



Hyperferritinemia is a potential marker of chronic chikungunya: A retrospective study on the Island of Curaçao during the 2014–2015 outbreak



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ARTICLE INFO

Article history:

Received 1 April 2016

Received in revised form

30 September 2016

Accepted 7 November 2016

Keywords:

Chikungunya virus

Curaçao

Ferritin

Neopterin

Chronic arthralgia

ABSTRACT

Background: Recently Chikungunya virus (CHIKV) outbreaks have been reported in the Caribbean. There is no data regarding the outbreak in Curaçao. In addition, to date there is no biomarker that could be used to predict chronic infection.

Objectives: To characterize the first CHIKV outbreak in Curaçao and to identify potential biomarkers for chronic infection.

Study design: A serological test and quantitative polymerase chain reaction (qPCR) were used on samples collected in Curaçao to confirm infection. Subsequently, six samples with high viral load were selected for phylogenetic analysis. Furthermore we investigated the association of macrophage-related biomarkers during CHIKV infection with chronic arthralgia/arthritis.

Results: 116 patients in Curaçao were diagnosed with CHIKV infection based on ELISA and 77% were tested positive for CHIKV by qPCR. Phylogenetic analysis showed that an Asian genotype was the cause of the outbreak. Elevated levels of ferritin and CRP were significantly associated with viraemia. In addition, elevated ferritin levels were significantly associated with chronic arthralgia.

Conclusions: The results showed that the presence of an Asian genotype of CHIKV in Curaçao for the first time. Moreover, we found an association between ferritin levels with chronic arthralgia.

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1. Background

Chikungunya virus (CHIKV) is an arthropod-borne alphavirus that cause dengue-like illness [1]. Three lineages of CHIKV have been identified to date, namely West African, Asian, and East-Central-South-Africa (ECSA) genotypes. In the past, CHIKV outbreaks have only been reported occasionally in Africa and Asia. An Asian lineage was introduced into the island of St Martin in 2013,

and quickly spread throughout the Caribbean, South and Central America [2].

Between 20% and 50% of CHIKV-infected patients may develop chronic arthralgia. To date there is no marker that could be used to predict chronic CHIKV infection. It is difficult to predict who will develop chronic disease and therefore finding biomarkers associated with disease is an important step in understanding pathogenesis and aiding the therapeutic decision-making to control the inflammation process early enough before any chronic arthritis occurs.

2. Objectives

Our aim was to describe the first CHIKV outbreak on the Island of Curaçao, validate the commercially available diagnostic kits to

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diagnose CHIKV infection, and identify markers associated with chronic infection.

3. Study design

3.1. Validation of serology for diagnostics of the clinical samples

Samples were obtained from the unit of clinical virology at the Department of Viroscience, Erasmus Medical Center. Fifty-two serum pairs were selected from 26 travelers. To study cross-reactivity of the serological assays, we selected sera from the same serum bank of patients with other viral infections; 12 patients diagnosed with an acute cytomegalovirus (CMV) infection, 17 patients with an acute Epstein-Barr virus (EBV) infection, three patients with an acute Ross River virus (RRV) infection and 20 patients with an acute DENV infection.

3.2. Clinical samples from Curaçao

Serum samples were collected during the 2014–2015 outbreak at the Medical Laboratory Services (MLS) headquarter and aliquoted for RNA isolation and serology. A selection of patients from the MLS database were selected for the retrospective study. Only patients for which paired serum samples were available were selected. Two groups were selected: 1) patients with laboratory confirmed CHIKV infection (based on ELISA) and 2) patients with other febrile illness (OFI). Febrile patients with a negative CHIKV ELISA in the acute and convalescent phase samples were diagnosed as OFI.

3.3. Chikungunya virus serology

CHIKV specific antibodies in sera from patients included in the cohort from Curaçao were determined using an IgM/IgG enzyme-linked immunosorbent assay (ELISA) kit (IBL, Germany). In addition, the IgM/IgG ELISA and the indirect immunofluorescence assay (IFA) for IgM and IgG from Euroimmun (Germany) were used for comparison. All the assays were performed according to the manufacturer's protocol. The sero-status of all samples for the validation study was confirmed in an in-house developed micro-neutralization assay as previously described [3].

3.4. Virus quantification by real time-PCR

The detection and quantification of CHIKV RNA in patient serum was determined by qRT-PCR as previously described [4,5]. Viral RNA load was determined using a standard curve. CHIKV envelope protein 1 (E1) RNA run-off transcripts were used as a standard curve and were prepared as previously described for West Nile virus [6].

3.5. Sequence and phylogenetic analyses of CHIKV genes

PCR reactions targeting a part of the E1, E2, and non-structural protein 2 (nsP2) were performed from samples of six patients with positive qRT-PCR results. Primers used for PCR and sequencing were derived from previous publications [7,8]. The phylogenetic trees were constructed using 522 nucleotides of the E1 gene, 429 nucleotides of the E2 gene and 483 nucleotides of the nsP2 gene. Sequences were aligned using MUSCLE software (v3.7) and the phylogenetic trees were reconstructed using the maximum likelihood implemented in the PhyML software (v3.0). Both softwares were used from the website, <http://phylogeny.lirmm.fr/as> previously described [9].

