaluated the combinatory effects of 6-TG with IFN α or ribavirin. Consistently, we confirmed that ribavirin and IFN α inhibit rotavirus replication at both RNA and protein levels (Fig. 6A and 6B). We found increased potency of IFN α in the presence of 6-TG. However, whether the combination of IFN α and 6-TG could be used to treat rotavirus infected patients remains to be further investigated.

In conclusion, this study has demonstrated that 6-TG effectively inhibits rotavirus replication with a high barrier to drug resistance development. We further identified the active form of Rac1 as an important host factor supporting rotavirus replication. 6-TG exerts its anti-rotavirus effects via the specific inhibition of Rac1 activation. Herein, this study provided important references for clinicians to optimize medications for organ recipients or IBD patients who are infected with rotavirus or at risk of rotavirus replication. These results may also help the development of new anti-rotavirus therapies.

Conflict of interest statement

All authors declare that they have no conflict of interest.

Acknowledgments

We would like to thank Professor Harry Greenberg (Stanford University School of Medicine, USA) for providing the mouse monoclonal antibody against rotavirus VP4 protein, Prof. Dr. Klemens Rottner and Dr. Anika Steffen (Helmholtz Centre for Infection Research) for providing the Rac1 If/If and Rac1 -/- MEFs cells, and Dr. Mauro Cozzolino (Italian National Research Council) for providing the active Rac1V12 and inactive Rac1N17 plasmids. This study was supported by the Dutch Digestive Foundation (MLDS) for a career development grant (No. CDG 1304 to Q. P.), the Erasmus MC Mrace grant (360525 to Q. P.), and the China Scholarship Council for funding PhD fellowship (201307720045 to Y. Y.).

References

- Apolloni, S., Parisi, C., Pesaresi, M.G., Rossi, S., Carri, M.T., Cozzolino, M., Volonte, C., D'Ambrosi, N., 2013. The NADPH oxidase pathway is dysregulated by the P2X7 receptor in the SOD1-G93A microglia model of amyotrophic lateral sclerosis. *J Immunol* 190, 5187-5195.
- Bosco, E.E., Mulloy, J.C., Zheng, Y., 2009. Rac1 GTPase: a "Rac" of all trades. *Cell Mol Life Sci* 66, 370-374.
- Bourguine, J., Garat, A., Allorge, D., Crunelle-Thibaut, A., Lo-Guidice, J.M., Colombel, J.F., Broly, F., Billaut-Laden, I., 2011. Evidence for a functional genetic polymorphism of the Rho-GTPase Rac1. Implication in azathioprine response? *Pharmacogenet Genomics* 21, 313-324.
- Cheng, K.W., Cheng, S.C., Chen, W.Y., Lin, M.H., Chuang, S.J., Cheng, I.H., Sun, C.Y., Chou, C.Y., 2015. Thiopurine analogs and mycophenolic acid synergistically inhibit the papain-like protease of Middle East respiratory syndrome coronavirus. *Antiviral Res* 115, 9-16.
- Chouchana, L., Narjoz, C., Beaune, P., Lorient, M.A., Roblin, X., 2012. Review article: the benefits of pharmacogenetics for improving thiopurine therapy in inflammatory bowel disease. *Aliment Pharmacol Ther* 35, 15-36.
- D'Ambrosi, N., Rossi, S., Gerbino, V., Cozzolino, M., 2014. Rac1 at the crossroad of actin dynamics and neuroinflammation in Amyotrophic Lateral Sclerosis. *Front Cell Neurosci* 8, 279.
- de Boer, N.K., Reinisch, W., Teml, A., van Bodegraven, A.A., Schwab, M., Lukas, M., Ochsenkuhn, T., Petritsch, W., Knoflach, P., Almer, S., van der Merwe, S.W., Herrlinger, K.R., Seiderer, J., Vogelsang, H., Mulder, C.J., Dutch, T.G.w.g., 2006. 6-Thioguanine treatment in inflammatory bowel disease: a critical appraisal by a European 6-TG working party. *Digestion* 73, 25-31.
- Dierkes, R., Warnking, K., Liedmann, S., Seyer, R., Ludwig, S., Ehrhardt, C., 2014. The Rac1 inhibitor NSC23766 exerts anti-influenza virus properties by affecting the viral polymerase complex activity. *PLoS One* 9, e88520.
- Fuhler, G.M., Drayer, A.L., Olthof, S.G., Schuringa, J.J., Coffey, P.J., Vellenga, E., 2008. Reduced activation of protein kinase B, Rac, and F-actin polymerization contributes to an impairment of stromal cell derived factor-1 induced migration of CD34+ cells from patients with myelodysplasia. *Blood* 111, 359-368.
- Grimwood, K., Buttery, J.P., 2007. Clinical update: rotavirus gastroenteritis and its prevention. *Lancet* 370, 302-304.
- Karjalainen, M., Kakkonen, E., Upla, P., Paloranta, H., Kankaanpaa, P., Liberali, P., Renkema, G.H., Hyypia, T., Heino, J., Marjomaki, V., 2008. A Raft-derived, Pak1-regulated entry participates in

- alpha2beta1 integrin-dependent sorting to caveosomes. *Molecular biology of the cell* 19, 2857-2869.
- Kolho, K.L., Klemola, P., Simonen-Tikka, M.L., Ollonen, M.L., Roivainen, M., 2012. Enteric viral pathogens in children with inflammatory bowel disease. *J Med Virol* 84, 345-347.
- Masclee, G.M., Penders, J., Pierik, M., Wolffs, P., Jonkers, D., 2013. Enteropathogenic viruses: triggers for exacerbation in IBD? A prospective cohort study using real-time quantitative polymerase chain reaction. *Inflamm Bowel Dis* 19, 124-131.
- Maybaum, J., Bainnson, A.N., Roethel, W.M., Ajmera, S., Iwaniec, L.M., TerBush, D.R., Kroll, J.J., 1987. Effects of incorporation of 6-thioguanine into SV40 DNA. *Mol Pharmacol* 32, 606-614.
- Mercer, J., Helenius, A., 2008. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science* 320, 531-535.
- Munshi, P.N., Lubin, M., Bertino, J.R., 2014. 6-thioguanine: a drug with unrealized potential for cancer therapy. *Oncologist* 19, 760-765.
- Parikh, K., Zhou, L., Somasundaram, R., Fuhler, G.M., Deuring, J.J., Blokzijl, T., Regeling, A., Kuipers, E.J., Weersma, R.K., Nuij, V.J., Alves, M., Vogelaar, L., Visser, L., de Haar, C., Krishnadath, K.K., van der Woude, C.J., Dijkstra, G., Faber, K.N., Peppelenbosch, M.P., 2014. Suppression of p21Rac signaling and increased innate immunity mediate remission in Crohn's disease. *Sci Transl Med* 6, 233ra253.
- Quetglas, J.I., Hernaez, B., Galindo, I., Munoz-Moreno, R., Cuesta-Geijo, M.A., Alonso, C., 2012. Small rho GTPases and cholesterol biosynthetic pathway intermediates in African swine fever virus infection. *J Virol* 86, 1758-1767.
- Sato, T., Clevers, H., 2013. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science* 340, 1190-1194.
- Shin, J.Y., Wey, M., Umutesi, H.G., Sun, X., Simecka, J., Heo, J., 2016. Thiopurine prodrugs mediate immunosuppressive effects by interfering with Rac1 protein function. *J Biol Chem* 291, 13699-13714.
- Tate, J.E., Burton, A.H., Boschi-Pinto, C., Parashar, U.D., 2016. Global, regional, and national estimates of rotavirus mortality in children <5 years of age, 2000-2013. *Clin Infect Dis* 62 Suppl 2, S96-S105.
- Tiede, I., Fritz, G., Strand, S., Poppe, D., Dvorsky, R., Strand, D., Lehr, H.A., Wirtz, S., Becker, C., Atreya, R., Mudter, J., Hildner, K., Bartsch, B., Holtmann, M., Blumberg, R., Walczak, H., Iven, H., Galle, P.R., Ahmadian, M.R., Neurath, M.F., 2003. CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. *J Clin Invest* 111, 1133-1145.
- Versteeg, H.H., Sorensen, B.B., Slofstra, S.H., Van den Brande, J.H., Stam, J.C., van Bergen en Henegouwen, P.M., Richel, D.J., Petersen, L.C., Peppelenbosch, M.P., 2002. VIIa/tissue factor interaction results in a tissue factor cytoplasmic domain-independent activation of protein synthesis, p70, and p90 S6 kinase phosphorylation. *J Biol Chem* 277, 27065-27072.
- Vesikari, T., Karvonen, A., Prymula, R., Schuster, V., Tejedor, J.C., Cohen, R., Meurice, F., Han, H.H., Damaso, S., Bouckennooghe, A., 2007. Efficacy of human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in European infants: randomised, double-blind controlled study. *Lancet* 370, 1757-1763.
- Watts, A., Bennett, W.E., Molleston, J.P., Gupta, S.K., Croffie, J.M., Waseem, S., McFerron, B.A., Steiner, S.J., Kumar, S., Vanderpool, C.P., Hon, E.C., Bozic, M.A., Subbarao, G.C., Pfefferkorn, M.D., 2017. The incidence of low seroimmunity to hepatitis B virus in children with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 65, 551-554.
- Yin, Y., Bijvelds, M., Dang, W., Xu, L., van der Eijk, A.A., Knipping, K., Tuysuz, N., Dekkers, J.F., Wang, Y., de Jonge, J., Sprengers, D., van der Laan, L.J., Beekman, J.M., Ten Berge, D., Metselaar, H.J., de Jonge, H., Koopmans, M.P., Peppelenbosch, M.P., Pan, Q., 2015a. Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. *Antiviral Res* 123, 120-131.
- Yin, Y., Metselaar, H.J., Sprengers, D., Peppelenbosch, M.P., Pan, Q., 2015b. Rotavirus in organ transplantation: drug-virus-host interactions. *Am J Transplant* 15, 585-593.

Yin, Y., Wang, Y., Dang, W., Xu, L., Su, J., Zhou, X., Wang, W., Felczak, K., van der Laan, L.J., Pankiewicz, K.W., van der Eijk, A.A., Bijvelds, M., Sprengers, D., de Jonge, H., Koopmans, M.P., Metselaar, H.J., Peppelenbosch, M.P., Pan, Q., 2016. Mycophenolic acid potently inhibits rotavirus infection with a high barrier to resistance development. *Antiviral Res* 133, 41-49.

Supplementary Tables & Figures

Table S1. Primers and shRNA sequences used in the study.

Sequence of rotavirus primers

	SA11 Rotavirus	Human Patient Rotavirus
Sense	TGGTTAAACGCAGGATCGGA	ACCATCTACACATGACCCTC
Anti-sense	AACCTTTCCGCGTCTGGTAG	CACATAACGCCCTATAGCC

Primer sequences of GAPDH

Primers	Human	Mouse
GAPDH-F	GTCTCCTCTGACTTCAACAGCG	TTCCAGTATGACTCCACTCACGG
GAPDH-R	ACCACCCTGTTGCTGTAGTAGCCAA	TGAAGACACCAGTAGACTCCACGAC

shRNA targeting sequences of Rac1

Primers	Human	Mouse
GAPDH-F	GTCTCCTCTGACTTCAACAGCG	TTCCAGTATGACTCCACTCACGG
GAPDH-R	ACCACCCTGTTGCTGTAGTAGCCAA	TGAAGACACCAGTAGACTCCACGAC

Table S2. 50% inhibition concentration (IC₅₀) of 6-TG, NSC23766, IFN α and ribavirin against SA11 rotavirus, 50% cytotoxic (CC₅₀) of against Caco2 cells, and corresponding selectivity index (SI, CC₅₀/IC₅₀).

Drugs	IC ₅₀ against SA11 rotavirus (M)	CC ₅₀ against Caco2 cells (M)	selectivity index (SI, CC ₅₀ /IC ₅₀)
6-TG	3.0×10^{-13} M	9.8×10^{-6} M	3.3×10^7
NSC23766	1.1×10^{-8} M	3.1×10^{-4} M	2.8×10^4
IFNα	3.1×10^{-5} IU	18706 IU	6.0×10^8
Ribavirin	1.6×10^{-7} M	3.02×10^{-2} M	1.9×10^5

Table S3. Patient characteristics.

Patient	Age (yrs)	Gender	Symptoms	Virus Detection							
				Enterovirus	Parechovirus	Norovirus genegroups I	Norovirus genegroups II	Adenovirus	Astrovirus	Sapovirus	Rotavirus
1	3.5	Female	Fever	No	No	No	No	No	No	No	Yes
2	74	Female	Congestive heart failure, myocarditis	No	No	No	No	No	No	No	Yes
3	27	Female	Fever, diarrhea, nausea, vomiting	No	No	No	No	No	No	No	Yes
4	67	Male	Fever, stomach ache, watery diarrhea (Kidney transplant)	No	No	No	No	No	No	No	Yes
5	28	Female	Nausea, stomach ache, watery diarrhea, fever, headache, vomiting	No	No	No	No	Yes	No	No	Yes

Supplementary Figures

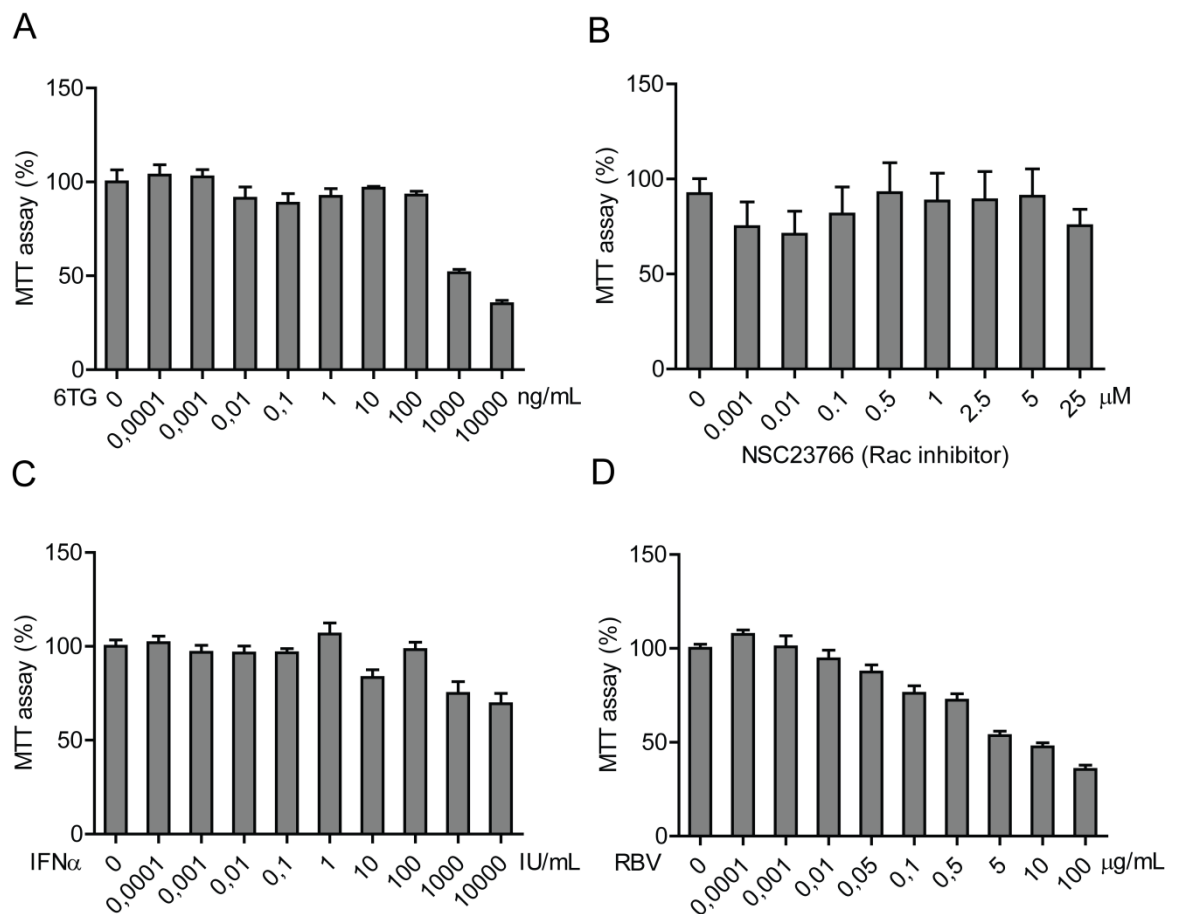


Fig. S1. Effect of 6-TG (A), NSC23766 (B), IFNα (C) and ribavirin (D) on host cell viability determined by MTT assays. Caco2 cells treated with all four drugs for 48 h.

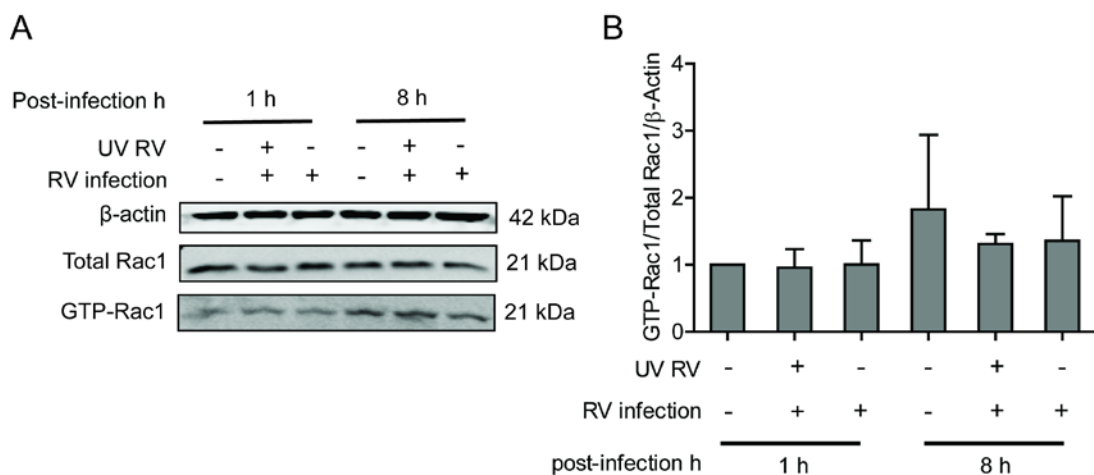


Fig. S2. The effect of inactive rotavirus by UV treatment, rotavirus and mock infection (1 and 8 h) on the expression of GTP-Rac1. (A) Western blot assay detected the expression of GTP-Rac1 after 1 and 8 h post-infection by inactive rotavirus (UV treatment), rotavirus and mock infection in Caco2 cells. (B) Quantification of the intensity of the immunoreactive bands of Rac1 (n = 4, means ± SEM, Mann-Whitney test) using Odyssey V 3.0 software.

Chapter 9

Significance of continuous rotavirus and norovirus surveillance in Indonesia

Mohamad S. Hakim^{1,2}, Hera Nirwati², Abu T. Aman², Yati Soenarto³, Qiuwei Pan¹

- 1 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, The Netherlands
- 2 Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
- 3 Department of Child Health, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

World Journal of Pediatrics 2018; 14(1): 4-12.

Abstract

Background: Diarrhea significantly contributes to the global burden of diseases, particularly in developing countries. Rotavirus and norovirus are the most dominant viral agents responsible for diarrheal disease globally. The aim of this review is to conduct a comprehensive assessment of rotavirus and norovirus study in Indonesia.

Data sources: Articles about rotavirus and norovirus surveillance in Indonesia were collected from databases, including PubMed and Google Scholar. Manual searching was performed to identify additional studies. Furthermore, relevant articles about norovirus diseases were included.

Results: A national surveillance of rotavirus-associated gastroenteritis has been conducted for years, resulting in substantial evidence about the high burden of the diseases in Indonesia. In contrast, norovirus infection received relatively lower attention and very limited data are available about the incidence and circulating genotypes. Norovirus causes sporadic and epidemic gastroenteritis globally. It is also emerging as a health problem in immunocompromised individuals. During post-rotavirus vaccination era, norovirus potentially emerges as the most frequent cause of diarrheal diseases.

Conclusions: Our review identifies knowledge gaps in Indonesia about the burden of norovirus diseases and the circulating genotypes. Therefore, there is a pressing need to conduct national surveillance to raise awareness of the community and national health authority about the actual burden of norovirus disease in Indonesia. Continuing rotavirus surveillance is also important to assess vaccine effectiveness and to continue tracking any substantial changes of circulating rotavirus genotypes.

Keywords: diarrhea; rotavirus; norovirus; Indonesia; surveillance

Introduction

Diarrheal diseases are among the leading causes of global disease burden with significant morbidity and mortality, especially in low income developing countries.^[1] In 2010, it was estimated that there were about 1.7 billion episodes of diarrhea in children aged less than five years, of which, 36 million progressed to severe diseases.^[1] In Indonesia, the incidence of diarrhea in all ages and in children aged less than five years are 3.5% and 10.2% respectively.^[2] In 2015, 5.0 billion episodes of diarrhea in all age groups are reported, as well as 21 diarrhea outbreaks with more than 1200 patients and 30 deaths (Case Fatality Rate [CFR] 2.47%).^[3]

Various bacteria, viruses, and parasites have been identified as the causes of diarrhea, mainly transmitted through contaminated food or water sources. A systematic review of articles published between 1990 and 2011 showed that rotavirus is the most dominant cause of diarrhea in children aged less than five years, followed by enteropathogenic *Escherichia coli* (EPEC) and caliciviruses (norovirus and sapovirus).^[4] Among pathogens commonly transmitted through contaminated food, norovirus is the most common. Norovirus causes 677 million diarrheal episodes, resulted in 213 000 deaths in all ages.^[5]

Since rotavirus and norovirus are the main viral pathogens causing diarrhea globally, we conducted a comprehensive review of the detection and surveillance of these viruses in Indonesia. Based on the available surveillance data, we identified the burden of the diseases in Indonesia as well as the circulating genotypes. Finally, we identified gaps in the previous surveillance to guide recommendations for improving the surveillance systems of these viruses in Indonesia.

Years of rotavirus surveillance and detection in Indonesia

Rotavirus is a non-enveloped, double stranded RNA (dsRNA) virus belongs to the *Reoviridae* family. Its genome consists of 11 dsRNA segments encoding six structural proteins (viral proteins [VPs]) and six non-structural proteins (NSPs). The viral particle comprises of three concentric protein layers. The outer layer is made of two neutralizing antigens, VP7 and VP4 proteins. These proteins are essential for binary classification of rotavirus into G- and P-

genotype, respectively.^[6] Rotavirus is the most important cause of hospitalization in children suffering from diarrhea. In 2013, it was estimated that 215 000 children aged less than five years died from rotavirus-associated diarrhea.^[7] Rotavirus-associated diseases also emerged in immunocompromised patients, including pediatric and adult organ transplant recipients.^[8]

In Indonesia, rotavirus was first visualized using electron microscopy in fecal specimens of infants and children with acute diarrhea in Yogyakarta collected during the year 1978-1979.^[9] Rotavirus was identified in 38% of the collected specimens.^[9] Rotavirus was also detected from fecal specimens collected from children with diarrhea during August 1979 to September 1981 in Jakarta and Medan.^[10] Strain characterization was performed by electropherotyping.^[11] A subsequent study successfully detected the rotavirus serotype by using monoclonal antibodies against VP7 protein.^[12]

To further investigate the burden and impact of rotavirus diseases, the Asian Rotavirus Surveillance Network (ARSN) was established.^[13] As a member of ARSN, the Indonesian Rotavirus Surveillance Network (IRSN) conducted a hospital-based surveillance based on World Health Organization (WHO) standard protocol.^[14-16] Rotavirus surveillance was also conducted by other institutions or research laboratories (Table 1).^[17-27] Rotavirus detection was based on enzyme immunoassay and characterization of rotavirus genotypes was performed by reverse transcriptase polymerase chain reaction (RT-PCR).^[15, 16]

These studies indicated a high incidence of rotavirus disease in children aged <5 years in Indonesia. Rotavirus detection rate in these studies was about 40%-50% (Table 1), in line with a recent analysis of worldwide studies from 2000-2013 (37%-40%).^[7] It is worth noting that the difference of detection rate between studies may be due to the difference of detection assays used and the surveillance period. Even though rotavirus was detected year-round, the incidence tends to be higher from June to August.^[14, 15] Rotavirus-positive children were at risk of developing severe clinical symptoms such as vomiting and dehydration.^[15, 23] Outbreaks have been reported from Papua and East Nusa Tenggara^[19, 22], underlying the urgency to control the diseases.

Rotavirus genotyping demonstrated that G1P[8], G1P[6] and G2P[4] strains were the most predominant strains circulating in Indonesia^[15, 16, 25, 26], in line with findings of worldwide studies.^[28] However, alterations of dominant strains in Indonesia was observed^[16],

supporting the importance of continuous surveillance in the country. Surveillance in Indonesia has identified novel genotypes such as G12 strain.^[29] Results of the observation highlight the importance of updating genotyping assays to cope with the rapid evolution of the virus that might result in typing failure.^[30]

Collectively, the surveillance system provides valuable data about the epidemiology and impact of rotavirus disease in Indonesia.^[15] Furthermore, it raises awareness about the magnitude of rotavirus disease burden. Together with the genotyping studies, government and public health experts could assess the prospect of introducing rotavirus vaccine into the national program to reduce the burden of diseases in Indonesia. It is expected that inclusion of rotavirus vaccines in the National Immunization Program will prevent 480 000 diarrhea cases in outpatient clinics, 176 000 hospitalizations and 8000 deaths of Indonesian children.^[31]

Decline of rotavirus diarrhea following rotavirus vaccine Introduction

Two commercially available oral rotavirus vaccines, Rotarix and RotaTeq, were licensed in 2006 and recommended for use by the World Health Organization in all countries, particularly those with a high incidence of severe rotavirus-associated gastroenteritis.^[32] Clinical trials of these vaccines demonstrated high efficacy against severe rotavirus disease in developed, high- and upper middle-income countries. However, a lower efficacy was observed in developing, lower middle-income countries.^[33]

These vaccines are live-attenuated vaccines that differ in their antigenic composition. Rotarix (GlaxoSmithKline Biologicals, Belgium) is a monovalent vaccine (RV1), containing G1P[8] strains derived from human rotavirus. RotaTeq (Merck, USA) is a pentavalent vaccine (RV5), derived from human-bovine reassortant viruses containing five most dominant strains, i.e. G1, G2, G3, G4, and P[8].^[34] Both vaccines are available in Indonesia. However, they are not included in the National Immunization Program due to financial constraints.^[35] Currently, there are no available data about the vaccination coverage and the impact of rotavirus vaccination in Indonesia.

Table 1. Rotavirus (RV) detection and surveillance in Indonesian population.

No	Regions	Study periods	Types of study	Population	No of cases tested*	RV positive samples (%)	Methods	References
1	Yogyakarta	June 1978 - June 1979	Hospital-based surveillance	Children age 0 – 12 years hospitalized due to acute diarrhea	334	126 (38%)	EM	[9]
2	Jakarta and Medan	August 1979 - September 1981	NS	Children with diarrhea.	41	16 (39%)	RPH	[10]
3	Jakarta	March 1997 - August 1999	Community- and hospital-based surveillance	Pediatric and adults patients reporting diarrhea	539	202 (37.5%)	EIA	[17]
4	Jakarta	March 1997 - June 1999	Hospital-based surveillance	Inpatient and outpatient reporting diarrhea	402	170 (42.3%)	EIA	[18]
5	Yogyakarta and Purworejo	August 2001 - April 2004	Hospital-based surveillance	Children <3 years admitted due to diarrhea	1321	705 (53%)	EIA	[13, 14]
6	Kupang, East Nusa Tenggara	August 2002	Outbreak investigation	Acute diarrhea cases during outbreak period (n=2.600)	27	13 (48%)	EIA	[19]
7	NS	February 2004 - February 2005	Hospital-based surveillance	Children <6 years of age with acute diarrhea	1660	755 (45.5%)	EIA or RT-PCR	[20]
8	Palembang, Jakarta, Bandung, Yogyakarta, Denpasar, Mataram	January - December 2006	Prospective surveillance in 6 teaching hospitals	Children aged <5 years with acute diarrhea (inpatient and outpatient)	2240 (inpatients) and 176 (outpatients)	1345 (60%) and 73 (41%)	EIA	[15]
9	Jakarta, Yogyakarta, Denpasar, Makassar, Mataram	January - April 2007	Hospital-based surveillance	Children <5 years old with acute diarrhea	421	257 (61%)	RT-PCR	[21]
10	Bandung, Yogyakarta, Mataram, Denpasar	2006, 2009, 2010	Hospital-based surveillance	Children aged <5 years with acute diarrhea	4235	2220 (52.4%)	EIA	[16]

11	Bintuni Bay, Papua	September - October 2008	Outbreak investigation in five villages	242 toddlers admitted to hospitals with massive diarrhea	15	10 (67%) or 12 (80%)	EIA or RT-PCR, respectively.	[22]
12	Denpasar	April 2009 - December 2011	Hospital-based surveillance	Children aged <5 years with acute diarrhea	656	327 (49.8%)	EIA	[23]
13	Bandung	April 2009 - December 2012	Hospital-based surveillance	Infants aged ≤6 months with acute diarrhea	134	60 (44.8%)	EIA	[24]
14	Surabaya	April - December 2013	Hospital-based surveillance	Children 1-60 months hospitalized due to acute diarrhea	220	88 (40%)	IC	[25]
15	Yogyakarta	February - August 2009	Hospital-based surveillance	Children aged <5 years with acute diarrhea	104	57 (54.8%)	EIA	[26]
16	Pekanbaru, Riau	January - July 2015	Hospital-based surveillance	Children aged 0-60 months	71	42 (59.2%) or 44 (62.0%)	EIA or RT-PCR, respectively.	[27]

*: for case-control studies, only number of tested cases were presented.

NS: not specified

IC: immunochromatographic assay

EIA: enzyme immunoassay

RT-PCR: reverse transcriptase polymerase chain reaction

EM: electron microscopy

RPH: reversed passive hemagglutination kits

By the end of 2013, Rotarix and RotaTeq had been included in the national immunization programs of more than 50 countries worldwide and showed a significant impact to reduce rotavirus diseases.^[33] In these countries, rotavirus vaccines proved to be safe and effective in reducing rotavirus-associated diarrhea cases, hospitalizations, and deaths.^[33] Interestingly, following a widespread implementation of rotavirus vaccination, a change in the epidemiology of viral gastroenteritis has been observed. With the decline of rotavirus disease, human norovirus infection has become more prevalent, especially in countries where universal rotavirus vaccination has been introduced. Reports from Bolivia^[36], Brazil^[37, 38], Nicaragua^[39], Finland^[40, 41], and the United States^[42, 43] indicated that norovirus has become more prevalent than rotavirus in causing gastroenteritis in children. As an example, during 2009-2010, norovirus was detected in 21% of young children with acute gastroenteritis, while rotavirus was detected in 12% of children at the same period in the United States.^[43] Although more comprehensive global epidemiological studies are needed, these initial reports clearly indicate that norovirus had emerged as the most predominant cause of gastroenteritis in children during post-rotavirus vaccination era.

Norovirus: an emerging and under-recognized human pathogen

Human norovirus is a linear, single-strand, positive RNA virus that is ~7.6 kb in length that belongs to the family of *Caliciviridae*. It is classified into at least 6 genogroups (genogroup 1 [G1] to GVI) and more than 40 genotypes. The prototype of human norovirus, Norwalk virus, is designated as GI, genotype 1 (GI.1). Human norovirus GII.4 is the most frequent cause of human infection, followed by GI and rarely, GIV. The other genogroup, GIII, GV, and GVI are bovine, murine and canine norovirus, respectively.^[44, 45] As an RNA virus, norovirus displays a great genetic diversity, particularly due to the error-prone nature of RNA dependent RNA polymerase (RdRp) and recombination between two related strains.^[46]

Norwalk virus is the first viral agent identified as the cause of gastroenteritis in human. The virus was visualized in 1972 from specimens collected during an outbreak of acute gastroenteritis in Norwalk, Ohio, the United States, and hence its name.^[47] However, its significance as human pathogen was under-recognized due to lack of a routine detection method.^[45] In addition, efforts to develop robust norovirus cell culture models that mimic the entire life-cycle in infected cells have been unsuccessful, hampering studies on the

molecular biology and development of specific anti-viral drugs.^[48] Along with the rapid development of diagnostic methodology based on quantitative real time PCR and its widespread availability, understanding of norovirus epidemiology and its disease burden have largely improved, particularly in developing countries.^[49]

The global burden of norovirus gastroenteritis

Norovirus diseases mainly affect children aged less than five years and older adults (greater than 65 years) in which it causes a high rate of hospitalization and death.^[49] Norovirus can infect general population, causing outbreaks and acute sporadic gastroenteritis, and also chronic infection in immunocompromised individuals (**Fig. 1**).

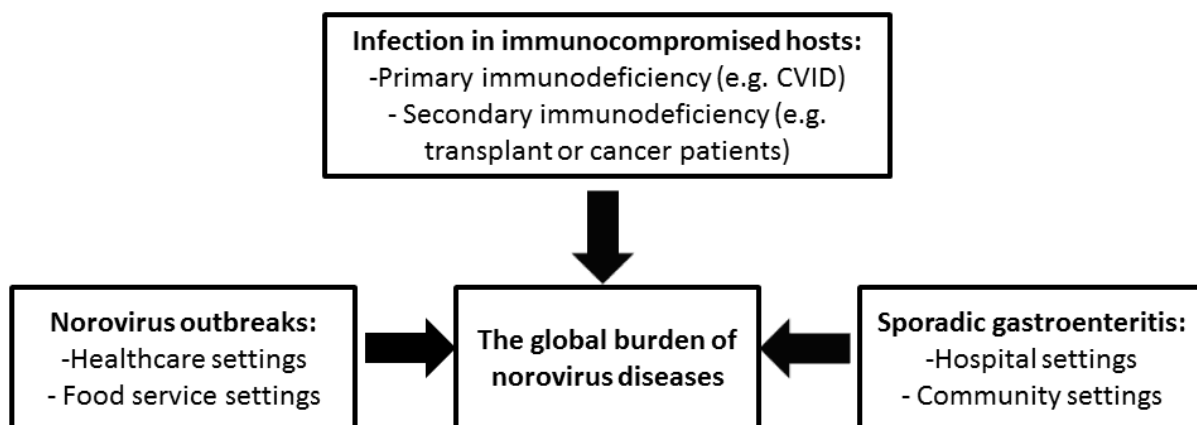


Figure 1. The global burden of norovirus gastroenteritis.

Norovirus outbreaks

Norovirus is the leading cause of acute gastroenteritis outbreaks globally. Indeed, most of our recent understandings about molecular epidemiology of norovirus come from the analysis of global outbreak samples.^[49] Several factors contribute to the high incidence of norovirus outbreaks, such as low infectious dose; prolonged fecal shedding that facilitate secondary transmission; viral stability in the environment; and lack of cross-protective and long-lasting immunity.^[45]

Norovirus is responsible for about 50% of all gastroenteritis outbreaks reported worldwide.^[50] In some countries, the incidence is even higher. As an example, an analysis of

fecal specimens taken from more than 300 outbreaks of nonbacterial gastroenteritis in the United States demonstrated that more than 90% of these outbreaks were attributable to norovirus.^[51, 52]

In a systematic analysis of norovirus outbreaks, the food service (e.g. restaurant) was the most common outbreak setting (35%), followed by health care (e.g. hospitals and long-term care facilities) (27%); leisure places (e.g. cruises, hotels and recreational activities) and school/daycare facilities (27%).^[53] For nosocomial outbreaks, the most frequently reported route of transmission was person-to-person transmission (18.5%). However, the majority of the transmission route of the nosocomial outbreaks (77.8%) is still unknown.^[54] Most importantly, a systematic analysis of published hospital outbreaks identified norovirus as the most common cause of a hospital wards closure.^[55] Altogether, the data underscore that the impact of norovirus disease should not be underestimated. Therefore, appropriate prevention and control measures are urgently required to prevent the occurrence of any future norovirus outbreaks.

Especially in healthcare settings, a highly virulent GII.4 norovirus strain has been recognized as the most common strain responsible for global norovirus outbreaks. The GII.4 strain is more likely to be associated with person-to-person transmission.^[56, 57] However, some of GII.4 outbreaks were attributable to foodborne transmission.^[58] A systematic review indicates that GII.4 outbreaks were associated with more severe clinical outcomes, independent of other factors.^[59] New variants of GII.4 strain frequently emerged in cycles of two to seven years, replacing the previously dominant variant to cause pandemic.^[57] As an RNA virus, the high mutation rate facilitates the virus to escape from the host's immune system, leading to a constantly susceptible population and widespread newly emerging strains.^[46] Interestingly, recent reports from Japan and China identified a novel strain, GII.17 norovirus, as the major cause of outbreaks and potentially replaces the previously dominating GII.4 strain.^[60]

Acute sporadic gastroenteritis

Beside outbreaks, norovirus is also an important agent of sporadic gastroenteritis. A systematic review of published studies between 1990 and 2008 documented that norovirus

was responsible for 12% of severe gastroenteritis in children aged less than five years worldwide, requiring emergency department visit and hospitalizations. Across all ages, norovirus accounted for 12% of mild and moderate diarrhea.^[61] More recent estimates indicate that it is accounted for 18% cases of acute gastroenteritis in children aged less than five years and mixed ages.^[62] This estimation suggests that norovirus is the most common cause of diarrhea across all ages. In children aged less than five years during pre-rotavirus vaccination era, it is the second leading cause of severe diarrhea, following rotavirus.

Infection in immunocompromised host

Due to the use of immunosuppressant agents, transplant recipients are at high risk of contracting norovirus infection. The prevalence of norovirus-associated diarrhea in hematopoietic stem cell transplant (HST) and solid organ transplant (SOT) recipients have been reported to be 18%.^[63] However, more thorough studies are required to confirm this finding. Importantly, norovirus infection in these patients was associated with more severe morbidity, such as prolonged viral shedding and recurrences of diarrhea episodes.^[63] Therefore, a reduction or withdrawal of immunosuppressant agents should be considered for transplant patients at risk of norovirus infections in order to enhance the immune response in fighting the infection.^[64] Patients with primary immunodeficiency, such as common variable immune deficiency (CVID), were also highly susceptible to develop chronic infections, leading to severe complications such as intestinal villous atrophy and malabsorption.^[65] Due to a prolonged phase of infection and an increase of viral mutation, immunocompromised and transplant patients may serve as potential norovirus reservoirs in the human population.^[66]

Relevance of norovirus surveillance in Indonesia

In contrast to rotavirus, norovirus surveillance and detection in Indonesian population, as well as in several other developing countries, are very limited, (Table 2). This suggests that norovirus received comparatively less concern than rotavirus, despite its significance in global contribution of all acute gastroenteritis cases. We found only four hospital-based surveillance identifying norovirus as the cause of acute gastroenteritis in Indonesia with a limited surveillance period and a relatively limited number of clinical sample tested. ^{[17, 18, 25,}

^{67]} Moreover, the surveillances were only conducted in two regions, three of which were conducted in the capital city of Jakarta. This is probably due to the requirement of RT-PCR for norovirus detection which is not widely available in Indonesia. Therefore, we can conclude that nationwide studies focused more on rotavirus diseases rather than any other type of viruses.^[14-16]

In these four studies, norovirus prevalence was about 18%-30% (Table 2), in accordance to the findings of global studies.^[62] One study identified norovirus as co-viral agent with rotavirus infection. Seventeen out of 88 rotavirus-infected patients (19.3%) were co-infected with human norovirus.^[25] Unfortunately, all previous studies (Table 2) did not report the genogroup and genotype of the infecting human norovirus. Consequently, genotyping data of norovirus circulating in Indonesia are not yet available. These findings clearly demonstrated the lack of national studies on the epidemiological burden and genetic diversity of human norovirus circulating in Indonesia.

Some countries have developed surveillance system to monitor norovirus incidence and outbreaks. NoroNet, led by the National Institute for Public Health and the Environment of the Netherlands (*Rijkinstituut voor Volksgezondheid en Milieu*, RIVM), is a collaborative network of international institutes maintaining a database of norovirus nucleotide sequences.^[60] In the United States, the Centers for Disease Control and Prevention (CDC) established CaliciNet on 2009.^[68] Both systems have proven successful in identifying the transmission routes and the emergence of norovirus' new strain variants.^[58, 60, 69] A reporting system to detect norovirus outbreaks was also established by the United Kingdom Department of Health through the Hospital Norovirus Outbreak Reporting System (HNORS). In this system, outbreak data are collected and summarized using a standardized paper and stored in a web-based database.^[70] This system was successful in increasing norovirus outbreak reports in hospitals. It also provided data about the burden and economic impact of norovirus outbreaks in hospitals.^[70] An efficient detection and surveillance system may be able to reduce the health and societal cost expenses due to norovirus infections and outbreaks.^[71]

Table 2. Norovirus (NoV) detection and surveillance in Indonesian population.

No	Regions	Study periods	Types of study	Population	No of cases tested	NoV positive samples (%)	Methods	References
1	Jakarta	March 1997 - June 1999	Hospital-based surveillance	Inpatient and outpatient reporting diarrhea	218	45 (20.6%)	RT-PCR	[18]
2	Jakarta	March 1997 - August 1999	Community- and hospital-based surveillance	Pediatric and adults patients reporting diarrhea	278	49 (18.0%)	RT-PCR	[17]
3	Jakarta	October 1997 - September 1999	Hospital-based study	Infants (0-1 year) and young children (aged 5-12 years) presenting acute diarrhea	102	31 (30%)	RT-PCR	[67]
4	Surabaya	April - December 2013	Hospital-based surveillance	Children 1-60 months hospitalized due to acute diarrhea	88	17 (19.3%)*	RT-PCR	[25]

*: as co-infection with rotavirus

Conclusions

Continuous surveillance is required to enhance our understanding of the burden and impact of rotavirus and norovirus gastroenteritis in Indonesia. During post-rotavirus vaccination era, improvement of active surveillance in Indonesia is necessary to assess the effectiveness of rotavirus vaccines and to enhance the early detection of any changes of circulating rotavirus genotypes.^[16] Continuous strain monitoring is pivotal to anticipate the emerging of novel or rare genotypes not included in the current vaccines, such as G12.^[72] The emergence of these genotypes may be due to vaccine-induced selective pressure. Subsequently, it may change the epidemiology of circulating rotavirus genotypes and influence the overall impact of rotavirus vaccines. The information is therefore useful for rotavirus vaccine development.

In addition, it is also necessary to include other patients in the surveillance, such as immunocompromised patients. In these patients, the incidence of rotavirus infection is considerably high and a prolonged diarrheal illness has been observed. These observations support the need of surveillance in these patients.^[8]

In contrast to rotavirus surveillance, norovirus surveillance in Indonesia is very limited. With the decline of rotavirus diseases following vaccine introduction, norovirus may emerge as a major cause of diarrhea in Indonesian children. Therefore, norovirus surveillance is crucial to investigate the burden of the disease and to characterize the genotypes. The surveillance system is of great importance to anticipate the emergence of novel, potentially pandemic strains such as GII.17 viruses.^[60] The data of surveillance could serve as the basis for vaccine development.^[49]

Acknowledgements

The authors thank to Noviarina Kurniawati, Risky Oktriani, and Deanna Camell for critical reading of this manuscript.