3.6. Levels of C-reactive protein, ferritin, anti-cyclic citrullinated peptide antibody, rheumatoid factor and neopterin in serum

Serum C-reactive Protein (CRP), ferritin, anti-cyclic citrullinated peptide (anti CCP) antibody and rheumatoid factor (RF) concentrations were determined using a multi-channel Roche Cobas 6000 analyzer. The threshold/normal values for the biomarkers were CRP (1 mg/dl), ferritin (man 30–400 ng/ml; woman 13–150 ng/ml), anti CCP (17 U/ml), and RF (20 IU/ml). A group of 80 patients were selected based on the ferritin serum levels for determination of serum neopterin, anti CCP and RF levels. Neopterin levels were measured using commercially available ELISA kit (IBL, Germany). The assay was performed according to the manufacturer's instructions.

3.7. Patient questionnaire

All the patients that were selected to determine neopterin levels were contacted and a questionnaire containing the following three questions was completed: 1) development of fever, 2) pain in the muscle(s) or joint(s) and 3) appearance of rash. Subsequently the patients were asked for how long after their visit to the general practitioner the clinical symptoms persisted.

3.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.01 software (Graphpad Software, USA). For comparison between continuous variables, Student's *t*-test or Mann Whitney test was performed depending on the distribution of the data. Differences in the proportion of patients were analysed by Chi-square test or Fischer's exact test depending on the normality of the data. *P* values ≤ 0.05 were considered to be statistically significant.

4. Results

4.1. Evaluation of diagnostic methods for detection of CHIKV antibodies

First, a virus neutralization test (VNT) was performed on 52 paired sera from 26 patients with suspicion of CHIKV infection. In 14 out of 26 patients (53.8%), neutralizing antibodies were detected in the acute samples of the paired sera. In order to check if we possibly missed early cases of infection by carrying out only neutralization assay, we additionally performed a CHIKV specific RT-PCR on all the acute samples of the paired sera. We detected CHIKV RNA in four patients that did not have neutralizing antibodies and thereby we confirmed the diagnosis of CHIKV on the acute samples.

The commercially available ELISAs and IFA were compared with the results of the VNT. The specificity of all the tests was above 90% for IgM as well as IgG, indicating that the number of false-positive results is very low. The sensitivity of all the tests was lower for both IgM and IgG, varying from 84% to 92% (Table 1). Subsequently, we analysed the cross-reactivity against RRV, EBV, CMV, and DENV of the three commercial tests. All three assays showed the same IgM cross-reactivity with RRV (33%) and different results were obtained with sera from acute EBV infections (11.8–47.1%). In addition, the Euroimmun ELISA showed a large amount of cross-reactivity with acute CMV infection (41.7%), whereas the other two assays did not show cross-reaction (Table 2). In addition, the reproducibility of the IFA is largely depending on the experience of the technician performing the test (data not shown).

Table 1

Performance of commercial serological methods versus virus neutralization test (VNT).

		Euroimmun IFA				Euroimmun ELISA				Novatec ELISA			
		IgG		IgM		IgG		IgM		IgG		IgM	
		pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
VNT	pos	22	3	23	2	21	4	23	2	22	3	23	2
	neg	0	27	1	26	1	26	0	27	2	25	1	26
	Sensitivity (%)	88		92		84		92		88		92	
	Specificity (%)	100		96.3		96.3		100		92.6		96.3	

Data are depicted as number unless otherwise stated. Abbreviations: VNT: virus neutralization test; IFA: indirect immunofluorescence assay; ELISA: enzyme-linked immunosorbent assay; pos: positive; neg: negative.

Table 2

Cross reactivity of commercial serological assays for CHIKV.

		Euroimmun IFA		Euroimmun ELISA		Novatec ELISA		Total no of patients (n)
VNT		IgG	IgM	IgG	IgM	IgG	IgM	
Acute CMV	0	0	0	0	41.7	0	0	12
Acute EBV	0	0	11.8	0	47.1	5.9	23.5	17
Acute RRV	0	67	33	0	33	0	33	3
Acute DENV	0	0	0	0	10	15	5	20

Data are depicted as percentage (%) unless otherwise stated. Abbreviations: VNT: virus neutralization test; IFA: indirect immunofluorescence assay; ELISA: enzyme-linked immunosorbent assay; CMV: cytomegalovirus; EBV: Epstein-Barr virus; RR: Ross River virus; DENV: dengue virus.

4.2. CHIKV diagnostic results of the 2014–2015 outbreak in Curaçao

In total 141 patients were included in the study. 116 patients were diagnosed with CHIKV infection based on the ELISA results. Viral RNA was detected in 77% of the samples and the highest viral RNA load that was found in this study was 10^8 viral copies/ml blood. In contrast, CHIKV RNA was not detected in any of the samples of the OFI group (n = 25).

4.3. Asian genotype in the outbreak in Curaçao

The sequences from the Curaçao viruses were obtained from the E1, E2 and nsP2 proteins (Fig. 1A–C). Sequences were deposited in GenBank under accession numbers KU727168–KU727181. Phylogenetic analyses of these partial sequences showed clustering of the Curaçao strains with the Asian Genotype. Closest relation of the E1 Asian genotype isolates was seen with available genomes from the Americas outbreak (from Brazil, Mexico and St Martin). Phylogenetic analyses of the other proteins also showed clustering of the isolates within the Asian genotype.