References

- 1 Walker CL, Rudan I, Liu L, Nair H, Theodoratou E, Bhutta ZA, et al. Global burden of childhood pneumonia and diarrhoea. *Lancet* 2013;381:1405-1416.
- 2 Badan Penelitian dan Pengembangan Kesehatan, Kementerian Kesehatan Republik Indonesia. Riset Kesehatan Dasar 2013.
<http://www.depkes.go.id/resources/download/general/Hasil%20Riskesdas%202013.pdf> (accessed July 31, 2017)
- 3 Kementerian Kesehatan Republik Indonesia. Profil Kesehatan Indonesia 2015.
<http://www.depkes.go.id/resources/download/pusdatin/profil-kesehatan-indonesia/profil-kesehatan-Indonesia-2015.pdf> (accessed July 31, 2017)
- 4 Lanata CF, Fischer-Walker CL, Olascoaga AC, Torres CX, Aryee MJ, Black RE, et al. Global causes of diarrheal disease mortality in children <5 years of age: a systematic review. *PLoS One* 2013; 8:e72788.
- 5 Pires SM, Fischer-Walker CL, Lanata CF, Devleesschauwer B, Hall AJ, Kirk MD, et al. Aetiology-specific estimates of the global and regional incidence and mortality of diarrhoeal diseases commonly transmitted through food. *PLoS One* 2015;10:e0142927.
- 6 Greenberg HB, Estes MK. Rotaviruses: from pathogenesis to vaccination. *Gastroenterology* 2009;136:1939-1951.
- 7 Tate JE, Burton AH, Boschi-Pinto C, Parashar UD, World Health Organization-Coordinated Global Rotavirus Surveillance Network. Global, regional, and national estimates of rotavirus mortality in children <5 years of age, 2000-2013. *Clin Infect Dis* 2016;62 Suppl 2:S96-S105.
- 8 Yin Y, Metselaar HJ, Sprengers D, Peppelenbosch MP, Pan Q. Rotavirus in organ transplantation: drug-virus-host interactions. *Am J Transplant* 2015;15:585-593.
- 9 Soenarto Y, Sebodo T, Ridho R, Alrasjid H, Rohde JE, Bugg HC, et al. Acute diarrhea and rotavirus infection in newborn babies and children in Yogyakarta, Indonesia, from June 1978 to June 1979. *J Clin Microbiol* 1981;14:123-129.
- 10 Hasegawa A, Inouye S, Matsuno S, Yamaoka K, Eko R, Suharyono W. Isolation of human rotaviruses with a distinct RNA electrophoretic pattern from Indonesia. *Microbiol Immunol* 1984;28:719-722.
- 11 Albert MJ, Soenarto Y, Bishop RF. Epidemiology of rotavirus diarrhea in Yogyakarta, Indonesia, as revealed by electrophoresis of genome RNA. *J Clin Microbiol* 1982;16:731-733.
- 12 Bishop RF, Unicom LE, Soenarto Y, Suwardji H, Ristanto, Barnes GL. Rotavirus serotypes causing acute diarrhoea in hospitalized children in Yogyakarta, Indonesia during 1978-1979. *Arch Virol* 1989;107:207-213.
- 13 Bresee J, Fang ZY, Wang B, Nelson EA, Tam J, Soenarto Y, et al. First report from the Asian Rotavirus Surveillance Network. *Emerg Infect Dis* 2004;10:988-995.
- 14 Wilopo SA, Soenarto Y, Bresee JS, Tholib A, Aminah S, Cahyono A, et al. Rotavirus surveillance to determine disease burden and epidemiology in Java, Indonesia, August 2001 through April 2004. *Vaccine* 2009;27 Suppl 5:F61-66.
- 15 Soenarto Y, Aman AT, Bakri A, Waluya H, Firmansyah A, Kadim M, et al. Burden of severe rotavirus diarrhea in Indonesia. *J Infect Dis* 2009;200 Suppl 1:S188-194.
- 16 Nirwati H, Wibawa T, Aman AT, Wahab A, Soenarto Y. Detection of group A rotavirus strains circulating among children with acute diarrhea in Indonesia. *Springerplus* 2016;5:97.
- 17 Oyofo BA, Subekti D, Tjaniadi P, Machpud N, Komalarini S, Setiawan B, et al. Enteropathogens associated with acute diarrhea in community and hospital patients in Jakarta, Indonesia. *FEMS Immunol Med Microbiol* 2002;34:139-146.
- 18 Subekti D, Lesmana M, Tjaniadi P, Safari N, Frazier E, Simanjuntak C, et al. Incidence of Norwalk-like viruses, rotavirus and adenovirus infection in patients with acute gastroenteritis in Jakarta, Indonesia. *FEMS Immunol Med Microbiol* 2002;33:27-33.

- 19 Corwin AL, Subekti D, Sukri NC, Willy RJ, Master J, Priyanto E, et al. A large outbreak of probable rotavirus in Nusa Tenggara Timur, Indonesia. *Am J Trop Med Hyg* 2005;72:488-494.
- 20 Putnam SD, Sedyaningsih ER, Listiyaningsih E, Pulungsih SP, Komalarini, Soenarto Y, et al. Group A rotavirus-associated diarrhea in children seeking treatment in Indonesia. *J Clin Virol* 2007;40:289-294.
- 21 Radji M, Putman SD, Malik A, Husrima R, Listiyaningsih E. Molecular characterization of human group A rotavirus from stool samples in young children with diarrhea in Indonesia. *Southeast Asian J Trop Med Public Health* 2010;41:341-346.
- 22 Pratiwi E, Setiawaty V, Putranto RH. Molecular characteristics of rotavirus isolated from a diarrhea outbreak in October 2008 in Bintuni Bay, Papua, Indonesia. *Virology (Auckl)* 2014; 5: 11-14.
- 23 Salim H, Karyana IP, Sanjaya-Putra IG, Budiarsa S, Soenarto Y. Risk factors of rotavirus diarrhea in hospitalized children in Sanglah Hospital, Denpasar: a prospective cohort study. *BMC Gastroenterol* 2014;14:54.
- 24 Prasetyo D, Sabaroedin IM, Ermaya YS, Soenarto Y. Association between severe dehydration in rotavirus diarrhea and exclusive breastfeeding among infants at Dr. Hasan Sadikin General Hospital, Bandung, Indonesia. *J Trop Med* 2015;2015:862578.
- 25 Sudarmo SM, Shigemura K, Athiyyah AF, Osawa K, Wardana OP, Darma A, et al. Genotyping and clinical factors in pediatric diarrhea caused by rotaviruses: one-year surveillance in Surabaya, Indonesia. *Gut Pathog* 2015;7:3.
- 26 Nirwati H, Hakim MS, Aminah S, Dwija IBNP, Pan Q, Aman AT. Identification of rotavirus strains causing diarrhoea in children under five years of age in Yogyakarta, Indonesia. *Malays J Med Sci* 2017;24:68-77.
- 27 Djojosugito FA, Savira M, Anggraini D, Putra AE. Identification of the P genotypes of rotavirus in children with acute diarrhea in Pekanbaru, Indonesia. *Malays J Microbiol* 2017;13:67-72.
- 28 Banyai K, Laszlo B, Duque J, Steele AD, Nelson EA, Gentsch JR, et al. Systematic review of regional and temporal trends in global rotavirus strain diversity in the pre rotavirus vaccine era: insights for understanding the impact of rotavirus vaccination programs. *Vaccine* 2012;30 Suppl 1:A122-130.
- 29 Wulan WN, Listiyaningsih E, Samsi KM, Agtini MD, Kasper MR, Putnam SD. Identification of a rotavirus G12 strain, Indonesia. *Emerg Infect Dis* 2010;16:159-161.
- 30 Nirwati H, Wibawa T, Aman AT, Soenarto Y. Genotyping of rotavirus by using RT-PCR methods. *I J Biotech* 2013;18:8-13.
- 31 Wilopo SA, Kilgore P, Kosen S, Soenarto Y, Aminah S, Cahyono A, et al. Economic evaluation of a routine rotavirus vaccination programme in Indonesia. *Vaccine* 2009;27 Suppl 5:F67-74.
- 32 World Health Organization. Rotavirus vaccines, WHO position paper - January 2013. *Wkly Epidemiol Rec* 2013;88:49-64.
- 33 Tate JE, Parashar UD. Rotavirus vaccines in routine use. *Clin Infect Dis* 2014;59:1291-1301.
- 34 Dennehy PH. Rotavirus vaccines: an overview. *Clin Microbiol Rev* 2008;21:198-208.
- 35 Suwantika AA, Zakiah N, Lestari K, Postma MJ. Accelerating the introduction of rotavirus immunization in Indonesia. *Expert Rev Vaccines* 2014;13:463-472.
- 36 McAtee CL, Webman R, Gilman RH, Mejia C, Bern C, Apaza S, et al. Burden of norovirus and rotavirus in children after rotavirus vaccine introduction, Cochabamba, Bolivia. *Am J Trop Med Hyg* 2016;94:212-217.
- 37 Sa AC, Gomez MM, Lima IF, Quetz JS, Havt A, Oria RB, et al. Group A rotavirus and norovirus genotypes circulating in the northeastern Brazil in the post-monovalent vaccination era. *J Med Virol* 2015; 87: 1480-1490.
- 38 Ferreira MS, Victoria M, Carvalho-Costa FA, Vieira CB, Xavier MP, Fioretti JM, et al. Surveillance of norovirus infections in the state of Rio De Janeiro, Brazil 2005-2008. *J Med Virol* 2010;82:1442-1448.
- 39 Bucardo F, Reyes Y, Svensson L, Nordgren J. Predominance of norovirus and sapovirus in Nicaragua after implementation of universal rotavirus vaccination. *PLoS One* 2014;9:e98201.

- 40 Hemming M, Rasanen S, Huhti L, Paloniemi M, Salminen M, Vesikari T. Major reduction of rotavirus, but not norovirus, gastroenteritis in children seen in hospital after the introduction of RotaTeq vaccine into the National Immunization Programme in Finland. *Eur J Pediatr* 2013;172:739-746.
- 41 Puustinen L, Blazevic V, Salminen M, Hamalainen M, Rasanen S, Vesikari T. Noroviruses as a major cause of acute gastroenteritis in children in Finland, 2009-2010. *Scand J Infect Dis* 2011;43:804-808.
- 42 Koo HL, Neill FH, Estes MK, Munoz FM, Cameron A, DuPont HL, et al. Noroviruses: The most common pediatric viral enteric pathogen at a large university hospital after introduction of rotavirus vaccination. *J Pediatric Infect Dis Soc* 2013;2:57-60.
- 43 Payne DC, Vinje J, Szilagyi PG, Edwards KM, Staat MA, Weinberg GA, et al. Norovirus and medically attended gastroenteritis in U.S. children. *N Engl J Med* 2013;368:1121-1130.
- 44 Robilotti E, Deresinski S, Pinsky BA. Norovirus. *Clin Microbiol Rev* 2015;28:134-164.
- 45 Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis. *N Engl J Med* 2009;361:1776-1785.
- 46 Rocha-Pereira J, Van Dycke J, Neyts J. Norovirus genetic diversity and evolution: implications for antiviral therapy. *Curr Opin Virol* 2016;20:92-98.
- 47 Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J Virol* 1972;10:1075-1081.
- 48 Karst SM, Wobus CE, Goodfellow IG, Green KY, Virgin HW. Advances in norovirus biology. *Cell Host Microbe* 2014;15:668-680.
- 49 Lopman BA, Steele D, Kirkwood CD, Parashar UD. The vast and varied global burden of norovirus: Prospects for prevention and control. *PLoS Med* 2016;13:e1001999.
- 50 Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention. Updated norovirus outbreak management and disease prevention guidelines. *MMWR Recomm Rep* 2011;60:1-18.
- 51 Fankhauser RL, Noel JS, Monroe SS, Ando T, Glass RI. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. *J Infect Dis* 1998;178:1571-1578.
- 52 Fankhauser RL, Monroe SS, Noel JS, Humphrey CD, Bresee JS, Parashar UD, et al. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. *J Infect Dis* 2002;186:1-7.
- 53 Matthews JE, Dickey BW, Miller RD, Felzer JR, Dawson BP, Lee AS, et al. The epidemiology of published norovirus outbreaks: a review of risk factors associated with attack rate and genogroup. *Epidemiol Infect* 2012;140:1161-1172.
- 54 Greig JD, Lee MB. A review of nosocomial norovirus outbreaks: infection control interventions found effective. *Epidemiol Infect* 2012;140:1151-1160.
- 55 Hansen S, Stamm-Balderjahn S, Zuschneid I, Behnke M, Ruden H, Vonberg RP, et al. Closure of medical departments during nosocomial outbreaks: data from a systematic analysis of the literature. *J Hosp Infect* 2007;65:348-353.
- 56 Vega E, Barclay L, Gregoricus N, Shirley SH, Lee D, Vinje J. Genotypic and epidemiologic trends of norovirus outbreaks in the United States, 2009 to 2013. *J Clin Microbiol* 2014;52:147-155.
- 57 Zheng DP, Widdowson MA, Glass RI, Vinje J. Molecular epidemiology of genogroup II-genotype 4 noroviruses in the United States between 1994 and 2006. *J Clin Microbiol* 2010;48:168-177.
- 58 Verhoef L, Hewitt J, Barclay L, Ahmed SM, Lake R, Hall AJ, et al. Norovirus genotype profiles associated with foodborne transmission, 1999-2012. *Emerg Infect Dis* 2015;21:592-599.
- 59 Desai R, Hembree CD, Handel A, Matthews JE, Dickey BW, McDonald S, et al. Severe outcomes are associated with genogroup 2 genotype 4 norovirus outbreaks: a systematic literature review. *Clin Infect Dis* 2012;55:189-193.
- 60 de Graaf M, van Beek J, Vennema H, Podkolzin AT, Hewitt J, Bucardo F, et al. Emergence of a novel GII.17 norovirus - End of the GII.4 era? *Euro Surveill* 2015;20.
- 61 Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinje J, Parashar UD. Systematic literature review of role of noroviruses in sporadic gastroenteritis. *Emerg Infect Dis* 2008;14:1224-1231.

-
- 62 Ahmed SM, Hall AJ, Robinson AE, Verhoef L, Premkumar P, Parashar UD, et al. Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis. *Lancet Infect Dis* 2014;14:725-730.
 - 63 Angarone MP, Sheahan A, Kamboj M. Norovirus in transplantation. *Curr Infect Dis Rep* 2016;18:17.
 - 64 Bok K, Green KY. Norovirus gastroenteritis in immunocompromised patients. *N Engl J Med* 2012;367:2126-2132.
 - 65 Woodward J, Gkrania-Klotsas E, Kumararatne D. Chronic norovirus infection and common variable immunodeficiency. *Clin Exp Immunol* 2017;188:363-370.
 - 66 Karst SM, Baric RS. What is the reservoir of emergent human norovirus strains? *J Virol* 2015;89:5756-5759.
 - 67 Subekti DS, Tjaniadi P, Lesmana M, Simanjuntak C, Komalarini S, Digdowirogo H, et al. Characterization of Norwalk-like virus associated with gastroenteritis in Indonesia. *J Med Virol* 2002;67:253-258.
 - 68 Vega E, Barclay L, Gregoricus N, Williams K, Lee D, Vinje J. Novel surveillance network for norovirus gastroenteritis outbreaks, United States. *Emerg Infect Dis* 2011;17:1389-1395.
 - 69 Centers for Disease C, Prevention. Emergence of new norovirus strain GII.4 Sydney--United States, 2012. *MMWR Morb Mortal Wkly Rep* 2013;62: 55.
 - 70 Harris JP, Adams NL, Lopman BA, Allen DJ, Adak GK. The development of web-based surveillance provides new insights into the burden of norovirus outbreaks in hospitals in England. *Epidemiol Infect* 2014;142:1590-1598.
 - 71 Bartsch SM, Lopman BA, Ozawa S, Hall AJ, Lee BY. Global economic burden of norovirus gastroenteritis. *PLoS One* 2016;11:e0151219.
 - 72 Neves MAO, Pinheiro HHC, Silva RSU, Linhares AC, Silva LD, Gabbay YB, et al. High prevalence of G12P[8] rotavirus strains in Rio Branco, Acre, Western Amazon, in the post-rotavirus vaccine introduction period. *J Med Virol* 2016;88:782-789.

Chapter 10

Norovirus and rotavirus infections in children less than five years of age hospitalized with acute gastroenteritis in Indonesia

Hera Nirwati^{1,#}, **Mohamad S. Hakim**^{1,2,#}, Yuli Mawarti³, Nenny S. Mulyani⁴, Aqsa Ikram^{2,5}, Abu T. Aman¹, Maikel P. Peppelenbosch², Yati Soenarto^{4,‡}, Qiuwei Pan^{2,‡}

- 1 Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
 - 2 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands
 - 3 Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
 - 4 Department of Child Health, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
 - 5 Atta-Ur-Rahman School of Applied Biosciences, National University of Science and Technology, Islamabad, Pakistan
- # Both authors contribute equally for this manuscript
- ‡ These authors share senior authorship

Archives of Virology (under revision).

Abstract

Rotavirus and norovirus are the most important viral causes of acute gastroenteritis in children. While most previous studies mainly focused on rotavirus, here we investigated the burden and epidemiology of norovirus and rotavirus diseases in Indonesia. Hospitalized children less than five years of age with acute gastroenteritis were enrolled in this study from January - December 2015 at three participating hospitals. Rotavirus was detected by enzyme immunoassay (EIA), followed by genotyping with reverse transcription PCR (RT-PCR). Norovirus genogroups were determined by TaqMan-based quantitative RT-PCR. Among 406 enrolled children, 75 (18.47%); 223 (54.93%) and 29 (7.14%) cases were positive for norovirus, rotavirus and both viruses (mixed infections), respectively. Most cases clinically presented with fever, diarrhea, vomiting and some level of dehydration. Identification of norovirus genogroup showed that the majority (n=69/75 [92%]) was genogroup II. Among 35 samples tested for rotavirus genotype, the most prevalent genotype was G3P[8] (n=30/35 [85.6%]). Our study suggests that the burden of norovirus diseases in Indonesian children should not be underestimated. It also shows the emergence of G3P[8] rotavirus genotype in Indonesia.

Keywords: acute gastroenteritis; children; Indonesia; norovirus; rotavirus

Introduction

Acute gastroenteritis (acute diarrhea) is one of the most important global health issues, especially in children less than five years of age [49]. It is clinically characterized by acute symptoms of fever, abdominal pain, vomiting and diarrhea. The mortality is mainly due to severe complications, including dehydration [13]. Control measures of diarrheal disease have resulted in a significant progress. The global diarrheal mortality across all ages has markedly declined, from an estimated 2.6 million annually in 1990 to about 1.3 million in 2013 [28].

Rotavirus and norovirus are the most common viral agents responsible for acute gastroenteritis in children less than five years of age [22]. Rotavirus is a segmented double stranded RNA (dsRNA) virus. Based on two outer layer structural proteins VP7 and VP4, it is classified into G- and P-genotype, respectively [14]. Our previous national surveillance studies found a high incidence of rotavirus diseases and identified G1P[8], G1P[6] and G2P[4] as the most common genotype circulating in Indonesia [30, 41, 51]. Reassortment between different strains contributes to rotavirus genetic diversity by creating a novel combination of G- and P-genotype [11]. Thus, continuous strain monitoring is greatly important to identify novel genotypes.

Norovirus is a positive, single strand RNA virus and considered as the second most common cause of severe gastroenteritis in children following rotavirus [33]. Globally, the prevalence is about 18% in children aged less than five years and mixed ages with acute gastroenteritis [1]. Norovirus genogroup I (GI), II (GII) and IV (GIV) are found to infect humans, with a total of more than 30 characterized genotypes within those genogroups. A single genotype, GII.4, is the most prevalent globally and responsible for most of norovirus-associated gastroenteritis outbreaks [7, 8]. Importantly, norovirus has emerged as the leading cause of acute gastroenteritis, especially in countries which have introduced universal rotavirus vaccination [21, 34].

Despite its importance as the leading cause of severe gastroenteritis, very limited data is available on the burden and epidemiology of norovirus diseases in children less than five years of age in Indonesia. Norovirus genogroup responsible for acute gastroenteritis cases in

hospitalized children in Indonesia is also unknown [15]. Therefore, the objectives of this study were to investigate the prevalence, seasonality, clinical characteristics and genotype distributions of norovirus and rotavirus infections in hospitalized children less than five years of age with acute gastroenteritis in Indonesia.

Materials and Methods

Sample and clinical data collection

This study used stool samples collected from children less than five years of age who were hospitalized with acute gastroenteritis in: 1) Mataram General Hospital, Nusa Tenggara Barat; 2) Dr. Sardjito General Hospital, Yogyakarta; and 3) Wates General Hospital (Kulon Progo District), Yogyakarta, during Indonesian Rotavirus Surveillance Study conducted on January - December 2015. Stool samples were collected within the first 48 hours after admission according to World Health Organization (WHO) protocol. Stool specimens were stored at 4 °C - 8 °C before they were transported to the Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Indonesia. The specimens were then aliquoted into several tubes and stored at -20 °C. The patients' clinical manifestation such as fever, vomiting, dehydration and diarrhea were obtained from the medical histories stored at Pediatric Research Office, Dr. Sardjito General Hospital Yogyakarta.

Severe dehydration was determined by two or more of the following signs: lethargy (unconsciousness), unable to drink or drinks poorly, sunken eyes, and skin pinch goes back very slowly (>2 seconds). Some dehydration was determined when two or more of the following signs were found: restlessness, irritability, sunken eyes, drinks eagerly, thirsty, and skin pinch goes back slowly [50].

Rotavirus detection and genotyping

All stool samples were examined for the presence of group A rotavirus by enzyme immunoassay (EIA) using the IDEIATM Rotavirus (DakoCytomation) kit according to the manufacturer's instructions. Rotavirus genotyping was performed as we described previously [31].

Norovirus genogroup detection

Norovirus RNA was extracted from stool samples by using QIAamp RNA stool minikit (Qiagen) according to the manufacturer's instructions. Determination of norovirus GI and GII were performed by TaqMan-based quantitative real time polymerase chain reaction (qRT-PCR) on the ABI 7000 (Applied Biosystem) according to Dung *et al* [9]. Primers and probes to identify norovirus genogroup were listed in Supplementary Table 1.

qRT-PCR reaction was conducted with a final volume of 25 µl containing 1 µl (10 µM) forward primer; 1 µl (10 µM) reverse primer; 0,3 µl (10 µM) probe; 12,5 µl 2X RT-PCR buffer; 1 µl 25X RT-PCR enzyme mix; 4,2 µl nuclease-free water and 5 µl RNA template. Reverse transcription for GI norovirus was performed at 61°C for 3 minutes and followed by an initial denaturation temperature of 95°C for 5 minutes; 45 cycles of 95°C for 5 seconds; 57°C for 45 seconds and 37°C for 60 seconds. Reverse transcription for GII norovirus was performed at 61°C for 3 minutes and followed by an initial denaturation temperature of 95°C for 5 minutes; 45 cycles of 95°C for 5 seconds; 60°C for 45 seconds and 37°C for 60 seconds.

Statistical analyses

Data were computed with Stata 13 SE. Characteristics of norovirus, rotavirus and mixed infections were presented in frequencies and percentages. Chi-square test was used to measure odds ratio and *p* value of etiology pathogens detected toward clinical manifestations of acute gastroenteritis of children less than five years of age in Indonesia.

Ethical approval

The research protocol was approved by the Ethical Committee of the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. Informed consent was provided by the parents or guardians of each child before the children were enrolled in the Indonesian Rotavirus Surveillance Study 2015.

Results

Study population

From January to December 2015, there were 406 stool samples collected from children less than five years of age hospitalized at three participating hospitals due to acute diarrhea. The samples were mostly collected from Mataram General Hospital (68.5%). The age of patients ranged from 0 to 59 months (median, 15 months). There were 261 (64.29%) male and 145 (35.71%) female patients; the sex ratio (male/female) was 1.8.

Virus detection rates and clinical characteristics

Of the 406 stool samples collected, 75 (18.47%) and 223 (54.93%) were positive for norovirus (as determined by qRT-PCR) and rotavirus (as determined by EIA), respectively. Mixed infections of rotavirus and norovirus were identified in 29 (7.14%) patients. Norovirus, rotavirus, and mixed infections were most commonly identified in children with acute diarrhea at Mataram General Hospital and were more frequently identified in male patients, which accounted for 66.7%, 65.5%, and 72.4% of cases, respectively (Table 1).

Fever, vomiting, diarrhea and dehydration were clinical features commonly observed in norovirus and rotavirus infections. Fever was more likely presented in children patients with acute diarrhea due to norovirus (OR = 6.78; $P = 0.009$) and rotavirus (OR = 4.07; $P = 0.044$) infections. Vomiting was only significantly associated with rotavirus infection (OR = 22.32; $P = <0.001$). Diarrhea as well as dehydration were not associated with norovirus, rotavirus or mixed infections (Table 2).