4.4. Biomarkers associated with viraemia and chronic arthralgia

For an analysis of biomarkers in relation to viral load, we first divided the included patients in viraemic (qRT-PCR positive) and non-viraemic (qRT-PCR negative) groups. As a negative control, we used serum samples from the OFI patients. We further divided the viraemic group in high viral load (VL) and low VL sub-groups based on arbitrary copy numbers ($\geq 10^4$ copies were considered high and $< 10^4$ copies low).

We found a significant increase in ferritin serum level in viraemic patients compared to non-viraemic patients ($p = 0.0026$) and patients with OFI ($p = 0.0042$) (Fig. 2A). In addition, the proportion of patients with prolonged chronic arthralgia (≥ 3 months) was significantly higher in the group with high ferritin levels ($p < 0.001$) compared to normal ferritin levels (Table 3). CRP levels were significantly elevated in viraemic group compared to non-viraemic ($p < 0.001$) and OFI group ($p < 0.001$) (Fig. 2C). Furthermore, we found significant differences in serum CRP levels when high VL

Table 3

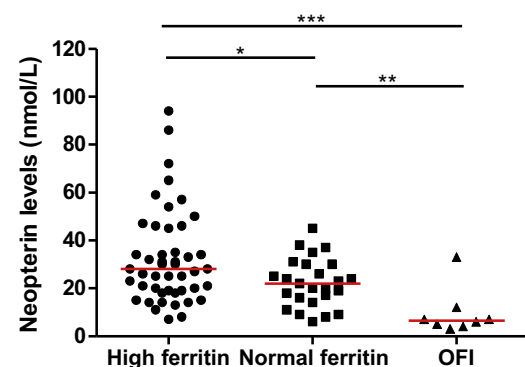
Comparison of clinical manifestations between high ferritin and normal ferritin levels patients.

	High ferritin levels (n = 47)	Normal ferritin levels (n = 25)	P
Fever	26 (55.3%)	12 (25%)	0.55
Rash	15 (31.9%)	8 (25%)	0.99
Joint(s)/muscle(s) pain	42 (89.4%)	7 (28%)	<0.001
<3 months	3 (6.4%)	1 (4%)	0.67
≥ 3 months	39 (83%)	6 (24%)	<0.001

Data are no (%) of patients within the group, unless otherwise indicated. Patients with elevated ferritin levels seem to have more chronic arthralgia ($p < 0.001$) which last more than 3 months ($p < 0.001$) compared to patients with normal ferritin levels.

was compared with low VL ($p = 0.0012$) and OFI groups ($p < 0.001$) (Fig. 2D).

To determine the neopterin levels, patients with normal or elevated ferritin levels were selected from the cohort. In total 80 CHIKV-infected patients were selected for neopterin ELISA. The group of patients with elevated ferritin levels had significantly higher levels of neopterin ($p = 0.03$) compared to patients with normal ferritin levels (Fig. 3).

**Fig. 3.** Correlation of serum neopterin levels with serum ferritin levels.

Serum neopterin levels in patients with elevated ferritin levels were significantly higher compared to the group with normal ferritin levels ($p < 0.03$). Horizontal red lines indicate the median of the groups. Abbreviations: OFI: other febrile illness. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.5. Biomarkers associated with rheumatoid diseases

To confirm whether the patients that experienced chronic arthralgia/arthritis did not have rheumatoid diseases, we measured anti-CCP and RF serum levels. None of the CHIKV patients showed elevated serum levels for both anti-CCP and RF (data not shown). These results suggest that the chronic disease was caused by CHIKV infection.

5. Discussion

This study reports the first outbreak of CHIKV in Curaçao, which was caused by an Asian genotype. The first CHIKV outbreak in the Caribbean was reported in late 2013 on the Island of St. Martin and shortly thereafter more cases were reported on the other Caribbean Islands [2]. The first suspected cases were detected approximately six months later in Curaçao. Afterwards the virus quickly spread throughout Curaçao. The majority of the patients described classic CHIKV symptoms (Table 3) [10].

Our study has several limitations. First, it is a retrospective study. Consequently, owing to the risk of recall bias we could not assess the whole spectrum of clinical symptoms. In addition, the cohort of patients was relatively small, but sufficient enough to justify the conclusions.

The evaluation of the commercial assays was carried out to ensure that the kit that was used in Curaçao is accurate and robust to diagnose CHIKV infection. Our study showed that all the tests evaluated had good sensitivity and specificity. The assays displayed high specificity, which is important in a population with high positivity rates. Sensitivity was lower for the three ELISA kits, which suggest an additional value of either PCR testing in the acute phase or testing follow up (convalescent) sample [11]. It is also important to always interpret the results of both IgM and IgG together, which can increase the reliability of the diagnosis. IFA was also tested in this study as several studies have shown that IFA tests have good sensitivity and specificity [12,13]. However, the necessity to have a well-equipped and adapted lab with experienced staff, and high costs limit the use of this assay in daily clinical settings [14]. Selection of the commercial kit should be made based on the sensitivity and specificity of the assay and also no/low cross-reactivity with other viruses. This is especially important for IgM, which is an important parameter to diagnose acute infection. Our study showed that all assays showed some degree of IgM cross-reactivity against CMV (Euroimmun ELISA), EBV (all kits), and DENV (Novatec and Euroimmun ELISA). Cross-reactivity of the commercially available ELISAs with other alphaviruses was also shown previously [14]. These results imply that careful anamneses and physical examinations of patients in addition to other