Seasonality and age distribution

Norovirus and rotavirus were identified in children less than five years of age with acute diarrhea throughout the year, following the diarrhea incidence. Norovirus seasonal distribution was peaked in January-February and July-August. Meanwhile, rotavirus seasonal distribution was mainly peaked during early May to the end of September, with slight decrease in July and another peak was identified in January (Figure 1). Although norovirus and rotavirus were identified in all age groups, rotavirus infection was clustered in children 7-24 months of age, while norovirus clustered in children 7-36 months of age (Figure 2).

Table 1. Characteristics of children less than five years old hospitalized with acute gastroenteritis [n=406].

	Norovirus + (n=75)	Rotavirus + (n=223)	Mixed Infections (n=29)
Detection rate (%)	18.47	54.93	7.14
Age, month [median (IQR)]	15 (8 – 23)	14 (9 – 24)	21 (13 – 30)
Sex:			
Male [n (%)]	50 (66.7%)	146 (65.5%)	21 (72.4%)
Female [n (%)]	25 (33.3%)	77 (34.5%)	8 (27.6%)
Case detection in each location:			
Dr. Sardjito Hospital Yogyakarta (n=39)	12 (16.0%)	15 (6.73%)	4 (13.8%)
Wates Hospital Yogyakarta (n=89)	17 (22.7%)	62 (27.8%)	10 (34.5%)
Mataram Hospital, Nusa Tenggara Barat (n=278)	46 (61.33%)	146 (65.47%)	15 (51.7%)

Table 2. Clinical features of norovirus and rotavirus infections among children less than five years old hospitalized with acute gastroenteritis [n=406].

	Norovirus + (n=75)	Rotavirus + (n=223)	Mixed Infections (n=29)
Clinical Features			
Fever [n (%)]	45/75 (60.0%)	170/223 (76.2%)	22/29 (75.%)
Diarrhea [n (%)]	74/75 (98.7 %)	222/223 (99.6%)	29/29 (100%)
Vomiting [n (%)]	66/75 (88.0 %)	209/223 (93.7%)	27/29 (93.1%)
No dehydration [n (%)]	14/75 (18.7%)	48/223 (21.5%)	5/29 (17.2%)
Some dehydration [n (%)]	59/75 (78.7%)	168/223 (75.3%)	24/29 (82.8%)
Severe dehydration [n (%)]	2/75 (2.7%)	7/223 (3.1%)	0/29 (0%)
The odds ratio of clinical features			
Fever [OR (P)]	6.78 (0.009)*	4.07 (0.044)*	0.21 (0.645)
Diarrhea [OR (P)]	0.44 (0.506)	0.57 (0.451)	0.23 (0.630)
Vomiting [OR (P)]	0.19 (0.665)	22.32 (<0.001)*	1.18 (0.277)
Dehydration [OR (P)]	1.55 (0.462)	1.48 (0.48)	2.03 (0.362)
*Statistically significant			

Figure 1. Seasonal distribution of norovirus (NV+) and rotavirus (RV+) infections during January – December 2015 [n=406].

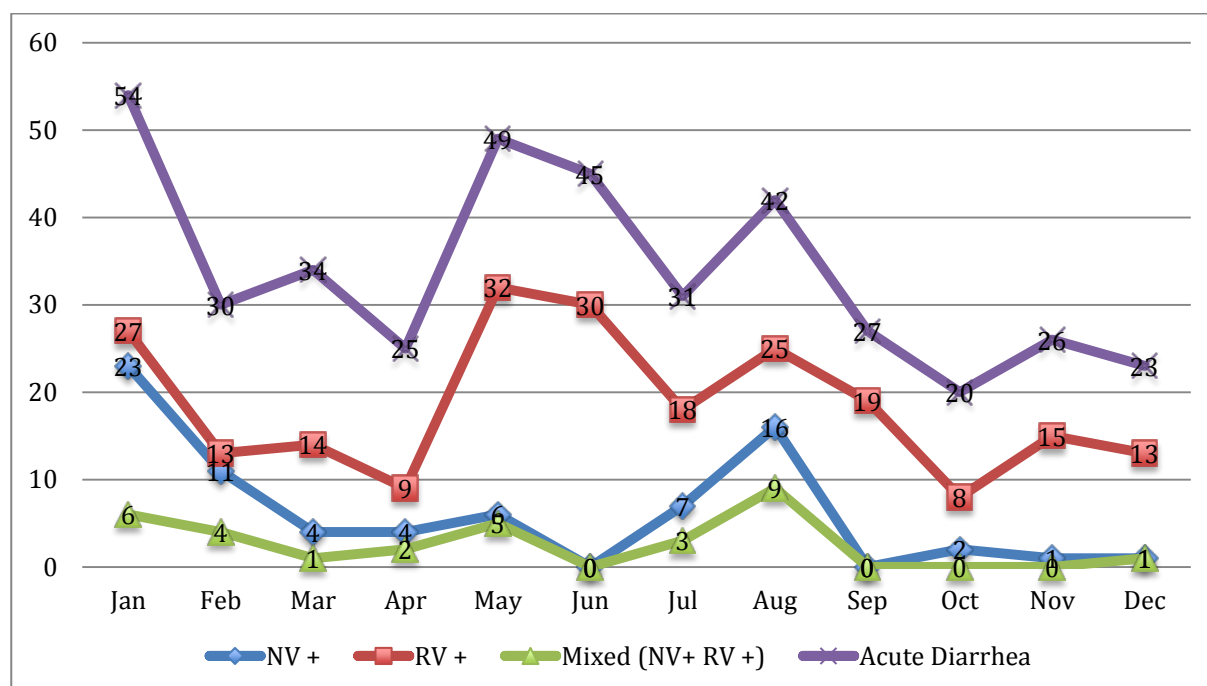
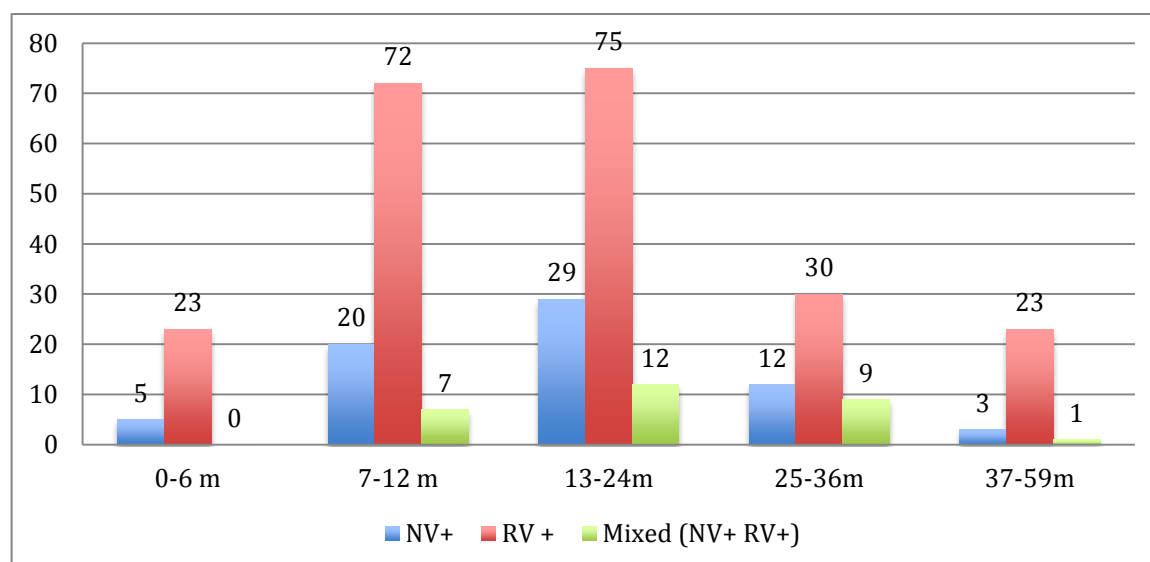


Figure 2. Age distribution of hospitalized children with norovirus (NV+) and rotavirus (RV+) infections [n=406].



Molecular epidemiology

Genogrouping of 75 norovirus-positive stool samples identified 6 (8%) and 69 (92%) of GI and GII norovirus, respectively. For rotavirus genotyping, G3P[8], G2P[6] and G9P[8] genotypes were identified in 30 (85.7%), 1 (2.9%) and 1 (2.9%) of 35 rotavirus-positive stool samples. Three of rotavirus genotypes (8.5%) were unidentified (Table 3).

Table 3. Genogroup of norovirus and genotypes of rotavirus identified in this study.

Viral Agent	n (%)
Norovirus (n = 75)	
Norovirus 1 (GI)	6 (8)
Norovirus 2 (GII)	69 (92)
Rotavirus (n = 35)	
G2P[6]	1 (2.9)
G3P[8]	30 (85.7)
G9P[8]	1 (2.9)
Untypeable	3 (8.5)

Discussion

Currently, there is a lack of nationwide studies to assess the burden of norovirus diseases in Indonesia, despite its importance as the main agent of acute gastroenteritis outbreaks globally [15]. Most of the previous studies have been conducted more than a decade ago in only two regions (Jakarta and Surabaya) and in a limited time of surveillance periods [15]. In addition, those studies did not characterize the genogroup and genotype of circulating norovirus in Indonesia [32, 43-45]. Consequently, there is lacking information about the burden of diseases, epidemiology, as well as norovirus diversity in Indonesia, similar with other developing countries [23].

Our study showed that the prevalence of norovirus in children less than five years of age hospitalized with acute gastroenteritis in Indonesia was 18.47%, similar with findings of worldwide studies [1]. Since the previous nationwide surveillance of acute gastroenteritis was mainly focused on rotavirus [41], we therefore suggest that the burden of norovirus

diseases in Indonesia should not be underestimated. In 2015, Ministry of Health [18] reported 21 diarrhea outbreaks in Indonesia with more than 1,200 affected patients and 30 deaths, resulting in a Case Fatality Rate (CFR) of 2.47%. Unfortunately, the etiological causes of these outbreaks were not further investigated. It is possible that a proportion of these reported outbreaks was attributable to norovirus as the most common cause of diarrhea in all ages. In fact, norovirus has been reported in diarrhea outbreaks in neighboring countries of Indonesia, including Singapore [29, 39] and Thailand [25, 35].

Based on our findings, norovirus and rotavirus infections commonly occurred between 7 and 24 months of age, with a median age of 15 and 14 months, respectively. Maternal antibodies could be the protective factor for children early in their life during breastfeeding period [4]. Administration of rotavirus vaccines scheduled at 2, 4 and 6 months should therefore reduce the prevalence of rotavirus diarrhea at the later ages. The overall clinical characteristics of norovirus and rotavirus infections are similar and therefore could not be easily distinguished in the clinics. With regard to seasonality, both viruses were detected throughout the year, with exception of norovirus which is not detected in June and September. We found a peak of rotavirus positive cases in the cool, dry season in May and June, consistent with our previous studies [41, 51]. In temperate countries of northern and southern hemispheres, peaks of norovirus and rotavirus cases were observed during winter season [20, 33]. Environmental factors, such as temperature and humidity, as well as population dynamics such as traveling and food consumption, all contribute to norovirus and rotavirus transmission in human.

We found that GII norovirus was much more prevalent (92%) than GI norovirus (8%). To our knowledge, this is the first study that identified norovirus genogroup in hospitalized children less than five years of age with acute gastroenteritis in Indonesia. Globally, GII.4 norovirus is the most prevalent strain in causing clinical diseases [8]. Interestingly, a recent study in Surabaya, Indonesia, reported norovirus shedding in asymptomatic healthy adults [48]. Norovirus genotyping found that most of the viruses was GII.2. Norovirus strains associated with outbreaks have also been detected, including GII.4 Sydney and GII.17 [48]. GII.4 Sydney has been detected in the gastroenteritis outbreaks of 2012-2013 in the United States [3], China [53], and Taiwan [52]. While GII.17 norovirus recently emerges as the predominant cause of gastroenteritis outbreaks in Japan and China and will potentially replace the

previously dominating GII.4 norovirus [5, 8]. Based on all these findings, characterization of norovirus genotypes in our patients should be the main focus of our future studies.

In our study, we did not investigate the source and route of transmission of the infecting norovirus. Norovirus genotypes differ in their mode of transmission. GI norovirus is associated with waterborne transmission. In contrast, GII.4 genotype is more often associated with person-to-person transmission than non GII.4 genotypes [8]. It has been previously hypothesized that immunocompromised individuals serve as reservoirs of the emerging norovirus strains which are then responsible for large outbreaks [19]. This hypothesis was challenged by a recent evidence suggesting that these individuals were unlikely to contribute at the epidemic level [10]. Noteworthy, multiple norovirus infections have been observed in asymptomatic healthy subjects with a relatively high viral load [48], questioning whether healthy asymptomatic individuals could potentially serve as reservoirs predisposing to sporadic cases and outbreaks. In nosocomial settings, however, norovirus transmission is more likely to occur from symptomatic patients [46, 47]. Therefore, these studies suggest that symptomatic individuals are also more likely to contribute in norovirus transmission in community settings. Definitely, an improved transmission route identification and effective prevention strategies are urgently required to reduce the prevalence of norovirus diseases in Indonesia.

Our study found a high incidence of rotavirus infection in Indonesia, similar with findings of our previous national surveillance conducted in 2006, 2009 and 2010 [30, 41]. The prevalence of rotavirus infection (54.93%) was indeed higher than norovirus (18.74%). These results were not surprising given the fact that rotavirus vaccines are not yet included in the National Immunization Program of Indonesia, although the parents can obtain them through private health facilities. However, the use of rotavirus vaccines was limited, because of limited knowledge of their importance and availability as well as financial constraints of our general population [40]. Noteworthy, in several countries where rotavirus vaccines have been universally introduced, norovirus infection become more prevalent than rotavirus as the cause of acute gastroenteritis in children [16, 21, 34, 37].

Rotavirus genotyping demonstrated that G3P[8] was the most common genotype. This finding was unexpected since G3P[8] was rarely detected in previous surveillance studies

conducted in several regions of Indonesia [30, 31, 36, 38, 41]. Worldwide surveillance data from 1996 to 2007 showed that G3P[8] genotype circulates at a lesser extent than G1P[8], G9P[8] and G2P[4] genotypes in human [2]. However, its prevalence has increased in the last decade in many countries and it has replaced the previously dominating G1P[8] genotype in some regions [6, 12, 17, 26, 27, 42]. Whether these changes were associated with mass vaccination or were simply the result of natural evolution of rotavirus remain to be investigated. Interestingly, G3 genotype has the largest host range among other human rotavirus group A. It has been detected in many species, including humans, pig, monkey, horse, cat, dog and rabbit [24]. Therefore, further sequence analysis is essential to determine the origin and evolution of G3P[8] genotype in Indonesia. These results also highlight the importance of continuous strain monitoring to find any alterations of circulating genotypes.

In conclusion, our study describes the considerably high burden of norovirus and rotavirus gastroenteritis in Indonesian children less than five years of age. Strengthening collaboration between the government, public health experts, virologist and research laboratories is highly important to assess the true burden of norovirus diseases in Indonesia, then formulate appropriate prevention and control strategies. In addition, norovirus genotyping should be performed in our future studies to provide databases of potentially emerging strains and to support the development of effective vaccines.

Acknowledgement

This study was financially supported by the Indonesia Endowment Fund for Education (LPDP) (to Mohamad S. Hakim) and Teuku Jacob Grants, Faculty of Medicine Universitas Gadjah Mada (to Hera Nirwati). The authors would like to thank Sri Fatmawati for her technical assistance.

Disclosures

The authors declare no conflict of interest.

References

1. Ahmed SM, Hall AJ, Robinson AE, Verhoef L, Premkumar P, Parashar UD, Koopmans M, Lopman BA (2014) Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis. *Lancet Infect Dis* 14:725-730.
2. Banyai K, Laszlo B, Duque J, Steele AD, Nelson EA, Gentsch JR, Parashar UD (2012) Systematic review of regional and temporal trends in global rotavirus strain diversity in the pre rotavirus vaccine era: insights for understanding the impact of rotavirus vaccination programs. *Vaccine* 30 Suppl 1:A122-130.
3. Centers for Disease C, Prevention (2013) Emergence of new norovirus strain GII.4 Sydney--United States, 2012. *MMWR Morb Mortal Wkly Rep* 62:55.
4. Chan J, Nirwati H, Triasih R, Bogdanovic-Sakran N, Soenarto Y, Hakimi M, Duke T, Buttery JP, Bines JE, Bishop RF, Kirkwood CD, Danchin MD (2011) Maternal antibodies to rotavirus: could they interfere with live rotavirus vaccines in developing countries? *Vaccine* 29:1242-1247.
5. Chan MCW, Hu Y, Chen H, Podkolzin AT, Zaytseva EV, Komano J, Sakon N, Poovorawan Y, Vongpunsawad S, Thanusuwannasak T, Hewitt J, Croucher D, Collins N, Vinje J, Pang XL, Lee BE, de Graaf M, van Beek J, Vennema H, Koopmans MPG, Niendorf S, Poljsak-Prijatelj M, Steyer A, White PA, Lun JH, Mans J, Hung TN, Kwok K, Cheung K, Lee N, Chan PKS (2017) Global Spread of Norovirus GII.17 Kawasaki 308, 2014-2016. *Emerg Infect Dis* 23:1359-1354.
6. Cowley D, Donato CM, Roczo-Farkas S, Kirkwood CD (2016) Emergence of a novel equine-like G3P[8] inter-genogroup reassortant rotavirus strain associated with gastroenteritis in Australian children. *J Gen Virol* 97:403-410.
7. de Graaf M, van Beek J, Vennema H, Podkolzin AT, Hewitt J, Bucardo F, Templeton K, Mans J, Nordgren J, Reuter G, Lynch M, Rasmussen LD, Iritani N, Chan MC, Martella V, Ambert-Balay K, Vinje J, White PA, Koopmans MP (2015) Emergence of a novel GII.17 norovirus - End of the GII.4 era? *Euro Surveill* 20.
8. de Graaf M, van Beek J, Koopmans MP (2016) Human norovirus transmission and evolution in a changing world. *Nat Rev Microbiol* 14:421-433.
9. Dung TT, Phat VV, Nga TV, My PV, Duy PT, Campbell JI, Thuy CT, Hoang NV, Van Minh P, Le Phuc H, Tuyet PT, Vinh H, Kien DT, Huy Hle A, Vinh NT, Nga TT, Hau NT, Chinh NT, Thuong TC, Tuan HM, Simmons C, Farrar JJ, Baker S (2013) The validation and utility of a quantitative one-step multiplex RT real-time PCR targeting rotavirus A and norovirus. *J Virol Methods* 187:138-143.
10. Eden JS, Chisholm RH, Bull RA, White PA, Holmes EC, Tanaka MM (2017) Persistent infections in immunocompromised hosts are rarely sources of new pathogen variants. *Virus Evol* 3:vex018.
11. Gentsch JR, Laird AR, Bielfelt B, Griffin DD, Banyai K, Ramachandran M, Jain V, Cunliffe NA, Nakagomi O, Kirkwood CD, Fischer TK, Parashar UD, Bresee JS, Jiang B, Glass RI (2005) Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs. *J Infect Dis* 192 Suppl 1:S146-159.
12. Gomez MM, Carvalho-Costa FA, Volotao Ede M, Rose TL, da Silva MF, Fialho AM, de Assis RM, Matthijssens J, Leite JP (2014) A decade of G3P[8] and G9P[8] rotaviruses in Brazil: epidemiology and evolutionary analyses. *Infect Genet Evol* 28:389-397.
13. Graves NS (2013) Acute gastroenteritis. *Prim Care* 40:727-741.
14. Greenberg HB, Estes MK (2009) Rotaviruses: from pathogenesis to vaccination. *Gastroenterology* 136:1939-1951.
15. Hakim MS, Nirwati H, Aman AT, Soenarto Y, Pan Q (2018) Significance of continuous rotavirus and norovirus surveillance in Indonesia. *World J Pediatr* 14(1): 4-12.
16. Hemming M, Rasanen S, Huhti L, Paloniemi M, Salminen M, Vesikari T (2013) Major reduction of rotavirus, but not norovirus, gastroenteritis in children seen in hospital after the introduction of RotaTeq vaccine into the National Immunization Programme in Finland. *Eur J Pediatr* 172:739-746.

17. Hull JJ, Teel EN, Kerin TK, Freeman MM, Esona MD, Gentsch JR, Cortese MM, Parashar UD, Glass RI, Bowen MD, National Rotavirus Strain Surveillance S (2011) United States rotavirus strain surveillance from 2005 to 2008: genotype prevalence before and after vaccine introduction. *Pediatr Infect Dis J* 30:S42-47.
18. Kementerian Kesehatan Republik Indonesia (Ministry of Health, Indonesia). Profil Kesehatan Indonesia 2015. <http://www.depkes.go.id/resources/download/pusdatin/profil-kesehatan-indonesia/profil-kesehatan-Indonesia-2015.pdf> (accessed January 4, 2018)
19. Karst SM, Baric RS (2015) What is the reservoir of emergent human norovirus strains? *J Virol* 89:5756-5759.
20. Kawai K, O'Brien MA, Goveia MG, Mast TC, El Khoury AC (2012) Burden of rotavirus gastroenteritis and distribution of rotavirus strains in Asia: a systematic review. *Vaccine* 30:1244-1254.
21. Koo HL, Neill FH, Estes MK, Munoz FM, Cameron A, DuPont HL, Atmar RL (2013) Noroviruses: The Most Common Pediatric Viral Enteric Pathogen at a Large University Hospital After Introduction of Rotavirus Vaccination. *J Pediatric Infect Dis Soc* 2:57-60.
22. Lanata CF, Fischer-Walker CL, Olascoaga AC, Torres CX, Aryee MJ, Black RE, Child Health Epidemiology Reference Group of the World Health O, Unicef (2013) Global causes of diarrheal disease mortality in children <5 years of age: a systematic review. *PLoS One* 8:e72788.
23. Lopman BA, Steele D, Kirkwood CD, Parashar UD (2016) The Vast and Varied Global Burden of Norovirus: Prospects for Prevention and Control. *PLoS Med* 13:e1001999.
24. Martella V, Banyai K, Matthijnssens J, Buonavoglia C, Ciarlet M (2010) Zoonotic aspects of rotaviruses. *Vet Microbiol* 140:246-255.
25. McCarthy KS, Guntapong R, Thattiyaphong A, Wangroongsarb P, Hall AJ, Olsen SJ, Holtz TH (2013) Outbreak of norovirus gastroenteritis infection, Thailand. *Southeast Asian J Trop Med Public Health* 44:409-416.
26. Medici MC, Tummo F, Martella V, Arcangeletti MC, De Conto F, Chezzi C, Magri A, Feher E, Marton S, Calderaro A, Banyai K (2016) Whole genome sequencing reveals genetic heterogeneity of G3P[8] rotaviruses circulating in Italy. *Infect Genet Evol* 40:253-261.
27. Mitui MT, Chan PK, Nelson EA, Leung TF, Nishizono A, Ahmed K (2011) Co-dominance of G1 and emerging G3 rotaviruses in Hong Kong: a three-year surveillance in three major hospitals. *J Clin Virol* 50:325-333.
28. Mortality GBD, Causes of Death C (2015) Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 385:117-171.
29. Ng TL, Chan PP, Phua TH, Loh JP, Yip R, Wong C, Liaw CW, Tan BH, Chiew KT, Chua SB, Lim S, Ooi PL, Chew SK, Goh KT (2005) Oyster-associated outbreaks of Norovirus gastroenteritis in Singapore. *J Infect* 51:413-418.
30. Nirwati H, Wibawa T, Aman AT, Wahab A, Soenarto Y (2016) Detection of group A rotavirus strains circulating among children with acute diarrhea in Indonesia. *Springerplus* 5:97.
31. Nirwati H, Hakim MS, Aminah S, Dwija I, Pan Q, Aman AT (2017) Identification of Rotavirus Strains Causing Diarrhoea in Children under Five Years of Age in Yogyakarta, Indonesia. *Malays J Med Sci* 24:68-77.
32. Oyofo BA, Subekti D, Tjaniadi P, Machpud N, Komalarini S, Setiawan B, Simanjuntak C, Punjabi N, Corwin AL, Wasfy M, Campbell JR, Lesmana M (2002) Enteropathogens associated with acute diarrhea in community and hospital patients in Jakarta, Indonesia. *FEMS Immunol Med Microbiol* 34:139-146.
33. Patel MM, Hall AJ, Vinje J, Parashar UD (2009) Noroviruses: a comprehensive review. *J Clin Virol* 44:1-8.
34. Payne DC, Vinje J, Szilagyi PG, Edwards KM, Staat MA, Weinberg GA, Hall CB, Chappell J, Bernstein DI, Curns AT, Wikswo M, Shirley SH, Hall AJ, Lopman B, Parashar UD (2013) Norovirus and medically attended gastroenteritis in U.S. children. *N Engl J Med* 368:1121-1130.