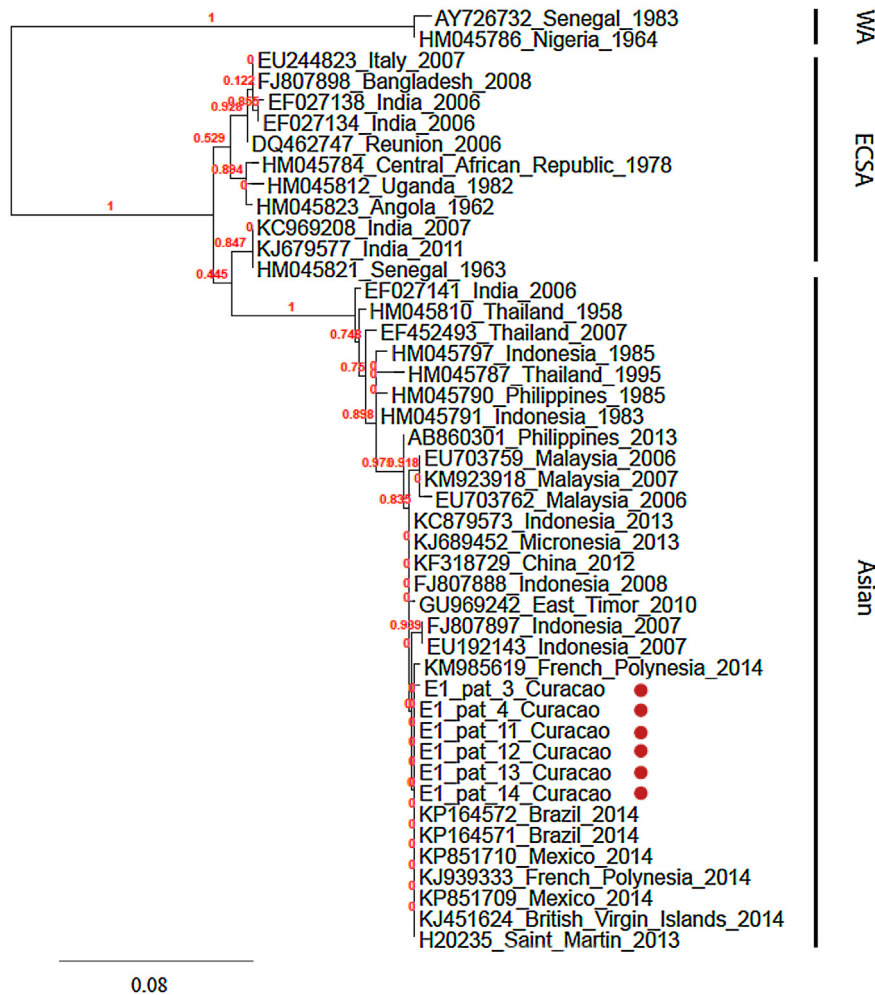


Fig. 1. Phylogenetic analyses of the CHIKV isolates. The sequences obtained in this study were marked with red dot. Panel A shows phylogenetic analysis of CHIKV isolates based on the E1 gene. Panel B shows phylogenetic analysis of CHIKV isolates based on the E2 gene. Panel C shows phylogenetic analysis of CHIKV isolates based on the nsP2 gene. The CHIKV isolates from Curaçao cluster with the strains isolated in the Americas and other strains belonging to the Asian genotype. Abbreviations: WA = West Africa; ECSA = East Central South Africa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