35. Phumpholsup T, Theamboonlers A, Wanlapakorn N, Felber JA, Suvaporn A, Puthanakit T, Chomto S, Payungporn S, Poovorawan Y (2015) Norovirus Outbreak at a Daycare Center in Bangkok, 2014. *Southeast Asian J Trop Med Public Health* 46:616-623.
36. Putnam SD, Sedyaningsih ER, Listyaningsih E, Pulungsih SP, Komalarini, Soenarto Y, Salim O, Subekti D, Riddle MS, Burgess TH, Blair PJ (2007) Group A rotavirus-associated diarrhea in children seeking treatment in Indonesia. *J Clin Virol* 40:289-294.
37. Puustinen L, Blazevic V, Salminen M, Hamalainen M, Rasanen S, Vesikari T (2011) Noroviruses as a major cause of acute gastroenteritis in children in Finland, 2009-2010. *Scand J Infect Dis* 43:804-808.
38. Radji M, Putman SD, Malik A, Husrima R, Listyaningsih E (2010) Molecular characterization of human group A rotavirus from stool samples in young children with diarrhea in Indonesia. *Southeast Asian J Trop Med Public Health* 41:341-346.
39. Raj P, Tay J, Ang LW, Tien WS, Thu M, Lee P, Pang QY, Tang YL, Lee KY, Maurer-Stroh S, Gunalan V, Cutter J, Goh KT (2017) A large common-source outbreak of norovirus gastroenteritis in a hotel in Singapore, 2012. *Epidemiol Infect* 145:535-544.
40. Seale H, Sitaresmi MN, Atthobari J, Heywood AE, Kaur R, MacIntyre RC, Soenarto Y, Padmawati RS (2015) Knowledge and attitudes towards rotavirus diarrhea and the vaccine amongst healthcare providers in Yogyakarta Indonesia. *BMC Health Serv Res* 15:528.
41. Soenarto Y, Aman AT, Bakri A, Waluya H, Firmansyah A, Kadim M, Martiza I, Prasetyo D, Mulyani NS, Widowati T, Soetjningsih, Karyana IP, Sukardi W, Bresee J, Widdowson MA (2009) Burden of severe rotavirus diarrhea in Indonesia. *J Infect Dis* 200 Suppl 1:S188-194.
42. Stupka JA, Degiuseppe JI, Parra GI, Argentinean National Rotavirus Surveillance N (2012) Increased frequency of rotavirus G3P[8] and G12P[8] in Argentina during 2008-2009: whole-genome characterization of emerging G12P[8] strains. *J Clin Virol* 54:162-167.
43. Subekti D, Lesmana M, Tjaniadi P, Safari N, Frazier E, Simanjuntak C, Komalarini S, Taslim J, Campbell JR, Oyofo BA (2002) Incidence of Norwalk-like viruses, rotavirus and adenovirus infection in patients with acute gastroenteritis in Jakarta, Indonesia. *FEMS Immunol Med Microbiol* 33:27-33.
44. Subekti DS, Tjaniadi P, Lesmana M, Simanjuntak C, Komalarini S, Digdowirogo H, Setiawan B, Corwin AL, Campbell JR, Porter KR, Oyofo BA (2002) Characterization of Norwalk-like virus associated with gastroenteritis in Indonesia. *J Med Virol* 67:253-258.
45. Sudarmo SM, Shigemura K, Athiyyah AF, Osawa K, Wardana OP, Darma A, Ranuh R, Raharjo D, Arakawa S, Fujisawa M, Shirakawa T (2015) Genotyping and clinical factors in pediatric diarrhea caused by rotaviruses: one-year surveillance in Surabaya, Indonesia. *Gut Pathog* 7:3.
46. Sukhrie FH, Teunis P, Vennema H, Copra C, Thijs Beersma MF, Bogerman J, Koopmans M (2012) Nosocomial transmission of norovirus is mainly caused by symptomatic cases. *Clin Infect Dis* 54:931-937.
47. Teunis PF, Sukhrie FH, Vennema H, Bogerman J, Beersma MF, Koopmans MP (2015) Shedding of norovirus in symptomatic and asymptomatic infections. *Epidemiol Infect* 143:1710-1717.
48. Utsumi T, Lusida MI, Dinana Z, Wahyuni RM, Yamani LN, Juniastuti, Soetjipto, Matsui C, Deng L, Abe T, Doan YH, Fujii Y, Kimura H, Katayama K, Shoji I (2017) Occurrence of norovirus infection in an asymptomatic population in Indonesia. *Infect Genet Evol* 55:1-7.
49. Walker CL, Rudan I, Liu L, Nair H, Theodoratou E, Bhutta ZA, O'Brien KL, Campbell H, Black RE (2013) Global burden of childhood pneumonia and diarrhoea. *Lancet* 381:1405-1416.
50. World Health Organization (WHO) (2006). Pocket book of hospital care for children: guidelines for the management of common illnesses with limited resources. Geneva: World Health Organization (WHO).
51. Wilopo SA, Soenarto Y, Bresee JS, Tholib A, Aminah S, Cahyono A, Gentsch JR, Kilgore P, Glass RI (2009) Rotavirus surveillance to determine disease burden and epidemiology in Java, Indonesia, August 2001 through April 2004. *Vaccine* 27 Suppl 5:F61-66.

52. Wu FT, Chen HC, Yen C, Wu CY, Katayama K, Park Y, Hall AJ, Vinje J, Huang JC, Wu HS (2015) Epidemiology and molecular characteristics of norovirus GII.4 Sydney outbreaks in Taiwan, January 2012-December 2013. *J Med Virol* 87:1462-1470.
53. Zheng QM, Zeng HT, Dai CW, Zhang SX, Zhang Z, Mei SJ, He YQ, Ma HW (2015) Epidemiological investigation of a norovirus GII.4 Sydney outbreak in a China elder care facility. *Jpn J Infect Dis* 68:70-74.

Supplementary Table

Supplementary Table 1. Primers and probes used in this study to identify the genogroup of norovirus.

Detection		Sequence
Norovirus I (GI)	Probe	AGATYGCGITCICCTGTCCA
	Primer (F)	CGYTGGATGCGITTYCATGA
	Primer (R)	CTTAGACGCCATCATCATTYAC
Norovirus II (GII)	Probe	TGGGAGGGCGATCGCAATCT
	Primer (F)	CARGARBCNATGTTYAGRTGGATGAG
	Primer (R)	TCGACGCCATCTTCATTCACA

Chapter 11

Identification of Rotavirus Strains Causing Diarrhoea in Children Under Five Years of Age in Yogyakarta, Indonesia

Hera Nirwati¹, Mohamad S. Hakim^{1,2}, Sri Aminah, Ida BNP Dwija⁴, Qiuwei Pan², Abu T.
Aman¹

- ¹ Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia
- ² Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, 3015 CE Rotterdam, The Netherlands
- ³ Department of Pediatric, Kodya Yogyakarta Hospital, 55162 Yogyakarta, Indonesia
- ⁴ Department of Microbiology, Faculty of Medicine, Udayana University, 80232 Denpasar, Indonesia

Malays J Med Sci 2017; 24(2): 68-77.

Abstract

Background: Rotavirus is an important cause of severe diarrhoea in children. The aims of this study were to identify the rotavirus strains that cause diarrhoea in children in Yogyakarta and to determine the association between rotavirus positivity and its clinical manifestations.

Methods: Clinical data and stool samples were collected from children hospitalised at Kodya Yogyakarta Hospital, Indonesia. Rotavirus was detected in stool samples using an enzyme immunoassay (EIA), which was followed by genotyping using reverse transcriptase polymerase chain reaction (RT-PCR). Electropherotyping was performed for the rotavirus-positive samples.

Results: In total, 104 cases were included in the study, 57 (54.8%) of which were rotavirus-positive. Based on a multiple logistic regression analysis, age group vomiting and stool mucous were associated with rotavirus positivity. Most of the 56 samples subjected to genotyping were classified as G1 (80.36%) and P[8] (69.64%) genotypes. The genotype combination G1P[8] was identified as the most prevalent strain (66.07%). Of the 19 samples subjected to electropherotyping, 17 G1 isolates and 1 G3 isolate had long patterns, and 1 G1 isolate had a short pattern.

Conclusion: G1P[8] was the most dominant strain of rotavirus causing diarrhoea in children in Yogyakarta. Age group, vomiting and stool mucous were associated with rotavirus positivity.

Keywords: rotavirus, acute diarrhoea, G-type, P-type, electropherotyping

Introduction

Rotavirus is an RNA virus that mainly infects the gastrointestinal tract. It is the most prevalent agent causing severe diarrhoea among infants and young children in both developed and developing countries, including Indonesia (1-3). It was recently estimated that rotavirus is responsible for 7,200,000–11,500,000 severe diarrhoea episodes and 133,000–284,000 deaths, most of which occur in developing countries (2). The incidence might be lower in countries that have introduced rotavirus vaccination (2).

Since rotavirus diarrhoea is prevalent among children in developed and developing countries, it is unlikely that improvements in sanitation and hygiene will be able to lower the incidence of the disease. Therefore, one of the best strategies for decreasing the global burden of disease is development and implementation of effective vaccines (1). The current strategy for developing rotavirus vaccines involves oral administration of live attenuated viruses that protection is expected to be similar to natural rotavirus infection (4, 5).

Rotavirus has three essential antigenic specificities, determined by the viral proteins VP6, VP7 and VP4. Based on the VP6 protein, rotavirus is classified into seven groups (A to G), yet only groups A, B and C infect humans. VP7, a glycoprotein, determines G-genotype, while VP4, which is protease-sensitive, determines P-genotype. VP7 and VP4 form the outer layer of rotavirus and elicit neutralising antibody responses. Therefore, they are implicated in the dual classification of rotavirus group A as G- and P-type (6).

The effectiveness of rotavirus vaccine can be enhanced by identification of the patterns of circulating virus serotypes and the role of these serotypes in causing clinical illness (7). During vaccine implementation, strain surveillance is necessary to monitor antibody-escape variants and changes in the circulating rotavirus strains among humans (8).

Two rotavirus vaccines, Rotarix and RotaTeq, have been developed by GSK and Merck, respectively. RotaTeq is a live attenuated pentavalent vaccine containing G1, G2, G3, G4 and P[8] rotavirus derived from reassortants of human and bovine strains. Rotarix is a live attenuated monovalent vaccine derived from the most common human rotavirus strain, G1P[8]. These vaccines have been proven to be highly effective for the global prevention of severe diarrhoea (9, 10). Before implementing the rotavirus vaccination programme in

Yogyakarta, it is crucial to perform a study to ensure that the vaccines used in the programme match with the most prevalent and clinically relevant rotavirus strain in Yogyakarta.

The aims of this study were to identify the rotavirus strains currently circulating in Yogyakarta and to determine the association between rotavirus positivity and the illness's clinical manifestation. This study provides accurate information about the rotavirus strains circulating in Yogyakarta before and after the implementation of the vaccination programme.

Materials and Methods

Study population and case definition

From February to August 2009, we enrolled 104 children under five years of age that were hospitalised at Kodya Yogyakarta General Hospital, Indonesia due to acute diarrhoea. We defined acute diarrhoea as 3 or more instances of loose stool within 24 hours for a duration of fewer than 2 weeks (11). We excluded patients whose faecal samples could not be obtained within 48 hours of admission to the hospital.

Clinical data collection

The patients' medical histories, including previous occurrences of blood and mucous in stool, fever, vomiting, dehydration and diarrhoea, were collected.

Sample collection and storage

Stool samples were collected within the first 48 hours after admission according to WHO protocol (12). Stool specimens were stored at 4–8⁰C before they were transported to the Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Indonesia. The specimens were then aliquoted into several tubes and stored at -20⁰C.

Detection of rotavirus in stool samples

All stool samples were examined for the presence of group A rotavirus by enzyme immunoassay (EIA) using the IDEIA™ Rotavirus (DakoCytomation) kit according to the manufacturer's instructions. Other possible causes of diarrhoea were not examined.

RT-PCR genotyping

Rotavirus RNA was extracted from rotavirus-positive faecal specimens using Trizol (Invitrogen) according to the manufacturer's instructions. Rotavirus RNA was analysed to determine the VP7 (G-type) and VP4 (P-type) genotypes using reverse transcriptase polymerase chain reaction (RT-PCR) as previously described (13-15). For G typing, the primers 9con1L (13) and VP7R (15) were used during the first round of RT-PCR (20 cycles) to amplify the 905-bp of the VP7 gene segment. Then, 9con1L was used in the second round of PCR (20 cycles) with the type-specific primers 9T1 (G1), 9T-2 (G2), 9T-3P (G3), 9T4 (G4) and 9T-9B (G9) (Table 1) (13). For P-typing, the primers con2 and con3 were used in the first-round of RT-PCR (20 cycles) to amplify the 877-bp fragment of the VP4 gene segment (14). Con3 was used in the second round of PCR (30 cycles) with the type-specific primers 1T-1D (P[8]), 2T-1 (P[4]), 3T-1(P[6]), 4T-1 (P[9]) and 5T-1 (P[10]) (Table 2) (14).

Samples that failed to yield a detectable PCR product from secondary amplification of the primer-specific PCR typing were re-typed with other primers using the methods described by Gouvea et al. (16) and Simmond et al. (8). For G typing, the consensus primers VP7F and VP7R were used in the first round of RT-PCR (30 cycles) to generate the 881-bp of the VP7 gene segment (15). VP7F was used in the second round of PCR (30 cycles) with the type-specific primers aBT1 (G1), aCT-2 (G2), G3 (G3), aDT4 (G4) and G9 (G9) (Table 1) (16). For P-typing, the consensus primers VP4F and VP4R were used in the first round of RT-PCR (30 cycles) to generate a 663-bp fragment of the VP4 gene segment (8). VP4F was used in the second round of PCR (40 cycles) with the type-specific primers 1T-1D (P[8]), 2T-1 (P[4]), 3T-1(P[6]), 4T-1 (P[9]) and 5T-1 (P[10]) (Table 2) (14). All PCR products were separated in 2% agarose gel and visualised under UV light after staining with ethidium bromide.

	Primer	Sequence (5'-3')	Position	PCR product (bp)	References
First amplification of G-typing as described by Das BK et al. (13) and Gomara MI et al. (15)					
	9con1L	TAG CTC CTT TTA ATG TAT GGT AT	37 - 59	895	Das BK et al. (13)
	VP7R	AAC TTG CCA CCA TTT TTT CC	914 - 932		Gomara MI et al. (15)
First amplification of G-typing as described by Gomara MI et al. (15)					
	VP7F	ATG TAT GGT ATT GAA TAT ACC AC	51 - 71	881	Gomara MI et al. (15)
	VP7R	AAC TTG CCA CCA TTT TTT CC	914 - 932		Gomara MI et al. (15)
Second amplification of G-typing as described by Das BK et al. (13)					
G1	9T-1	TCT TGT CAA AGC AAA TAA TG	176 - 195	158	Das BK et al. (13)
G2	9T-2	GTT AGA AAT GAT TCT CCA CT	262 - 281	244	Das BK et al. (13)
G3	9T-3P	GTC CAG TTG CAG TGT AGC	484 - 501	464	Das BK et al. (13)
G4	9T-4	GGG TCG ATG GAA AAT TCT	423 - 440	403	Das BK et al. (13)
G9	9T-9B	TAT AAA GTC CAT TGC AC	131 - 147	110	Das BK et al. (13)
Second amplification of G-typing as described by Gouvea et al. (16)					
G1	aBT1	CAA GTA CTC AAA TCA ATG ATG G	314 - 335	618	Gouvea et al. (16)
G2	aCT2	CAA TGA TAT TAA CAC ATT TTC TGT G	411 - 435	521	Gouvea et al. (16)
G3	G3	ACG AAC TCA ACA CGA GAG G	250 - 259	682	Gouvea et al. (16)
G4	aDT4	CGT TTC TGG TGA GGA GTT G	480 - 498	452	Gouvea et al. (16)
G8	aAT8	GTC ACA CCA TTT GTA AAT TCG	178 - 198	754	Gouvea et al. (16)
G9	aFT9	CTT GAT GTG ACT AYA AAT AC	757 - 776	179	Gouvea et al. (16)

Table 1. Primers used for VP7 genotyping of rotavirus strains

	Primer	Sequence (5'-3')	Position	PCR product (bp)	References
First amplification of P-typing as described by Gentsch et al. (14)					
	Con-3	TGG CTT CGC TCA TTT ATA GAC A	11 - 32	876	Gentsch et al. (14)
	Con-2	ATT TCG GAC CAT TTA TAA CC	868 - 887		Gentsch et al. (14)
First amplification of P-typing as described by Simmond et al. (8)					
	VP4F	TAT GCT CCA GTN AAT TGG	132 - 149	663	Simmond et al. (8)
	VP4R	ATT GCA TTT CTT TCC ATA ATG	775 - 795		Simmond et al. (8)
Second amplification of P-typing as described by Gentsch et al. (14)					
P[4]	2T-1	CTA TTG TTA GAG GTT AGA GTC	474 – 494	483	Gentsch et al. (14)
P[6]	3T-1	TGT TGA TTA GTT GGA TTC AA	259 – 278	267	Gentsch et al. (14)
P[8]	1T-1D	TCT ACT TGG ATA ACG TGC	339 – 356	345	Gentsch et al. (14)
P[9]	4T-1	TGA GAC ATG CAA TTG GAC	385 – 402	391	Gentsch et al. (14)
P[10]	5T-1	ATC ATA GTT AGT AGT CGG	575 – 594	583	Gentsch et al. (14)

Table 2. Primers used for VP4 genotyping of rotavirus strains

Electropherotyping

Rotavirus RNA was extracted using Minikit (Qiagen) according to the manufacturer's instructions. RNA was subjected to polyacrylamide gel electrophoresis to separate the 11 segments of dsRNA, which were then visualised by silver staining.

Statistical analyses

The variables were described using frequencies and percentages. Chi-square tests and logistic regression analyses were used to determine the association between rotavirus infections and clinical manifestations using STATA 13. The variables that had *P* values of less than 0.25 according to the chi-square tests were entered into a logistic regression analysis model. Age group and all the clinical symptoms analysed (vomiting, stool mucous, bloody stool and fever) had *P* values of less than 0.25 (Table 3). Bloody stool was omitted because the chi-square test revealed cells with a count of zero. Therefore, age group, vomiting, stool mucous and fever were examined in the multiple logistic regression analysis. The fit of the model was checked using the Hosmer-Lemeshow test. The results were presented as crude and adjusted odds ratios with a 95% confidence interval. *P* values of less than 0.05 were considered statistically significant.

Ethical approval

The research protocol was approved by the Ethical Committee of the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. Informed consent was provided by the parents or guardians of each child before the children were enrolled in the study.

Results

From February to August 2009, 104 children under five years of age who were hospitalised at Kodya Yogyakarta General Hospital, Indonesia due to acute diarrhoea were enrolled in the study. There were 70 (67.3%) male and 34 (32.7%) female patients (Table 3). Of the 104 samples collected, 57 (54.8%) were positive for rotavirus, as determined by EIA.

Rotavirus diarrhoea particularly affects young children; 70.2% of the cases involved children under two years of age. Most cases involved children aged 12–23 months, with the next largest group comprised of children aged 24–59 months (Table 3). Additionally, the chi-square tests revealed significant differences in vomiting, stool mucous and bloody stool between rotavirus-positive and rotavirus-negative patients (Table 3).

We present the final model of the multiple logistic regression analysis in Table 4. There are four variables associated with rotavirus positivity: age group, including the 0–11-month-old group (adjusted OR = 0.31; CI 95% = 0.10 to 0.93; $P = 0.04$) and 12–23-month-old group (adjusted OR = 3.46; CI 95% 1.03 to 11.60; $P = 0.04$), stool mucous (adjusted OR = 0.24; CI 95% = 0.07 to 0.79; $P = 0.02$), and vomiting (adjusted OR = 3.97; CI 95% = 1.32 to 11.97; $P = 0.01$). In our study, only one patient had mild dehydration, and all the others ($n = 103$) had no dehydration.

Of the 57 rotavirus-positive samples, 56 faecal specimens were characterised as G or P genotypes. G1 was the most prevalent genotype (80.36%), followed by G2 (16.07%) and G3 (3.57%) (Table 5). Regarding P-typing, they were classified as P[8] (69.64%), P[4] (17.86%), P[6] (7.14%) or untypeable (5.36%) (Table 5). The identified G- and P-type combinations were G1P[8] (66.07%), G2P[4] (16.07%), G1P[6] (7.14%), G1P[untypeable] (5.36%), G3P[8] (3.57%) and G1P[4] (1.79%) (Table 5).

A number of rotavirus-positive stool samples did not have sufficient volume for polyacrylamide gel electrophoresis (PAGE) analysis. Only 24 samples were subjected to PAGE analysis, and 19 isolates had good results. Eighteen samples were identified as G1, and one sample was identified as G3. All of them showed the migration pattern of the genome of rotavirus group A: 4-2-3-2. The results of electropherotyping showed that 17 of 18 G1 isolates and 1 G3 isolate had long patterns, while 1 G1 isolate had a short pattern (Figure 1).

Variable		n (%) of patients with rotavirus-positive diarrhoea (n=57)	n (%) of patients with rotavirus-negative diarrhoea (n=47)	n	χ^2 -Statistic ^a (df)	P value ^a
Gender	Male	41 (71.9)	29 (61.7)	70	1.22 (1)	0.27
	Female	16 (28.1)	18 (38.3)	34		
Age groups	0–11	11 (19.3)	25 (53.2)	36	4.57 (1)	0.03*
	12–23	29 (50.9)	9 (19.1)	38	2.96 (1)	0.08
	24–59	17 (29.8)	13 (27.7)	30		
Clinical manifestation	Vomiting	46 (80.7)	27 (57.4)	73	6.66(1)	0.01*
	Stool mucous	6 (10.5)	17 (36.2)	23	9.83 (1)	0.00*
	Bloody stool	0 (0)	11 (23.4)	11	14.92 (1)	0.00*
	Fever	18 (31.6)	23 (48.9)	41	3.25 (1)	0.07

^a Chi-square test for independence

Table 3. Characteristics and clinical symptoms of children under five years of age hospitalised at Kodya Yogyakarta General Hospital (n = 104).

Variable	<i>b</i>	Adjusted OR (95% CI)	P value ^a
Age group: 0–11 months vs. 24–59 months ^b	-1.17	0.31 (0.10 - 0.93)	0.04
Age group: 12–23 months vs. 24–59 months ^b	1.24	3.46 (1.03 - 11.60)	0.04
Vomiting	1.38	3.97 (1.32 - 11.97)	0.01
Stool mucous	-1.41	0.24 (0.07 - 0.79)	0.02

OR = odds ratio. ^a Likelihood ratio test; ^b reference category.

Table 4. Association between age groups, clinical symptoms and rotavirus positivity revealed by multiple logistic regression.

Rotavirus strains	Total (%) (n=56)
G genotype	
G1	45 (80.36)
G2	9 (16.07)
G3	2 (3.57)
P genotype	
P[8]	39 (69.64)
P[4]	10 (17.86)
P[6]	4 (7.14)
P[untypeable]	3 (5.36)
G- and P-type combinations	
G1P[8]	37 (66.07)
G2P[4]	9 (16.07)
G1P[6]	4 (7.14)
G1P[untypeable]	3 (5.36)
G3P[8]	2 (3.57)
G1P[4]	1 (1.79)

Table 5. Rotavirus genotype distribution in Kodya Yogyakarta Hospital.

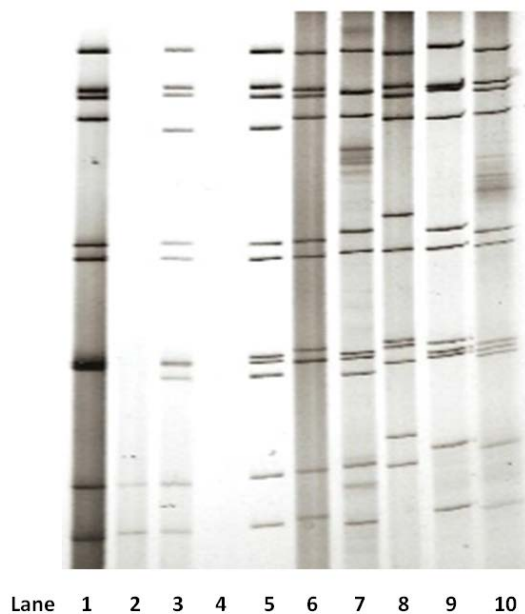


Figure 1. Electropherotype profile of rotavirus RNA genome. Lanes 1–5: sample RW13 (G3P[8]) - long pattern; sample RW22 - RNA insufficient; sample RW23 (G1P[8]) - long pattern; sample RW26 - no RNA; sample RW30 (G1P[8]) - long pattern. Lanes 6–10 are control strains: RV3 (G3P[6]) - long pattern; RV4 (G1P[8]) - long pattern; RV5 (G2P[4]) - short pattern; F45 (G9P[8]) - long pattern; ST3 (G4P[6]) - long pattern.

Discussion

This hospital-based study described a high incidence of rotavirus infection in Yogyakarta, Indonesia. Rotavirus infection was identified in 54.8% of 104 children hospitalised with acute diarrhoea. Compared to previous hospital-based studies performed in Indonesia in the late 1970s and 1990s, the incidence rate was similarly high. Bishop et al. (17) reported rotavirus infection in 38% of children with severe acute diarrhoea in Yogyakarta. Subekti et al. (18), who conducted a study in Jakarta from 1997–1999, showed that 170 of 236 (72%) children with diarrhoea were infected with rotavirus. Putnam et al. (19), who conducted a study in Medan, Makassar, Jakarta, Yogyakarta, Surabaya, Bali and Nusa Tenggara, found that 748 of 1,660 (45.1%) children under five years of age suffered from diarrhoea due to rotavirus. Moreover, Soenarto et al. (20), who conducted a study of children under five years of age in Yogyakarta, Jakarta, Bandung, Mataram, Denpasar and Palembang in 2006, showed that 60% of the 2,240 children studied suffered from diarrhoea due to rotavirus. These findings support the need for continuous surveillance of the disease in various locations.

Our study found a high incidence of rotavirus infection in Yogyakarta, Indonesia, which is comparable to the findings of similar studies in surrounding countries, such as Malaysia (38%) (21), Thailand (43%) (22), Cambodia (56%) (23) and Myanmar (57%) (24). Our study provides evidence that rotavirus infection gives a substantial burden to the diarrhoeal diseases and it is critical to develop an effective approach to preventing infection.

Rotavirus infection in children clinically manifests with several symptoms, such as fever, vomiting and watery, bloodless diarrhoea (25, 26). These clinical manifestations are more severe than those of other causes of viral gastroenteritis (27). In our study, vomiting was associated with rotavirus positivity (Table 4), which aligns with the findings of other studies (20, 28). Those studies also reported that rotavirus-positive patients were less likely to have bloody diarrhoea (20, 28), in agreement with our study.

Severe complications of rotavirus infection, such as bloody diarrhoea associated with necrotizing enterocolitis (NEC) and bowel perforation, have been described (25, 26, 29). Even though they have not yet been fully elucidated, several factors were associated with the pathogenesis of the disease (30). However, an enterotoxin protein, NSP4, and the ability

of rotavirus to trigger the apoptosis of intestinal cells might be the factors that most affect the manifestation of the disease (31).

Our study demonstrated that the percentage of children aged 0–11 months who suffered from rotavirus diarrhoea was lower than that of children aged 12–23 and 24–59 months (Table 3). This phenomenon is probably due to the presence of anti-rotavirus immunoglobulin G (IgG) and IgA antibodies in breast milk (32). In their study of children in Yogyakarta, Chan et al. (33) found a high concentration of anti-rotavirus IgG and IgA in colostrum as well as in breast milk. Moreover, Clemens et al. (34) found that exclusive breastfeeding confers better protection against severe rotavirus infection than partial or no breastfeeding. However, further study is required to fully address this issue in our patient population.

The genetic diversity of circulating rotavirus strains may have a crucial role in the initiation of the vaccination programme and its clinical evaluation. Some studies have been conducted to identify the rotavirus strains circulating in Indonesia. Using samples collected in Yogyakarta from 1978–1979, Bishop et al. (17) identified G1 (2%); G2 (9%), G3 (53%) and G4 (36%) strains. However, Soenarto et al. (20) reported that the G1 and G9 genotypes were the most prevalent, accounting for 73 (30%) and 69 (29%) of the 240 samples, respectively, followed by G2, which accounted for 34 samples (14%). Putnam et al. (19) reported that the rotavirus strains causing diarrhoea in Indonesia were P[4] (16.2%), P[6] (11.3%), P[8] (24.7%), P[9] (0.7%), P[10] (1.3%), P[11] (0.5%), mixed infection (3.6%) and untypeable (1.6%). However, Soenarto et al. (20) found that the P-type rotavirus strains causing diarrhoea were distributed differently: P[4] (16.4%), P[6] (55.6%), P[8] (17.5%), mixed infection (9%) and untypeable (1.6%).

We found that G1P[8] was the most dominant strain of rotavirus causing diarrhoea in children under five years of age hospitalised at Kodya Yogyakarta Hospital. G1P[8] was also the predominant strain identified in Malaysia in a 1996 study (35). However, G9P[8] genotypes became the most common strains in Malaysia from 2001–2003 and in 2007 (21, 35).

In our study, G2P[4], G1P[6], G3P[8] and G1P[4] were also common causative agents. Since the majority of rotavirus strains circulating in Yogyakarta are similar to those circulating

globally, we suggest that the current rotavirus vaccines can effectively reduce the prevalence of rotavirus diarrhoea in children under five years of age in Yogyakarta.

Rotavirus has several mechanisms to generate genetic diversity. One is accumulation of point mutation (genetic drift), which is due to the error-prone nature of RNA-dependent RNA polymerase (RdRp) enzyme (36). Such mutations could be introduced at the primer binding sites and result in typing failure (8, 15). Another important mechanism of rotavirus evolution is genetic shift due to reassortment of the segmented RNA genome. This event could lead to a new combination of G and P types (36) and introduce novel genotypes through zoonotic transmission (37). Both mechanisms may explain our finding that the P-type of about 5% of samples could not be identified. The rapid evolution of rotavirus may increase the number of strains that cannot be genotyped with currently available assays. Therefore, our study underscores the importance of regularly updating typing methods to successfully detect the currently circulating rotavirus strains.

Continuous monitoring is important for identifying circulating rotavirus strains before and after the introduction of the vaccine. It will provide information about the impact of the vaccine on genotype distribution and the possible emergence of less common rotavirus strains circulating in certain geographical areas. It is also crucial to assess the effectiveness of the vaccination programme against rotavirus diarrhoea.

According to the migration of its 11 RNA gene segments, rotavirus can also be classified as either long or short RNA electropherotype using polyacrylamide gels. The short electropherotype occurs as a result of partial duplication in gene segment 11. This gene runs more slowly than gene segment 10; because of its standard size, gene segment 11 in long-electropherotype strains migrates faster than segment 10 (38). In this study, we found that most G1 and G3 isolates had a long electropherotype. Long electropherotypes usually belong to the Wa genogroup, whereas short electropherotypes typically belong to the DS-1 genogroup (36).

Conclusions

We found that rotavirus caused acute diarrhoea in 54.8% of children under five years of aged hospitalised at Kodya Yogyakarta General Hospital. The majority (66.07%) was G1P[8], the most prevalent strain identified globally. Age group, vomiting and stool mucous were associated with rotavirus positivity.

Acknowledgements

This research was supported by the grant from the Indonesian Government (RISBINIPTEKDOK 2009/2010). Also supported by the Indonesia Endowment Fund for Education (LPDP) for funding PhD fellowship to Mohamad S. Hakim. We thank to Murdoch Children Research Institute Melbourne, Australia, for providing facilities to conduct G- and P-genotyping; Abdul Wahab (Universitas Gadjah Mada) for providing assistance in data analysis; Linda Oktabriana (Universitas Gadjah Mada), Nada Bogdanovic-Sakran and Carl D. Kirkwood (Murdoch Children Research Institute) for their technical support; and the parents of the children involved in this study for their willingness to participate.

Conflict of interest

The authors declare no conflict of interest.

References

1. Tate JE, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD, et al. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. *Lancet Infect Dis*. 2012; **12(2)**:136–141. doi: 10.1016/S1473-3099(11)70253-5.
2. Walker CL, Rudan I, Liu L, Nair H, Theodoratou E, Bhutta ZA, et al. Global burden of childhood pneumonia and diarrhoea. *Lancet*. 2013; **381(9875)**:1405–1416. doi: 10.1016/S0140-6736(13)60222-6.
3. Parashar UD, Gibson CJ, Bresee JS, Glass RI. Rotavirus and severe childhood diarrhea. *Emerg Infect Dis*. 2006; **12(2)**:304–306. doi: 10.3201/eid1202.050006.
4. Bresee JS, Hummelman E, Nelson EA, Glass RI. Rotavirus in Asia: the value of surveillance for informing decisions about the introduction of new vaccines. *J Infect Dis*. 2005; **192 Suppl 1**:S1–5. doi: 10.1086/431515.
5. Glass RI, Bresee JS, Turcios R, Fischer TK, Parashar UD, Steele AD. Rotavirus vaccines: targeting the developing world. *J Infect Dis*. 2005; **192 Suppl 1**:S160–166. doi: 10.1086/431504.
6. Dennehy PH. Rotavirus vaccines: an overview. *Clin Microbiol Rev*. 2008; **21(1)**:198–208. doi: 10.1128/CMR.00029-07.
7. Bern C, Unicomb L, Gentsch JR, Banul N, Yunus M, Sack RB, et al. Rotavirus diarrhea in Bangladeshi children: correlation of disease severity with serotypes. *J Clin Microbiol*. 1992; **30(12)**:3234–3238. doi: 10.1128/JCM.30.12.3234-3238.1992.
8. Simmonds MK, Armah G, Asmah R, Banerjee I, Damanka S, Esona M, et al. New oligonucleotide primers for P-typing of rotavirus strains: Strategies for typing previously untypeable strains. *J Clin Virol*. 2008; **42(4)**:368–373. doi: 10.1016/j.jcv.2008.02.011.
9. Desai R, Oliveira LH, Parashar UD, Lopman B, Tate JE, Patel MM. Reduction in morbidity and mortality from childhood diarrhoeal disease after species A rotavirus vaccine introduction in Latin America - a review. *Mem Inst Oswaldo Cruz*. 2011; **106(8)**:907–911.
10. Parashar U, Steele D, Neuzil K, Quadros C, Tharmaphornpilas P, Serhan F, et al. Progress with rotavirus vaccines: summary of the Tenth International Rotavirus Symposium. *Expert Rev Vaccines*. 2013; **12(2)**:113–117. doi: 10.1586/erv.12.148.
11. Neves MA, Pinheiro HH, Silva RS, Linhares AC, Silva LD, Gabbay YB, et al. High prevalence of G12P[8] rotavirus strains in Rio Branco, Acre, Western Amazon, in the post-rotavirus vaccine introduction period. *J Med Virol*. 2016; **88(5)**:782–789. doi: 10.1002/jmv.24404.
12. World Health Organization (2002) Generic protocol for (i) hospital-based surveillance to estimate the burden of rotavirus gastroenteritis in children and (ii) a community-based survey on utilization of health care services for gastroenteritis in children. Field test version. Geneva, Switzerland: Department of Vaccines and Biologicals, World Health Organization.
13. Das BK, Gentsch JR, Cicirello HG, Woods PA, Gupta A, Ramachandran M, et al. Characterization of rotavirus strains from newborns in New Delhi, India. *J Clin Microbiol*. 1994; **32(7)**:1820–1822.
14. Gentsch JR, Glass RI, Woods P, Gouvea V, Gorziglia M, Flores J, et al. Identification of group A rotavirus gene 4 types by polymerase chain reaction. *J Clin Microbiol*. 1992; **30(6)**:1365–1373.
15. Gomara MI, Cubitt D, Desselberger U, Gray J. Amino acid substitution within the VP7 protein of G2 rotavirus strains associated with failure to serotype. *J Clin Microbiol*. 2001; **39(10)**:3796–3798. doi: 10.1128/JCM.39.10.3796-3798.2001.
16. Gouvea V, Glass RI, Woods P, Taniguchi K, Clark HF, Forrester B, et al. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J Clin Microbiol*. 1990; **28(2)**:276–282.
17. Bishop RF, Unicomb LE, Soenarto Y, Suwardji H, Ristanto, Barnes GL. Rotavirus serotypes causing acute diarrhoea in hospitalized children in Yogyakarta, Indonesia during 1978–1979. *Arch Virol*. 1989; **107(3–4)**:207–213.

18. Subekti D, Lesmana M, Tjaniadi P, Safari N, Frazier E, Simanjuntak C, et al. Incidence of Norwalk-like viruses, rotavirus and adenovirus infection in patients with acute gastroenteritis in Jakarta, Indonesia. *FEMS Immunol Med Microbiol*. 2002; **33(1)**:27–33.
19. Putnam SD, Sedyaningsih ER, Listiyaningsih E, Pulungsih SP, Komalarini, Soenarto Y, et al. Group A rotavirus-associated diarrhea in children seeking treatment in Indonesia. *J Clin Virol*. 2007; **40(4)**:289–294. doi: 10.1016/j.jcv.2007.09.005.
20. Soenarto Y, Aman AT, Bakri A, Waluya H, Firmansyah A, Kadim M, et al. Burden of severe rotavirus diarrhea in Indonesia. *J Infect Dis*. 2009; **200 Suppl 1**:S188–194. doi: 10.1086/605338.
21. Hung LC, Wong SL, Chan LG, Rosli R, Ng AN, Bresee JS. Epidemiology and strain characterization of rotavirus diarrhea in Malaysia. *Int J Infect Dis*. 2006; **10(6)**:470–474. doi: 10.1016/j.ijid.2006.05.008.
22. Jiraphongsa C, Bresee JS, Pongsuwanna Y, Kluabwang P, Poonawagul U, Arpornitip P, et al. Epidemiology and burden of rotavirus diarrhea in Thailand: results of sentinel surveillance. *J Infect Dis*. 2005; **192 Suppl 1**:S87–93. doi: 10.1086/431508.
23. Nyambat B, Meng CY, Vansith K, Vuthy U, Rin E, Kirkwood C, et al. Hospital-based surveillance for rotavirus diarrhoea in Phnom Penh, Cambodia, March 2005 through February 2007. *Vaccine*. 2009; **27 Suppl 5**:F81–84. doi: 10.1016/j.vaccine.2009.08.085.
24. Moe K, Thu HM, Oo WM, Aye KM, Shwe TT, Mar W, et al. Genotyping of rotavirus isolates collected from children less than 5 years of age admitted for diarrhoea at the Yangon Children's Hospital, Myanmar. *Vaccine*. 2009; **27 Suppl 5**:F89–92. doi: 10.1016/j.vaccine.2009.08.068.
25. Koukou D, Chatzichristou P, Trimis G, Siahaniidou T, Skiathitou AV, Koutouzis EI, et al. Rotavirus Gastroenteritis in a Neonatal Unit of a Greek Tertiary Hospital: Clinical Characteristics and Genotypes. *PLoS One*. 2015; **10(7)**:e0133891. doi: 10.1371/journal.pone.0133891.
26. Sharma R, Hudak ML, Premachandra BR, Stevens G, Monteiro CB, Bradshaw JA, et al. Clinical manifestations of rotavirus infection in the neonatal intensive care unit. *Pediatr Infect Dis J*. 2002; **21(12)**:1099–1105. doi: 10.1097/01.inf.0000040560.26682.88.
27. Chen SY, Chang YC, Lee YS, Chao HC, Tsao KC, Lin TY, et al. Molecular epidemiology and clinical manifestations of viral gastroenteritis in hospitalized pediatric patients in Northern Taiwan. *J Clin Microbiol*. 2007; **45(6)**:2054–2057. doi: 10.1128/JCM.01519-06.
28. Salim H, Karyana IP, Sanjaya-Putra IG, Budiarsa S, Soenarto Y. Risk factors of rotavirus diarrhea in hospitalized children in Sanglah Hospital, Denpasar: a prospective cohort study. *BMC Gastroenterol*. 2014; **14**:54. doi: 10.1186/1471-230X-14-54.
29. Dearlove J, Latham P, Dearlove B, Pearl K, Thomson A, Lewis IG. Clinical range of neonatal rotavirus gastroenteritis. *Br Med J (Clin Res Ed)*. 1983; **286(6376)**:1473–1475.
30. Greenberg HB, Estes MK. Rotaviruses: from pathogenesis to vaccination. *Gastroenterology*. 2009; **136(6)**:1939–1951. doi: 10.1053/j.gastro.2009.02.076.
31. Yin Y, Metselaar HJ, Sprengers D, Peppelenbosch MP, Pan Q. Rotavirus in organ transplantation: drug-virus-host interactions. *Am J Transplant*. 2015; **15(3)**:585–593. doi: 10.1111/ajt.13135.
32. Yolken RH, Peterson JA, Vonderfecht SL, Fouts ET, Midthun K, Newburg DS. Human milk mucin inhibits rotavirus replication and prevents experimental gastroenteritis. *J Clin Invest*. 1992; **90(5)**:1984–1991. doi: 10.1172/JCI116078.
33. Chan J, Nirwati H, Triasih R, Bogdanovic-Sakran N, Soenarto Y, Hakimi M, et al. Maternal antibodies to rotavirus: could they interfere with live rotavirus vaccines in developing countries? *Vaccine*. 2011; **29(6)**:1242–1247. doi: 10.1016/j.vaccine.2010.11.087.
34. Clemens J, Rao M, Ahmed F, Ward R, Huda S, Chakraborty J, et al. Breast-feeding and the risk of life-threatening rotavirus diarrhea: prevention or postponement? *Pediatrics*. 1993; **92(5)**:680–685.
35. Zuridah H, Kirkwood CD, Bishop RF, Bogdanovic-Sakran N, Yap KL. Molecular characterization and epidemiology of rotavirus isolates obtained from children with diarrhoea in Malaysia. *Med J Malaysia*. 2009; **64(3)**:193–196.
36. Gentsch JR, Laird AR, Bielfelt B, Griffin DD, Banyai K, Ramachandran M, et al. Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs. *J Infect Dis*. 2005; **192 Suppl 1**:S146–159. doi: 10.1086/431499.

37. Martella V, Banyai K, Matthijnssens J, Buonavoglia C, Ciarlet M. Zoonotic aspects of rotaviruses. *Vet Microbiol.* 2010; **140(3–4)**:246–255. doi: 10.1016/j.vetmic.2009.08.028.
38. Matsui SM, Mackow ER, Matsuno S, Paul PS, Greenberg HB. Sequence analysis of gene 11 equivalents from "short" and "super short" strains of rotavirus. *J Virol.* 1990; **64(1)**:120–124.