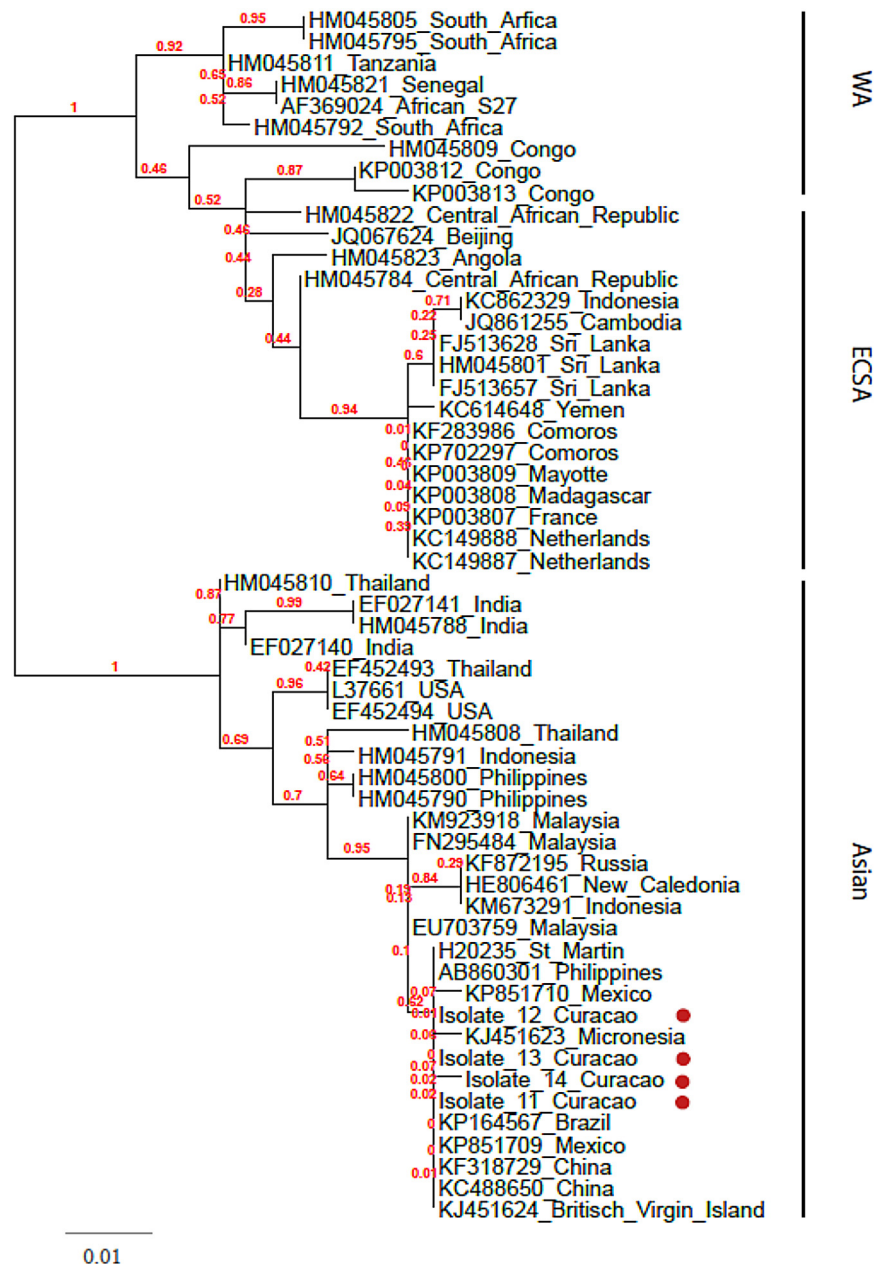


Fig. 1. (Continued)

diagnostic tools are important to obtain an accurate diagnosis in selected cases.

Phylogenetic analyses of the CHIKV genes were in line with other reports coming from the Americas. The fact that the CHIKV strain circulating is closely related to the strains currently circulating in the Americas suggests that the Curaçao outbreak was a result of an introduction of the virus from one of the neighboring Islands. Recently a study reported the emergence of ECSA strain in Brazil from a patient that returned from Angola [15]. It has been shown that the A226V mutation in the viral E1 protein can increase viral transmission in *A. albopictus*, which was associated with the large CHIKV epidemic in the Indian Ocean [16,17]. Given the presence of *A. albopictus* in many latin American countries and the extensive travelling between the islands in the region, there is a realistic risk for introduction of the Indian Ocean sub-lineage in the future.

We found significantly elevated serum levels of CRP in CHIKV patients with viraemia compared to non-viraemic and OFI groups. C-reactive protein is an acute-phase protein that is synthesized by the liver. Several studies showed that monocytes/macrophages and hepatocytes are the target cell of CHIKV infection [18–20]. It has been shown that CHIKV interactions with monocytes induced a robust and rapid innate immune responses with the production of cytokines and chemokines [18]. It is possible that hepatocytes are indirectly activated by pro-inflammatory mediators and/or activated immune cells to synthesize high amounts of CRP. Additionally, direct infection of hepatocytes could also initiate the production of CRP. Our study is in line with previous studies that also found increased CRP levels in patients infected with DENV, another arbovirus, which also infects macrophages and hepatocytes [21,22].