Chapter 12

Summary and Discussion

Hepatitis E virus infection

The existence of HEV has been suggested since the early 1980s [1]. However, little efforts were invested to determine its global burden and epidemiology, especially when compared to other viral hepatitises. Therefore **in chapter 2**, I systematically reviewed the burden of HEV outbreaks by analyzing data from all global HEV outbreaks reported in the literature. Most outbreaks occurred in developing countries of the Asian and African continents and involved HEV genotypes 1 and 2. Recently, HEV outbreaks were reported from several countries, including India [2], Bangladesh [3], Nepal [4], Sudan [5,6], China [7], Australia [8], Japan [9], and Spain [10]. I thus suggest that HEV poses a serious threat for global public health in the future. Unfortunately, there are no effective prevention and treatment strategies available for HEV. To control HEV outbreaks, vaccinations are required to prevent its spread. Definitely, the development of effective vaccine and treatment mainly depends on our understanding of innate and adaptive immunity against HEV infection. **In chapter 3**, I comprehensively discuss our recent progress on understanding of immunity against HEV. Identification of RIG-I as a potent anti-HEV ISG provides proof-of-concept of the notion that RIG-I agonists may constitute novel HEV therapy and excitingly it is to be expected that these agonist will exhibit more limited side effects as compared to the current IFN or RBV therapy [11]. Manipulation of T cell immunity is also a potentially successful approach for prophylactic and therapeutic HEV vaccines [12,13].

Off-label treatment, such as ribavirin, has been useful for managing some chronic cases of HEV infection, although ribavirin treatment failure has been reported in a subset of patients as well [14]. Therefore, those patients require alternative therapies to clear HEV. A study reported that sofosbuvir is valuable in inhibiting HEV replication and has an additive effect in combination with ribavirin [15]. Sofosbuvir was originally developed to target the HCV NS5B RNA-dependent RNA polymerase. **In chapter 4**, I demonstrate that sofosbuvir potently inhibits HCV, but not HEV in cell culture models and that observation has proven in line with several case reports published by others [16-18]. I thus conclude that sofosbuvir is not likely to be the novel drug of choice for chronic HEV treatment.

Immune responses and antiviral treatments provide selective pressure to virus infections, including HEV. Viral adaptation is thus an important strategy of viruses to overcome the host

immune defense mechanisms and antiviral therapy and ultimately, to maintain viral fitness and replication competency [19]. As a single strand RNA virus, HEV replication is clearly error-prone due to the lack of proofreading activity of HEV RNA-dependent RNA polymerase. In **chapter 5**, we investigated the nature of adaptive response of HEV to the host immune system by performing a comprehensive bioinformatics study of characteristic mutations in the HEV genome. I demonstrated a genotype-specific prevalence and evolution of the specific mutations and their impact on the reduced B and T cell epitope reactivity. My study gives the insight that HEV genetic variants were probably associated with disease pathogenesis and clinical progression. The presence of such pronounced molecular evolution, adapting to antiviral mechanisms bodes ill for efforts directed at counteracting the virus.

Drugs and immune-based treatments against rotavirus infection

IFN-mediated response is known as the first defense mechanism against virus infection. IFN executes its antiviral effect via induction of hundreds of IFN-stimulated genes (ISGs) [20]. To understand the innate responses against rotavirus infection, I showed in **chapter 6** that rotavirus infection predominantly induces type III (IFN λ 1) rather than type I (IFN α and IFN β) IFN responses, without functional production (secretion) of IFN proteins. Consequently, these responses are not sufficient to inhibit rotavirus replication. Instead, basal IFN signaling maintained by the ISGF3 complex appears to restrict rotavirus replication. Furthermore, I demonstrated that exogenous treatments by all types of IFNs can inhibit the replication of both rotavirus SA11 and human patient-derived rotaviruses in Caco-2 cell line as well as intestinal organoids through JAK-mediated induction of known ISGs. Since ISGs are the ultimate effectors of IFN-mediated antiviral responses, further studies are needed to identify specific anti-rotavirus ISGs to improve our understanding of immunity against rotavirus infections.

It is known that rotavirus infection can induce the production of TNF- α in dendritic cells [21]. However, the exact role of TNF- α in rotavirus infection remains unexplored. In **chapter 7**, we demonstrated that TNF- α could potentially inhibit rotavirus infection. Mechanistically, the effect was achieved by NF κ B-regulated genes via the activation of classical nuclear factor κ B (NF- κ B) signaling (**chapter 7**). Our findings expand the previously known anti-viral properties of TNF- α against many other viruses, including influenza, HCV, HEV and poxviruses. In the

clinic, TNF- α inhibitors have been widely used as treatments in many immune-mediated inflammatory diseases, including inflammatory bowel disease (IBD) [22]. Whether the use of TNF- α inhibitors in IBD patients were associated with an increased risk of rotavirus infection requires further investigation.

Gastrointestinal infections, including rotavirus, have been recognized as a potential factor of exacerbation and induction of flares in IBD. In IBD patients, 6-TG has been effectively used for the clinical treatment [23]. Interestingly, we found that 6-TG can inhibit rotavirus infection. Furthermore, I demonstrated that the inhibitory effect of 6-TG is achieved through its inhibition of the active form of Rac1 protein (GTP-Rac1) (**chapter 8**). Based on my findings, it is reasonable to investigate the incidence of rotavirus infection in IBD patients undergoing 6-TG treatment as compared to other medications. My findings also suggest that IBD patients with a high risk of contracting rotavirus infections may benefit from 6-TG therapy.

The burden of rotavirus and norovirus infections in Indonesia

Rotavirus and norovirus are the most important viral pathogens in causing acute gastroenteritis (acute diarrhea) globally. The incidence of acute diarrhea in Indonesia is considerably high. Diarrhea is the leading cause of infant mortality in Indonesia [24]. Therefore, both viruses should be targeted for routine surveillance. In **chapter 9**, I first comprehensively reviewed rotavirus and norovirus surveillance in Indonesia. I identified the knowledge gaps in Indonesia, in which norovirus surveillances and detections were very limited, as compared with rotavirus.

Globally, norovirus is associated with 18% (95% CI: 17%-20%) of diarrheal diseases [25]. The burden of norovirus infections is substantially high in all regions, including in high-, middle-, and low-income countries. Strikingly, norovirus became the most common cause of pediatric gastroenteritis attending the medical care in high- and middle-income countries with successful national rotavirus vaccination programs [26]. Therefore, norovirus should be the main focus for targeted prevention and control measure strategies. Unfortunately, currently there are limited data available on the burden, epidemiology and strain diversity of norovirus in middle- and low-income countries, particularly in Indonesia (**chapter 9**). In contrast with rotavirus, for which effective vaccines have been introduced, norovirus

vaccines remain in the development pipeline. Immunity against norovirus infection is known to be type-specific. Given the fact that norovirus is constantly evolving, addressing the data gap on molecular epidemiology of norovirus in middle- and low-income countries is important, especially through ongoing surveillance programs and it is relevant to support the vaccine development.

Therefore, in **chapter 10**, we performed a one-year surveillance of rotavirus and norovirus infection in children under five years of age in three participating hospitals in Indonesia. I found that the prevalence of norovirus and rotavirus in children less than five years of age hospitalized with acute gastroenteritis in Indonesia were 18.47% and 54.93%, respectively. For norovirus, GII norovirus was much more prevalent (92%) than GI norovirus (8%), similar with findings of worldwide studies. Since norovirus typing is sequence-based, experiments are now ongoing to determine the genotypes.

An unexpected findings of the 2015 surveillance was that I found that G3P[8] was the most prevalent rotavirus genotype (**chapter 10**). A previous surveillance conducted in 2009 (**chapter 11**) demonstrated that G1P[8] was the most dominant genotype. As a segmented RNA virus, rotavirus is also continuously evolving. Consequently, rotavirus displays a great diversity because of frequent reassortment, interspecies transmission, point mutation and gene rearrangement events and ultimately, novel genotypes and unusual G/P type combinations are emerging in human populations [27]. All of these factors emphasize the need for continuous rotavirus surveillance to support rotavirus vaccination programs, as I have previously discussed in **chapter 9**.

Final remarks

Combating virus diseases in humans, including hepatitis E, rotavirus and norovirus, requires close and extensive collaborations between virologist, immunologist, epidemiologist, clinicians and many other expertises to comprehend the biology, evolution, pathogenesis, transmission and epidemiology of virus diseases to finally develop effective vaccines and antiviral therapy. Only then can the burden of severe viral infections be significantly reduced. This thesis is a little example on how we can apply different approaches in understanding the epidemiology, immunity and antiviral therapy of gastrointestinal viruses. However, we

have to acknowledge that more work should be done in the future to reduce the burden of viral infections.

References

1. Khuroo MS. Study of an epidemic of non-A, non-B hepatitis. Possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. *Am J Med* 1980;68(6):818-24.
2. Singh MP, Majumdar M, Goyal K, Lakshmi PV, Bhatia D, Ratho RK. Investigation of suspected viral hepatitis outbreaks in North West India. *Diagn Microbiol Infect Dis* 2016;84(4):309-14.
3. Haque F, Banu SS, Ara K, et al. An outbreak of hepatitis E in an urban area of Bangladesh. *J Viral Hepat* 2015;22(11):948-56.
4. Shrestha A, Lama TK, Karki S, et al. Hepatitis E epidemic, Biratnagar, Nepal, 2014. *Emerg Infect Dis* 2015;21(4):711-3.
5. Elduma AH, Zein MM, Karlsson M, Elkhidir IM, Norder H. A single lineage of hepatitis E virus causes both outbreaks and sporadic hepatitis in Sudan. *Viruses* 2016;8(10).
6. Ahmed SS, Soghaier MA, Mohammed S, Khogali HS, Osman MM, Abdalla AM. Concomitant outbreaks of yellow fever and hepatitis E virus in Darfur States, Sudan, 2012. *J Infect Dev Ctries* 2016;10(1):24-9.
7. Zhang L, Yan B, Xu A. A hepatitis E outbreak by genotype 4 virus in Shandong province, China. *Vaccine* 2016;34(33):3715-8.
8. Pokorny AM, Pokorny CS. Local outbreak of hepatitis E: a rare cause of viral hepatitis in Australia. *Intern Med J* 2015;45(12):1300-2.
9. Ishida S, Matsuura K, Yoshizumi S, et al. Hepatitis E outbreak at a nursing home for aged people in Hokkaido, Japan, between February and March 2016. *J Clin Virol* 2018;101:23-8.
10. Rivero-Juarez A, Frias M, Martinez-Peinado A, et al. Familial hepatitis E outbreak linked to wild boar meat consumption. *Zoonoses Public Health* 2017;64(7):561-5.
11. Xu L, Wang W, Li Y, et al. RIG-I is a key antiviral interferon-stimulated gene against hepatitis E virus regardless of interferon production. *Hepatology* 2017;65(6):1823-39.
12. Hakim MS, Ikram A, Zhou J, Wang W, Peppelenbosch MP, Pan Q. Immunity against hepatitis E virus infection: implications for therapy and vaccine development. *Rev Med Virol* 2018;28(2):e1964.
13. Hakim MS, Spaan M, Janssen HL, Boonstra A. Inhibitory receptor molecules in chronic hepatitis B and C infections: novel targets for immunotherapy? *Rev Med Virol* 2014;24(2):125-38.
14. Peters van Ton AM, Gevers TJ, Drenth JP. Antiviral therapy in chronic hepatitis E: a systematic review. *J Viral Hepat* 2015;22(12):965-73.
15. Dao Thi VL, Debing Y, Wu X, et al. Sofosbuvir inhibits hepatitis E virus replication in vitro and results in an additive effect when combined with ribavirin. *Gastroenterology* 2016;150(1):82-5 e4.
16. Todesco E, Mazzola A, Akhavan S, et al. Chronic hepatitis E in a heart transplant patient: sofosbuvir and ribavirin regimen not fully effective. *Antivir Ther* 2018; doi: 10.3851/IMP3227. [Epub ahead of print]
17. Donnelly MC, Imlach SN, Abravanel F, et al. Sofosbuvir and daclatasvir anti-viral therapy fails to clear HEV viremia and restore reactive T cells in a HEV/HCV co-infected liver transplant recipient. *Gastroenterology* 2017;152(1):300-1.
18. van der Valk M, Zaaier HL, Kater AP, Schinkel J. Sofosbuvir shows antiviral activity in a patient with chronic hepatitis E virus infection. *J Hepatol* 2017;66(1):242-3.
19. Taubenberger JK, Kash JC. Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host Microbe* 2010;7(6):440-51.

20. Wang W, Xu L, Su J, Peppelenbosch MP, Pan Q. Transcriptional regulation of antiviral interferon-stimulated genes. *Trends Microbiol* 2017;25(7):573-84.
21. Mesa MC, Rodriguez LS, Franco MA, Angel J. Interaction of rotavirus with human peripheral blood mononuclear cells: plasmacytoid dendritic cells play a role in stimulating memory rotavirus specific T cells in vitro. *Virology* 2007;366(1):174-84.
22. Ben-Horin S, Kopylov U, Chowers Y. Optimizing anti-TNF treatments in inflammatory bowel disease. *Autoimmun Rev* 2014;13(1):24-30.
23. Meijer B, Mulder CJ, Peters GJ, van Bodegraven AA, de Boer NK. Efficacy of thioguanine treatment in inflammatory bowel disease: a systematic review. *World J Gastroenterol* 2016;22(40):9012-21.
24. Agtini MD, Soeharno R, Lesmana M, et al. The burden of diarrhoea, shigellosis, and cholera in North Jakarta, Indonesia: findings from 24 months surveillance. *BMC Infect Dis* 2005;5:89.
25. Ahmed SM, Hall AJ, Robinson AE, et al. Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis. *Lancet Infect Dis* 2014;14(8):725-30.
26. Hakim MS, Nirwati H, Aman AT, Soenarto Y, Pan Q. Significance of continuous rotavirus and norovirus surveillance in Indonesia. *World J Pediatr* 2018;14(1): 4-12.
27. Jain S, Vashistt J, Changotra H. Rotaviruses: is their surveillance needed? *Vaccine* 2014;32(27):3367-78.

Chapter 13

Nederlandse Samenvatting

Dutch Summary

Samenvatting voor de leek

Bacteriële infecties kunnen worden behandeld met antibiotica maar tot nu toe bestaat er voor de meeste virale infecties geen doeltreffend geneesmiddel. De symptomen, die een virale infectie veroorzaakt, zijn afhankelijk van de plaats waar de infectie zich bevindt, de uitgebreidheid van de infectie en het soort virus. Afhankelijk van de ernst en de behandelmogelijkheden kan een virale infectie zeer lichte verschijnselen van een zich niet al te prettig voelen veroorzaken, tot juist een extreem beroerd zijn, met rillingen, hoge koorts en verwardheid. In dit proefschrift concentreer ik mij op drie verschillende virale infecties, ziekte veroorzaakt door het hepatitis E virus, rotavirus en norovirus. Van al deze ziektes probeer ik het klinisch/epidemiologisch probleem in kaart te brengen, terwijl ik voor het hepatitis E virus en het rotavirus ook middels onderzoek van hun interactie met geneesmiddelen en het menselijk immuunsysteem ook een bijdrage probeer te leveren voor het ontwikkelen van nieuwe wegen voor rationele behandeling van ziekte. **Hoofdstuk 1** geeft een uitgebreide introductie, rationale en rechtvaardiging van de in dit proefschrift beschreven studies.

Hepatitis E

Eén van de verwekkers van virale hepatitis is het hepatitis E virus. Het hepatitis E virus komt via gastro-intestinale kanaal de bloedsomloop binnen en repliceert zich dan in de lever. Het beloop van de acute HEV-infectie kent meerdere fases: van subklinisch, naar acuut en uiteindelijk fulminant. Hepatitis E, wat in toenemende mate wordt gezien als een “public health concern”, is bij 0.2 – 1.0 % van de patiënten dodelijk, maar dit percentage kan in het laatste trimester van hun zwangerschap bij zwangere vrouwen stijgen tot een fataliteitspercentage van 20-25%. Daarnaast is het virus gevaarlijk voor transplantatiepatiënten. Dergelijke patiënten ontvangen immuunsysteem remmende medicijnen die mogelijk de weerstand tegen het hepatitis E virus zouden kunnen verminderen. Het gevolg is dat zulke patiënten vatbaar zijn voor Hepatitis E. Opvallenderwijze was de last die mensheid meestorst als het gevolg van Hepatitis E nog nooit goed gekarakteriseerd. In **Hoofdstuk 2** doe ik dit wel en concludeer dat het Hepatitis E virus de belangrijkste oorzaak van acute virale leverontsteking (wereldwijd) en geef ik een uitgebreid overzicht waar te wereld welk

Hepatitis E virus ziekte uitbraak heeft veroorzaakt. In **Hoofdstuk 3** ga ik dan uitgebreid in op de afweermechanismen die ons immuunsysteem gebruikt om te vechten, maar ik laat ook de belofte zien van juist preventieve vaccinatiestrategieën. De gedachte dat preventie de weg vooruit is met betrekking tot Hepatitis E wordt verder versterkt door de studies gepresenteerd in **Hoofdstuk 4** en **Hoofdstuk 5**. In hoofdstuk 4 onderzoek de recent gedane suggestie dat medicatie die nuttig is bij het behandelen van Hepatitis C ook een gunstig effect heeft bij de behandeling van Hepatitis E. Met celkweekproeven die ik deed, kon ik helaas deze suggestie niet bevestigen, de eerder gepubliceerde data lijken een artefact te zijn. In hoofdstuk 5 onderzoek ik het vermogen van het Hepatitis E virus om zich aan te passen aan antivirale strategieën. Dit blijkt jammer genoeg erg groot te zijn en het virus verandert gemakkelijk van structuur om zowel immuunsysteem als farmacologische behandeling te omzeilen. Zoals ook bediscussieerd in **Hoofdstuk 12** zijn we dus nog ver weg wat betreft effectieve behandeling en is verder wetenschappelijk onderzoek urgent nodig.

Rotavirus en Norovirus

Buikloop of diarree kenmerkt zich door een hoofdzakelijk dunne, brijige tot waterige, soms slijmerige, ontlasting die gepaard kan gaan met ernstige uitdroging of zelfs ondervoeding. Het woord diarree is afgeleid van het Grieks [$\delta\iota\alpha$ ["dia"] = door, $\rho\epsilon\omega$ ["reo"] = stromen]. De oorzaken van diarree zijn divers, zo zijn er toxische, bacteriologische en virale oorzaken. In dit proefschrift concentreer ik mij op virale oorzaken en met name op de zogenaamde rotavirussen en norovirussen. Een rotavirus is vooral bekend van dat het bij jonge kinderen overgeven en diarree veroorzaakt. Het is een van de ernstigere diarreeverwekkers in deze leeftijdsgroep, omdat de diarree die het veroorzaakt zowel erg besmettelijk als hevig is, en vaak ook lang aanhoudend. In ontwikkelingslanden overlijden naar schatting 600.000 kinderen per jaar aan een rotavirus infectie. In Nederland is een rotavirus veel minder bedreigend door de betere uitgangstoestand (voeding, weerstand) van de patiëntjes en de mogelijkheid om spoedig in een ziekenhuis door middel van een infuus te worden gerehydrateerd, waardoor uitdroging wordt voorkomen. Toch is het ook bij ons een belangrijke oorzaak van ziekenhuisopnamen wegens diarree bij kinderen. Vrijwel ieder kind maakt echter in zijn of haar leven minstens één rotavirusinfectie door en ziekenhuisopname is gelukkig maar zelden noodzakelijk. Ook norovirus veroorzaakt diarree. In dit proefschrift onderzoek ik de beschikbare informatie over het klinisch probleem geassocieerd met

rotavirussen norovirussen in Indonesië. Voor rotavirus blijkt veel informatie voorhanden, echter wat betreft norovirus is er schokkend weinig bekend (**hoofdstuk 9**). Ik ben vervolgens het voorkomen van rotavirussen en norovirussen in een Indonesisch ziekenhuis gaan onderzoeken (**hoofdstuk 10**). Ik vond dat in kleine kinderen (opgenomen in het ziekenhuis vanwege buikloop) in 18.47 % van de gevallen besmet waren met norovirus terwijl in 54.93 % van de gevallen er sprake was van een rotavirusinfectie. In **hoofdstuk 11** kijk ik ook nog goed welke stammen betrokken zijn bij deze infecties. Het klinisch probleem opgeworpen door deze virussen is dus inderdaad heel groot.

Antivirale mechanismen

Het is dus belangrijk dat het lichaam en met name het epitheel van de darm zich kan verdedigen tegen rotavirusinfectie. Een belangrijke gedachte hierbij is dat de zogenaamde interferonen hierbij een belangrijke rol spelen. In **hoofdstuk 6** laat ik zien dat dergelijke interferonen weliswaar zeer potent zijn in het aanzwengelen van antivirale immuniteit in het darmepitheel, maar dat de betrokken mechanismen al constitutief continu aanwezig zijn en helpen de darm te vrijwaren van virale infectie. Bovendien laat ik in **hoofdstuk 7** dat dezelfde mechanismen ook geactiveerd kunnen worden door andere niet-interferon ontstekingshormonen. Dus waar interferonen wel effectief in het bestrijden van virussen blijft hun rol in de normale afweer vooralsnog onduidelijk. Mijn werk werd nog verder op meer fundamenteel niveau uitgebouwd door mijn studies (**hoofdstuk 8**) met 6-thioguanine (een immunosuppressivum dat wordt gebruikt na orgaantransplantatie maar vooral bij auto-immuunziekten zoals de ziekte van Crohn). Het bleek dat deze medicatie een sterk remmend effect had op het rotavirus en haar infectie van cellen. Ook bij deze patiënten zou men dus moeten kiezen voor een behandelstrategie waarin deze medicatie snel wordt ingezet bij die patiënten die een verhoogd risico lopen op rotavirusinfectie.

Conclusie

Samenvattend kan ik zeggen dat het probleem van virale infectie wellicht nog groter is dan gedacht en dat mechanismen waarmee mensen zich verdedigen tegen virale infectie op belangrijke details anders werken dan voorheen werd gedacht. Ik roep dus op tot verder onderzoek naar nieuwe wijzen om virusinfecties te bestrijden.

Appendix

Acknowledgements

Publications

PhD Portfolio

Curriculum Vitae

Acknowledgements

I took the liberty to express my gratitude towards the followings, who kind assistance and endearing support along the way of my four year PhD program in Rotterdam.

Firstly, towards my promoter, Prof. Dr. Maikel Peppelenbosch. I am still amazed by your seemingly limitless knowledge and experience in the biological field. You know everything, you know every single component of any signaling pathway. I always learn new ideas and new techniques from you to address a specific scientific question. You are also a humorous person, you can always make us laugh during our regular meeting. Surprisingly, that fun approach towards our regular meeting turned out to be contributing factors that increase the effectiveness and the efficiency of the meeting itself, well at least it is for me. At first, you were seemed unreachable, but time and time again, I gained insight to see your golden heart beyond what meets the eyes. Thank you very much for your critical comments of my manuscript and also towards this thesis. My papers are much more “colorful” with your “sophisticated” and “unpredicted” sentences that I myself may never thought of.

Dr. Qiuwei Pan (Abdullah), my deepest gratitude for all of your support, motivation and encouragement during my PhD journey. During the first year, you always guide me to become an independent PhD student, who can develop idea and solve the research problem ourselves. I know that you want me to be mature as a good scientist in the future. I am deeply inspired and touched by your passion in science, but on the other hand, I am also amazed that you always prioritize Allah The Almighty and also your family. You always pray in time, I am really happy to have you in my PhD journey. I learned a lot from you how to write a manuscript and how to present my results. Thank you for inviting me to have dinner in your house, time and time again and meet your friends from China and Indonesia, and sometimes you even kindly share your lunch when you see that I have only two pieces of bread for my lunch. I will always remember the time we visit our brothers in France to deliver some food for them in order to help them survive in the refugee camps. I therefore learned two big words from you: science and humanity.

The inner committee, Prof. dr. M.J. Bruno, Prof. W.J. de Jonge and Prof. dr. B. van Hoek, thank you very much for all of your valuable comments and suggestions to improve my thesis.

Dr. Andre Boonstra, thank you very much for giving me an opportunity to conduct my research project during my master study. I learn cellular immunology fields from you, especially in HBV and HCV immunology. I remember that we always have a regular weekly meeting to discuss immunology papers that I do not understand well. Then, you explain me everything beyond the paper itself. In addition, you always supervise me in the lab, that is very important in order to build

up my confidence in performing the next experiments independently. You show me how to work hard despite weekends and motivated me to have a high-impact publication during my master and finally I can make it! Again, thank you very much.

Dr. Gwenny Fuhler, thanks for your help for my project. You are so generous to share your patient materials with us. I always learn from you how to raise a good and critical question during MDL seminars. Thank you so much for revising my rotavirus paper.

Dr. Ron Smits, thank you for your kind assistance when I had problems with my experiments. You are the first teacher in the lab I meet when I had problems with my experiments. Thank you for teaching us how to work safely and properly in the MLI and MLII lab.

Dr. Dave Sprengers, thank you for all of your suggestions during the regular meeting. Since you are a real clinician, you always think in a clinical way to bridge our research with the clinical relevance for our patients. That is very important for me as a medical doctor as well.

Prof. Osterhaus, Dr. Jan Nouwen and Dr. Frank van Fliet, thanks for giving me an opportunity to pursue my master study in the Research master program of Infection and Immunity. I enjoy so much and hope that we can continue our collaboration in the future.

Dr. Abu Tholib Aman, my dearest teacher in Indonesia. Many times you convince me that I can pursue my postgraduate degree abroad. You motivates me to improve my English when you saw me that my English was very bad. You were always there for me when I was about to give up pursuing scholarships. You convinced me that I can make my dream come true, playing with viruses and mastering molecular techniques. Thank you very much, your words will never be forgotten.

To Wenshi, you are both a very smart person and a talented researcher as well. You show me around the lab, teach me patiently so many different techniques. I always come to you when I had difficulties in my research project. I am inspired with your way of thinking and doing experiments in a such efficient way to results in a high-impact publication. I believe that in the future you will be one of the leading scientist in biological field. Your motto were, no pain no gain. Wished you happiness with your lovely wife.

To Wen, we are in the same year and we always make it fun in the lab and in the office. You are a hard-working student and also good at cooking. We sometimes have a discussion about politics, family religion and even love life. I will never forget that! Thanks for your help to my norovirus experiments. Good luck for your next endeavour.

To Ying Ying, we have been friends since we pursue our master study in Rotterdam. I learned from you how to guide intern students doing her experiments. Sometimes I saw you work during the weekend in the lab with your son, Andrew. You are a supermom for him. Wish you the best for your future.

To Changbo, thanks for helping me with my experiments, I wish you success with your publication. Never give up!

To Aqsa, thank you so much with all of your effort to help me finishing my rotavirus paper. You also help me to make figures for my HEV immunology review. Thanks!

To Suk Yee, thanks for always helping me when I got my “nose attack”. Success with your PhD project.

To Sunrui, thank you for helping me with organoids and TCID experiments.

To Anthonie, I will never forget your kindness to teach me how flow cytometry works.

To Gertine, I first learned how to do cell culture from you, to titrate the antibody. I also come to you when the flow cytometry machine doesn't work. You motivate me to try speaking Dutch, but I am very sorry that I am not good in learning Dutch, I'm not even that good in English.

To my roommate Na 10-01, Thijmen, Vincent, Pauline, Adriaan, Pauline, Ruby, Jorke, Ivo, Katheryna, Alessandra, Rachid, we always share our happiness, our sadness and our frustration. We congratulate each other when our paper get accepted, we motivate each other when our paper get rejection, sometimes we spend our weekend to have dinner. Wish all of you guys the best for your future!

Jiaye, thank you for our discussion about your research; Juan Li, thank you for helping me with my systematic review paper, you motivate me that I can complete the paper; Buyun, thanks for sharing your STAT1 KO huh7 cell lines; Wanlu, congratulations for your Gastroenterology paper, you make it! It is really inspiring; Meng Li, sometimes we do our MLII lab duty together and we can manage well, also thanks for helping me with the confocal; Pengyu, success with your experiments. You always have your experiments in the weekend, which makes me not feel lonely; Shihao, thanks for your help for my project; Shaoshi, I really enjoy your explanation about BMI and obesity in China; Wenhui, you are also a supermom, wish you the best for your family; Rattana, hopefully I can visit Bangkok someday.

Jan, thank you for your warm smiling and greetings whenever I meet you; Petra, thanks for helping me with my sofosbuvir paper and also sharing your Huh-ET replicon cell lines; Raymond, thanks for solving my computer and software problems; Leonie, thanks for organizing ordering agents, primers and everything, you are really helpful; Auke, thank you for helping me with ordering of molecular stuff; Marieke, thanks for teaching me how cell sorter works.

All MDL members who have left and who are still present: Jaap, Luc, Hanneke, Andrea, Thomas, Sonja, Marcel, Patrick, Marla, Ksenia, Shanta, Jun Hou, Sharida, Cynthia, Shan Li, Ling Wang, Yang Li, Zhijiang Miao, Xumin Qu, Yunlong Li, Qin Yang, Janine, Michiel, Aafke, Yuebang, Lei, Zhouhong, Guoying, Menggeng Liu, Lauke, Noe, Gülce, Kim, Aniek, Paula, Monique de Beijer, Monique Verstegen, Kelly, Natasha, Buddy, Martine, Frances, Henk, Floris, Jasmijn, Mehjabeen,

Lisanne, Jasper, Dowty, Michelle, Wouter, Angela, Renee, Hugo, Rik, Martijn, Arjan, Rajesh, Wesley, Kan, Yijin, Cindy, Pratika, Greta, Ishaku, and so on. I want to thank you for helping me during the last six years. I have indeed enjoy my time with all of you in the lab and in the office. Best wishes to all of you.

All the co-authors of my papers, thank you for your contribution to my thesis and my publications.

To my Nijmegen family: bunda and your lovely husband Ibrahim, Ismail, Deasi Asiyah, mas Agung and your lovely wife. Being with you makes my life in the Netherlands so colourful. Hopefully, all of us can be a good moslem.

To my Indonesian friends in Rotterdam: om Ajo, mas Pugo, mbak Novika, mbak Titi, bang Fasha, Salma, bang Andie, mbak Vicky, mas Fuad, mbak Diah, mas Akram, mas Mikhta, mbak Rara, mbak Maeyta, pak Ahmad Mansur, Widagdo, Bri, bu Yulia Rosa, mbak Rina, mbak Dewi Santosaningsih, my housemate mas Adi Nugroho, pak Harry, mbak Dwi and Rafa, Fikru, Adhit Filan, Helmi Aziz, Noorma, mas Andi Rofyan, pak Yono (and ISR family), and many others. Moreover, “The absurdity gentleman” family: mas Iwan, Ferdi, Syarif, Kevin and Ario: many thanks for our togetherness during Ramadhan 1436 H. Your presence makes me feel at my home country though I live far away from Indonesia.

To my Wageningen (“Pengajian Dua Pekan”) family: Adhitya Wira, Haru, Deslak, Azril, Muhajir, Kholik, mbak Vivi Aryati, Acha, Fitria Rizkyka, mbak Hikmah Hassan, Reni, Ilya, Frita, Thalitha, Eka Novia, mbak Eva Johan, mbak Fatima, Aliya, Dissa, Rahmawati Moti, Amy Rachmawati, Ines, Tika, Arin, Intan, Rizka Oktarianti, Fitri, Ami Gusri, mbak Rovani (and family), and others: Be with those who make the road to Jannah easier.

To KAGAMA chapter Netherlands, mas Hero, mas Aji Putra, mbak Tiwi, mbak Aryan, mbak Inna, mas Erlis, mbak Erda, mas Dhadhang, mas Aji, mas Sunu, mbak Mila Indrastuti, mbak Dita Prasetyanti, mbak Aryanti, mbak Dian Paramita, Niken, mbak Fero, mbak Uun, mas Tulus, mas Iwan, and many others, thank you for all of your supports.

To my colleague at the Department of Microbiology, Faculty of Medicine UGM, Prof. Tri, pak Praseno, pak Mustafa, bu Titik, bu Hera, pak Nur, pak Ludhang, bu Ning, bu Susi, mbak Lutvia, thank you so much for all of your supports.

I would also like to express my gratitude towards my sister and brothers: mbak Nurul, mas Yusuf and Habib. As we grew up together, I’ve always know that it is impossible for me to grow apart from you all. You gave me strength and perseverance to keep on living upon mom’s and dad’s demise. It is saddening enough to be orphaned in such young age, but I realized that it could’ve been worse: being orphan without siblings. You three are true blessings without disguise that I should forever be grateful for, *alhamdulillah ‘ala kulli hal*, all praise be to Allah.

To my beautiful daughters Sarah and Shafiya, you are the candles of my eyes, the flowers of our future garden of *jannah*. It broke my heart, Sarah, when you wept and cried upon my last departure back to the Netherlands. I knew you both didn't understand yet regarding my struggle now, but suffice it for me to say, this is me, taking part of global effort to create a better world for you both and your generation to live. My love for you both extends beyond the very existence of my physique *per se*.

Last but not least, towards my dearest wife, Nina. It still lingers in my mind the day that we went to Kokap about eight years ago. The travel there alone took forever, and we're on your two wheeled Yamaha Vega R, but as we both believed that we were on our way up, didn't matter how long and how hard the journey was, for the sake of your final year paper, and our future together, it was something we need to accomplish together as a team. And here we are now, as we climbed this uphill journey that may end up as my 'permanent head damage' (PhD), one journey that I know would feel much heavier and less enjoyable without you by my side. I love you more than before because of Allah.

Publications

1. **Hakim MS**, Ikram A, Zhou J, Wang W, Peppelenbosch M, Pan Q. Immunity against hepatitis E virus infection: implications for therapy and vaccine development. *Reviews in Medical Virology* 2018; 28(2): e1964.
2. **Hakim MS**, Wang W, Bramer WM, Geng J, Huang F, de Man RA, Peppelenbosch MP, Pan Q. The global burden of hepatitis E outbreak: A systematic review. *Liver International* 2017; 37(1): 19-31.
3. Wang W*, **Hakim MS***, Nair VP, Ruiter PE, Huang F, Sprengers D, van der Laan LJW, Peppelenbosch MP, Surjit M, Pan Q. Distinct antiviral potency of Sofosbuvir against Hepatitis C and E viruses. *Gastroenterology* 2016; 151(6): 1251-1253.
4. **Hakim MS**, Spaan M, Janssen HLA, Boonstra A. Inhibitory receptor molecules in chronic hepatitis B and C infections: novel targets for immunotherapy? *Reviews in Medical Virology* 2014; 24(2): 125-138.
5. **Hakim MS**, Nirwati H, Aman AT, Soenarto Y, Pan Q. Significance of continuous rotavirus and norovirus surveillance in Indonesia. *World Journal of Pediatrics* 2018; 14(1): 4-12.
6. **Hakim MS**, Zhou X, Wang Y, Peppelenbosch MP, Pan Q. Anti-diabetic drugs, insulin and metformin, have no direct interaction with hepatitis C virus infection or anti-viral interferon response. *AIMS Molecular Science* 2014; 2(1): 49-58.
7. Ikram A, **Hakim MS**, Zhou JH, Wang W, Peppelenbosch M, Pan Q. Genotype-specific acquisition, evolution and adaptation of characteristic mutations in hepatitis E virus. *Virulence* 2018; 9(1): 121-132.
8. Nirwati H, **Hakim MS**, Aminah S, Dwija IBNP, Pan Q, Aman AT. Identification of rotavirus strains causing diarrhoea in children under five years of age in Yogyakarta, Indonesia. *Malays J Med Sci* 2017; 24(2): 68-77.
9. Wang W, Xu L, Brandsma JH, Wang Y, **Hakim MS**, Zhou X, Yin Y, Fuhler GM, van der Laan LJ, van der Woude CJ, Sprengers D, Metselaar HJ, Smits R, Poot RA, Peppelenbosch MP, Pan Q. Convergent transcription of interferon-stimulated genes by TNF- α and IFN- α augments antiviral activity against HCV and HEV. *Scientific Reports* 2016; 6: 25482. doi: 10.1038/srep25482.
10. Wang W, Wang Y, Qu C, Wang S, Zhou J, Cao W, Xu L, Ma B, **Hakim MS**, Yin Y, Li T, Peppelenbosch MP, Zhao J, Pan Q. The RNA genome of hepatitis E virus robustly triggers antiviral interferon response. *Hepatology* 2018; 67(6): 2096-2112.
11. Nirwati H*, **Hakim MS***, Mawarti Y, Mulyani NS, Ikram A, Aman AT, Peppelenbosch MP, Soenarto Y[#], Pan Q[#]. Norovirus and rotavirus infections in children less than five years of age hospitalized with acute gastroenteritis in Indonesia. *Archives of Virology* (Under revision)
12. Nirwati H*, **Hakim MS***, Ikram A*, Donato CM, Wibawa T, Aman AT, Peppelenbosch MP, Kirkwood CD, Pan Q[#], Soenarto Y[#]. Phylogenetic and immunoinformatic analysis of VP4, VP7 and NSP4 genes of rotavirus strains circulating in children with acute gastroenteritis in Indonesia. (in preparation)
13. **Hakim MS**, Ding S, Chen S, Yin Y, Su J, van der Woude CJ, Fuhler GM, Peppelenbosch MP, Pan Q, Wang W. TNF- α exerts potent anti-rotavirus effects via the activation of classical NF- κ B pathway. *Virus Research* 2018; 253: 28-37.

14. **Hakim MS**, Chen S, Ding S, Yin Y, Ikram A, Ma X, Wang W, Peppelenbosch MP, Pan Q. Basal interferon signaling and therapeutic use of interferons in controlling rotavirus infection in human intestinal cells and organoids. *Scientific Reports* 2018; 8(1): 8341.
15. Yin Y*, Chen S*, **Hakim MS***, Wang W, Xu L, Dang W, Qu C, Verhaar AP, Su J, Fuhler GM, Peppelenbosch MP, Pan Q. 6-thioguanine potently inhibits rotavirus infection through suppression of Rac1 GDP/GTP cycling. *Antiviral Research* 2018; 156: 92-101.
16. Soloviov SO, **Hakim MS**, Nirwati H, Aman AT, Soenarto Y, Pan Q, Dzyublyk IV, Andreeva TI. Quality of symptom-based diagnosis of rotavirus infection based on mathematical modeling. In: Hu Z., Petoukhov S., Dychka I., He M. (eds) *Advances in Computer Science for Engineering and Education*. ICCSEE 2019. **Advances in Intelligent Systems and Computing**, Vol 754. Springer, Cham.
17. Dang W, Xu L, Yin Y, Chen S, Wang W, **Hakim MS**, Chang KO, Peppelenbosch MP, Pan Q. IRF-1, RIG-I and MDA5 display potent antiviral activities against norovirus coordinately induced by different types of interferons. *Antiviral Research* 2018; 155: 48-59.
18. Qu C, Zhang S, Wang W, Li M, Wang Y, Shokrollahi E, **Hakim MS**, Raat NJH, Peppelenbosch MP, Pan Q. Mitochondrial electron transport chain complex III sustains hepatitis E virus replication and represents an antiviral target. *The FASEB Journal* 2018; doi: 10.1096/fj.201800620R. [Epub ahead of print]
19. Miao Z, Zhang S, Ma Z, **Hakim MS**, Wang W, Peppelenbosch MP, Pan Q. Recombinant identification, molecular classification and proposed reference genomes for hepatitis delta virus. (Under revision)
20. Zhou J, Ou X, Ikram A, **Hakim MS**, Peppelenbosch MP, Hu Y, Pan Q. Genotype and host-dependent patterns of codon usage in hepatitis E virus. (Submitted)
21. Nirwati H, **Hakim MS**, Darma S, Mustafa M, Nuryastuti T. Detection of *bla_{OXA}* genes and identification of biofilm-producing capacity of *Acinetobacter baumannii* in a Tertiary Teaching Hospital, Klaten, Indonesia. *Med J Malaysia* 2018; accepted.
22. Soloviov SO, **Hakim MS**, Nirwati H, Soenarto Y, Pan Q, Dzyublyk I, Kovalyuk O, Andreeva T. Analytical support in pharmaceutical care of patients with rotavirus infection in Indonesia. (Under review)
23. Dang W, Yu P, **Hakim MS**, Bramer WM, Peppelenbosch MP, Pan Q. Norovirus infection in hematopoietic stem cell and solid organ transplant recipients: a systematic review. (Under review)
24. Su J, Wang W, **Hakim MS**, Ma Z, Peppelenbosch MP, Pan Q. Interactions of the gut bacterial microbiota and virome: Implications in diarrhea. (in preparation)

* These authors contributed equally as the first author.

These authors share senior authorships.

PhD Portfolio

Name of PhD Student	Mohamad Saifudin Hakim
Erasmus MC Department	Gastroenterology and Hepatology
PhD Period	October 2014 – September 2018
Promotor	Prof. Dr. Maikel P. Peppelenbosch
Copromotor	Dr. Qiuwei Pan

PhD Training	Year	ECTS
Seminars		
Weekly MDL seminar program in experimental gastroenterology and hepatology (attending) (42 weeks/year; @1,5 h)	2014-2018	9
Weekly MDL seminar program in experimental gastroenterology and hepatology (presenting) (preparation time 16 h; 2 times/year)	2014-2018	4,6
Biweekly research group education (attending) (20 times/year; @1,5 h)	2014-2018	4,3
Biweekly research group education (presenting) (preparation time 8 h; 4 times/year)	2014-2018	4,6
General Courses and Workshops		
Study Design Course, Netherlands Institute for Health Sciences (NIHES)	2011	2,0
The Course Biomedical Research Techniques	2011	1,6
The Basic Human Genetic Course: Genetics for Dummies	2011	0,5
The Basic Introduction Course on SPSS for Research MSc and PhD	2011	0,6
The 3 rd Symposium and Workshops on Microbial Pathogenesis	2011	1,0
Population Dynamics in Infection and Immunity	2012	1,8
The Workshop Browsing Genes and Genomes with UCSC	2012	0,4
The Introductory Course on Statistics and Survival Analysis	2012	0,5
The Course on Molecular Medicine	2012	0,7
The Course in Virology	2012	1,8
The Course on Molecular Diagnostic	2012	1,0
The 5 th Symposium and Master Classes on Mucosal Immunology	2013	0,5
The Workshop on Photoshop and Illustrator CS5	2013	0,3
Workshop Systematic Literature Retrieval in Pubmed and Other	2015	1,0

Databases, Erasmus MC Medical Library		
The EndNote Workshop, Erasmus MC Medical Library	2015	0,2
The Course on Topics in Meta-Analysis, Netherlands Institute for Health Sciences (NIHES)	2015	0,7
The Workshop on NCBI & other open source software	2016	1,0
Introduction in GraphPad Prism version 6	2018	0,3
The Course in Virology, MolMed	2018	1,8
The Workshop on Microsoft Access 2010: Basic	2018	0,3
The CPO Course Patient Oriented Research: Design, Conduct and Analysis	2018	0,3
The Course Research Integrity	2018	0,3
Course Microbiomics I	2018	0,3
National and International Conferences		
15th International Congress of Immunology (ICI 2013), International Union of Immunological Societies (IUIS), Milan, Italy.	2013	1,8
Annual Day of the Molecular Medicine, Postgraduate School Molecular Medicine, Erasmus MC (attending).	2012, 2013, 2016, 2017, 2018	1,5
Annual Day of the Molecular Medicine, Postgraduate School Molecular Medicine, Erasmus MC (three poster presentation). (preparation time: 3 posters @ 8 h)	2018	0,8
Reviewing for Scientific Journals (@ 8h)		
<ul style="list-style-type: none"> One paper for <i>BioMed Research International</i>. One paper for <i>BMC Infectious Diseases</i>. One paper for <i>Journal of Infection</i>. One paper for <i>PLoS ONE</i>. 	2016-2018	1,2
Teaching activities		
Aqsa Ikram (research internship: December 2016 – April 2017)		2
Academic Awards		
International Postgraduate Scholarships, Directorate General of Higher Education (DIKTI), Ministry of Education and Culture, Indonesia	2011-2013	-
Infection and Immunity Fund, Erasmus MC Rotterdam, the Netherlands	2013	-
The Indonesia Endowment Fund for Education (LPDP) Scholarship, Ministry of Finance, Indonesia	2014-2018	-

Curriculum Vitae

Mohamad Saifudin Hakim was born in Rembang, Central Java, Indonesia, on February 22, 1985. He was raised by his beloved parents (*late* Munasir and Suhatmidah) and grew up together with his one sister (Nurul) and two brothers (Yusuf and Habib).

In 2003, he completed his high school (SMA Taruna Nusantara, Magelang, Central Java) and started his medical school at the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta Indonesia (2003-2007). After two years of clinical internship (2007-2009), he obtained his medical degree (MD) in 2009. In 2011, he started his research master in the Research Master Program of Infection and Immunity (RM I&I), Postgraduate School of Molecular Medicine, Erasmus Medical Center (EMC) Rotterdam, the Netherlands with financial support from International Postgraduate Scholarship, Directorate General of Higher Education (DIKTI), Ministry of Education and Culture, Indonesia. He performed his research on immunology of hepatitis B and C virus, under the supervision of Dr. Andre Boonstra at the Department of Gastroenterology and Hepatology, EMC. He obtained his master degree in 2013.

Then in 2014, with the support of the Indonesia Endowment Fund for Education (LPDP), Ministry of Finance Indonesia, he got an opportunity to start his PhD research at the Department of Gastroenterology and Hepatology, EMC Rotterdam, the Netherlands under the supervision of Prof. Dr. Maikel P. Peppelenbosch and Dr. Qiuwei Pan. His PhD research focus on virus-drug-host interactions, especially enteric (rotavirus and norovirus) and hepatitis E viruses. Currently, he is an academic staff at the Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta Indonesia. He got married with his lovely wife, Nina, and has two daughter, Sarah and Shafiyah.