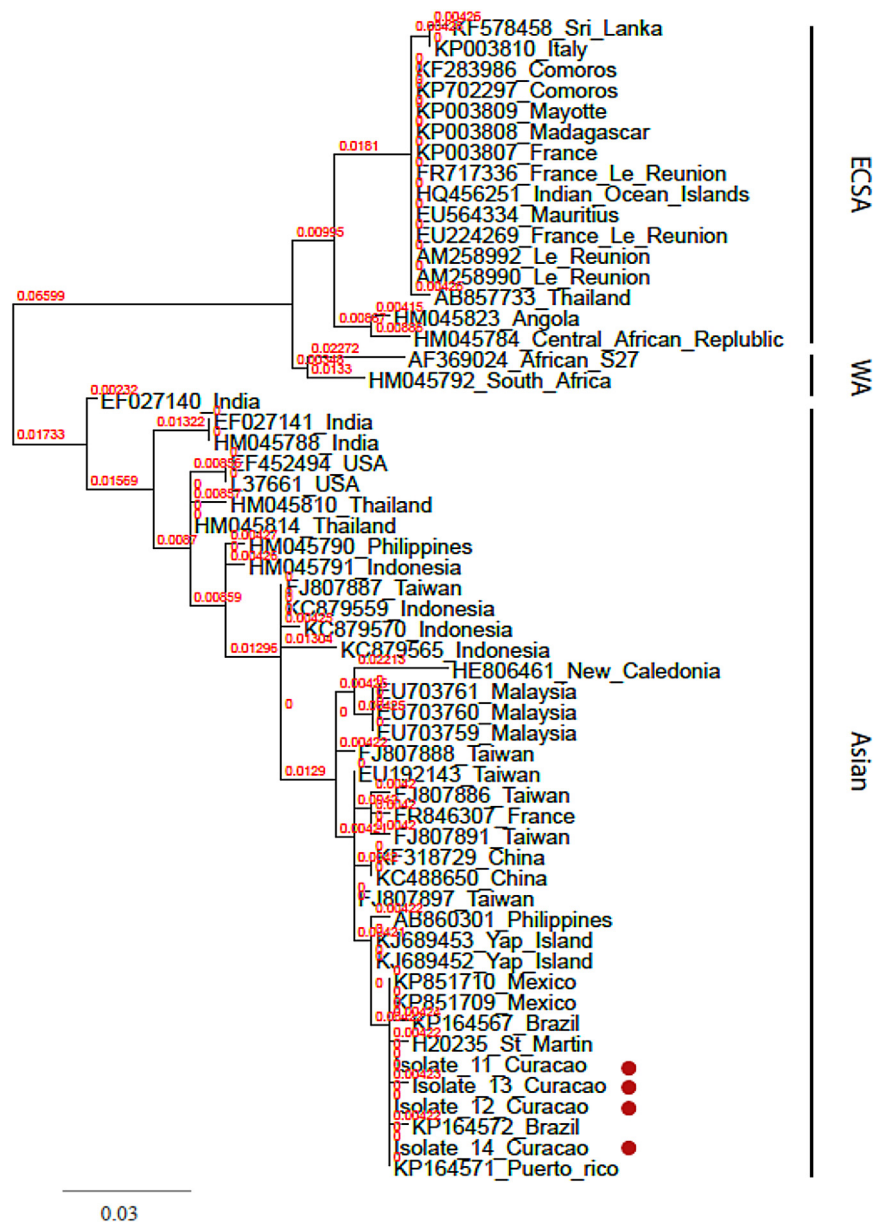


Fig. 1. (Continued)

We also found elevated serum levels of ferritin and neopterin, which are markers of activated macrophages. Studies have shown that synthesis of ferritin can be triggered by cytokines and iron [23,24]. In this study, increased concentrations of ferritin were significantly associated with viraemia. Macrophage and hepatocyte are important producers of the secreted form of ferritin and therefore direct infection and subsequent viral replication in these cells may activate them and increase the production of ferritin. We also found that a significant number of patients with elevated ferritin levels in the acute serum sample developed chronic arthralgia. Hyperferritinaemia has been shown to be associated with other arboviral infections and autoimmune disorders like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [25–27]. We did not find positive rheumatoid markers (anti-CCP and RF) in our cohort, which suggest that elevated ferritin levels and chronic arthralgia/arthritis were not caused by rheumatoid diseases. In this study we also found an association between elevated ferritin levels with production of neopterin, which is a specific marker of

activated macrophages. Elevated serum levels of both molecules were also shown to be associated with the severity of DENV infection [27,28]. The results presented in the manuscript support the hypothesis that macrophages might play a role in the development of chronic CHIKV infection. In addition, our results suggest that ferritin could serve as a potential prognostic marker for development of chronic CHIKV infection.

6. Conclusions

This study is the first report of CHIKV emergence in Curaçao. We identified the etiological strain associated with the CHIKV outbreaks in Curaçao, which belongs to the Asian genotype. We found an association between hyperferritinaemia with chronic chikungunya. Further studies are needed to evaluate whether ferritin could serve as a potential prognostic markers for chronic chikungunya.

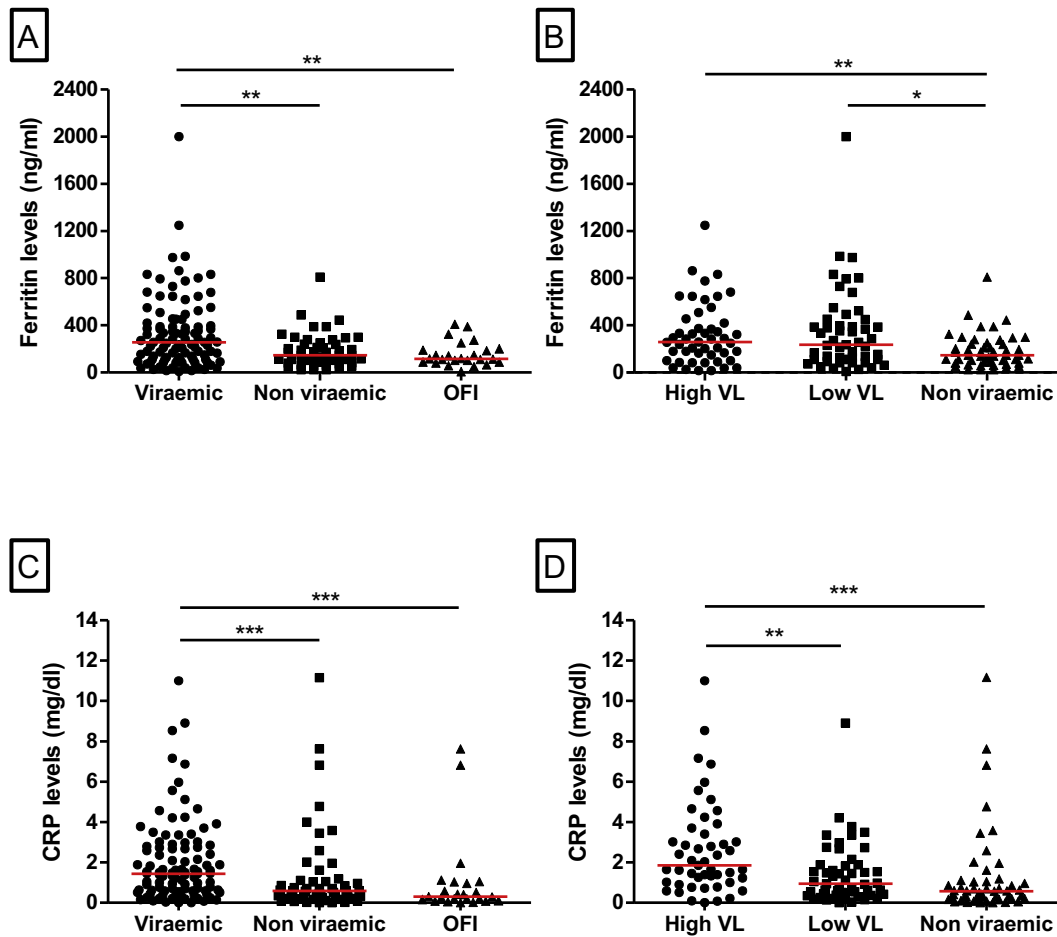


Fig. 2. Ferritin and C-reactive protein (CRP) serum levels in patients infected with CHIKV.

Samples selected in the Curaçao 2014–2015 cohort were divided in qPCR positive (viraemic) and qPCR negative (non-viraemic) groups. (A) Ferritin serum levels were significantly elevated in viraemic patients compared to non-viraemic ($p=0.0026$) and OFI group ($p=0.0042$) (B) No significant difference in ferritin levels was observed between viraemic samples when divided into high VL and low VL ($p=0.929$), indicating that the presence of viraemia rather than viral load determined the serum ferritin levels. (C) Serum CRP levels were significantly increased in viraemic patients compared to non-viraemic ($p<0.001$) and OFI group ($p<0.001$). CRP levels of patients classified in high VL group was significantly elevated compared to low VL ($p=0.0012$) and non viraemic group ($p<0.001$). Horizontal red lines indicate the median of the groups. Abbreviations: OFI: other febrile illness; VL: viral load. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Competing interest

The authors declare that they have no competing interest apart from ADMEO who is a part time employee of Viroclinics BV. The stated competing interest does not influence the author's adherence to the policies on sharing data and materials.

Ethical approval

Ethical approval of this study was obtained from the Medical Ethics Committee in Curaçao with reference number 2014-003.

Funding

FA was supported by the Directorate of Higher Education (DIKTI) PhD grant of the Ministry of Research, Technology and Higher Education of the Republic of Indonesia. LP was supported by Medical Laboratory Services, Curaçao. The funders had no role in study design, data collection and analysis, preparation of the manuscript, and decision to publish.

Acknowledgements

The authors would like to thank the physicians and the patients in Curaçao for their help and support in this study. We thank Henk-Jan van den Ham for his help with the statistical analyses. We also thank Janienne Klaasse for her excellent technical assistance on the validation study experiments.

References

- [1] C. Caglioti, E. Lalle, C. Castilletti, F. Carletti, M.R. Capobianchi, L. Bordini, Chikungunya virus infection: an overview, *New Microbiol.* 36 (2013) 211–227.
- [2] S. Cassadou, S. Boucau, M. Petit-Sinturel, P. Huc, I. Leparc-Goffart, M. Ledrans, Emergence of chikungunya fever on the French side of Saint Martin island, October to December 2013, *Euro Surveill.* 19 (2014).
- [3] P. van den Doel, A. Volz, J.M. Roose, V.D. Sewbalaksing, G.P. Pijlman, I. van Middelkoop, et al., Recombinant modified vaccinia virus Ankara expressing glycoprotein E2 of Chikungunya virus protects AG129 mice against lethal challenge, *PLoS Negl. Trop. Dis.* 8 (2014) e3101.
- [4] P. Laurent, K. Le Roux, P. Grivard, G. Bertil, F. Naze, M. Picard, et al., Development of a sensitive real-time reverse transcriptase PCR assay with an internal control to detect and quantify chikungunya virus, *Clin. Chem.* 53 (2007) 1408–1414.
- [5] M. Goeijenbier, G. Aron, F. Anfasa, A. Lundkvist, J. Verner-Carlsson, C.B. Reusken, et al., Emerging viruses in the Republic of Suriname: retrospective and prospective study into chikungunya circulation and suspicion of Human

- Hantavirus Infections, 2008–2012 and 2014, Vector Borne Zoonotic Dis. 15 (2015) 611–618.
- [6] S.M. Lim, P. Koraka, A.D. Osterhaus, B.E. Martina, Development of a strand-specific real-time qRT-PCR for the accurate detection and quantitation of West Nile virus RNA, J. Virol. Methods 194 (2013) 146–153.
 - [7] K.P. Niyas, R. Abraham, R.N. Unnikrishnan, T. Mathew, S. Nair, A. Manakkadan, et al., Molecular characterization of Chikungunya virus isolates from clinical samples and adult *Aedes albopictus* mosquitoes emerged from larvae from Kerala, South India, Virol. J. 7 (2010) 189.
 - [8] E. Sreekumar, A. Issac, S. Nair, R. Hariharan, M.B. Janki, D.S. Arathy, et al., Genetic characterization of 2006–2008 isolates of Chikungunya virus from Kerala, South India, by whole genome sequence analysis, Virus Genes 40 (2010) 14–27.
 - [9] A. Dereeper, V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, et al., Phylogeny.fr: robust phylogenetic analysis for the non-specialist, Nucleic Acids Res. 36 (2008) W465–W469.
 - [10] S.C. Weaver, M. Lecuit, Chikungunya virus and the global spread of a mosquito-borne disease, N. Engl. J. Med. 372 (2015) 1231–1239.
 - [11] M. Niedrig, H. Zeller, I. Schuffenecker, C. Drosten, P. Emmerich, L. Rumer, et al., International diagnostic accuracy study for the serological detection of chikungunya virus infection, Clin. Microbiol. Infect. 15 (2009) 880–884.
 - [12] G. Yap, K.Y. Pok, Y.L. Lai, H.C. Hapuarachchi, A. Chow, Y.S. Leo, et al., Evaluation of Chikungunya diagnostic assays: differences in sensitivity of serology assays in two independent outbreaks, PLoS Negl. Trop. Dis. 4 (2010) e753.
 - [13] N. Litzba, I. Schuffenecker, H. Zeller, C. Drosten, P. Emmerich, R. Charrel, et al., Evaluation of the first commercial chikungunya virus indirect immunofluorescence test, J. Virol. Methods 149 (2008) 175–179.
 - [14] C.M. Prat, O. Flusin, A. Panella, B. Tenebray, R. Lanciotti, I. Leparc-Goffart, Evaluation of commercially available serologic diagnostic tests for chikungunya virus, Emerg. Infect. Dis. 20 (2014) 2129–2132.
 - [15] M.R. Nunes, N.R. Faria, J.M. de Vasconcelos, N. Golding, M.U. Kraemer, L.F. de Oliveira, et al., Emergence and potential for spread of Chikungunya virus in Brazil, BMC Med. 13 (2015) 102.
 - [16] K.A. Tsatsarkin, R. Chen, R. Yun, S.L. Rossi, K.S. Plante, M. Guerbois, et al., Multi-peaked adaptive landscape for chikungunya virus evolution predicts continued fitness optimization in *Aedes albopictus* mosquitoes, Nat. Commun. 5 (2014) 4084.
 - [17] K.A. Tsatsarkin, D.L. Vanlandingham, C.E. McGee, S. Higgs, A single mutation in chikungunya virus affects vector specificity and epidemic potential, PLoS Pathog. 3 (2007) e201.
 - [18] Z. Her, B. Malleret, M. Chan, E.K. Ong, S.C. Wong, D.J. Kwek, et al., Active infection of human blood monocytes by Chikungunya virus triggers an innate immune response, J. Immunol. 184 (2010) 5903–5913.
 - [19] K. Labadie, T. Larcher, C. Joubert, A. Mannioui, B. Delache, P. Brochard, et al., Chikungunya disease in nonhuman primates involves long-term viral persistence in macrophages, J. Clin. Invest. 120 (2010) 894–906.
 - [20] T. Couderc, F. Chretien, C. Schilte, O. Disson, M. Brigitte, F. Guivel-Benhassine, et al., A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease, PLoS Pathog. 4 (2008) e29.
 - [21] M. Juffrie, G.M. Meer, C.E. Hack, K. Haasnoot, A.J. Sutaryo Veerman, et al., Inflammatory mediators in dengue virus infection in children: interleukin-6 and its relation to C-reactive protein and secretory phospholipase A2, Am. J. Trop. Med. Hyg. 65 (2001) 70–75.
 - [22] C.C. Chen, I.K. Lee, J.W. Liu, S.Y. Huang, L. Wang, Utility of C-reactive protein levels for early prediction of dengue severity in adults, Biomed Res. Int. 2015 (2015) 936062.
 - [23] M. Fahmy, S.P. Young, Modulation of iron metabolism in monocyte cell line U937 by inflammatory cytokines: changes in transferrin uptake, iron handling and ferritin mRNA, Biochem. J. 296 (Pt 1) (1993) 175–181.
 - [24] T.N. Tran, S.K. Eubanks, K.J. Schaffer, C.Y. Zhou, M.C. Linder, Secretion of ferritin by rat hepatoma cells and its regulation by inflammatory cytokines and iron, Blood 90 (1997) 4979–4986.
 - [25] C. Rosario, G. Zandman-Goddard, E.G. Meyron-Holtz, D.P. D'Cruz, Y. Shoenfeld, The hyperferritinemic syndrome: macrophage activation syndrome, Still's disease, septic shock and catastrophic antiphospholipid syndrome, BMC Med. 11 (2013) 185.
 - [26] C. Rosario, Y. Shoenfeld, The hyperferritinemic syndrome, Isr. Med. Assoc. J. 16 (2014) 664–665.
 - [27] C.A. van de Weg, R.M. Huits, C.S. Pannuti, R.M. Brouns, R.W. van den Berg, H.J. van den Ham, et al., Hyperferritinaemia in dengue virus infected patients is associated with immune activation and coagulation disturbances, PLoS Negl. Trop. Dis. 8 (2014) e3214.
 - [28] C.A. van de Weg, P. Koraka, E.C. van Gorp, A.T. Mairuhu, M. Supriatna, A. Soemantri, et al., Lipopolysaccharide levels are elevated in dengue virus infected patients and correlate with disease severity, J. Clin. Virol. 53 (2012) 38–42.