
Facing Antimicrobial Resistance in Gastrointestinal Bacteria
ROBERT-JAN HASSING

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in Gastrointestinal Bacteria

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Facing Antimicrobial Resistance in Gastrointestinal Bacteria

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PROMOTIECOMMISSIE

Promotoren:	Prof.dr. B.H.Ch. Stricker
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CHAPTER 1

General introduction

Gastrointestinal bacterial pathogens

Symptomatic gastrointestinal infections are frequently diagnosed illnesses, and an important cause of morbidity and mortality worldwide. In the Netherlands about 4.5 million episodes of symptomatic gastroenteritis per year are diagnosed with an estimated incidence of 11 cases per 1000 people per year in a general practitioner's population[1]. In developed countries gastrointestinal infections are mostly caused by bacterial pathogens, such as *Campylobacter, Salmonella, Shigella* and *Yersinia* species and viral pathogens such as Norwalk-like virus, Rotavirus, Adenovirus and Astrovirus[1,2]. *Campylobacter, Salmonella, Shigella* and *Yersinia* are rod-shaped bacteria of the *Enterobacteriaceae* family. Parasitic infections are also responsible for a large burden of gastrointestinal disease, but occur mainly in developing countries[3]. Typhoidal *Salmonella* species are bacterial gastrointestinal pathogens, endemic in many developing countries with poor hygiene and sanitation[4,5]. In contrast with other bacterial gastrointestinal pathogens, which are usually self-limiting diseases, typhoidal *Salmonella* infections usually require antimicrobial treatment. In the past decades, antimicrobial treatment options have been complicated by emergence of antimicrobial resistance[5,6]. This thesis concerns studies on risk factors for gastroenteritis and antimicrobial resistance, and focusses on epidemiology and antimicrobial resistance of typhoidal *Salmonella* species.

Risk factors for gastroenteritis

Gastroenteritis is mostly associated with foodborne transmission. Risk factors for *Campylobacter* infections include travelling abroad, eating undercooked chicken, environmental sources, and direct contact with farm animals[7]. Travel, eating raw eggs, and eating in restaurants have been associated with *Salmonella* infection[8]. An association between proton pump inhibitor (PPI) therapy and bacterial gastroenteritis has been suggested as well as contradicted[9-17].

There is also an increased incidence of gastroenteritis in patient with HIV[18]. HIV-infected patients also have an increased risk of more severe disease course of bacterial gastroenteritis[18]. For example, long term carriage or bacteremia after gastroenteritis caused by *Campylobacter* of *Salmonella* species are more often observed in patient with HIV[18,19]. HIV infection does not seem to be a risk factor for Shigella bacteremia20. Moreover, since 1994 remarkable outbreaks of *Shigella* infection have been observed in HIV-infected men who have sex with men, as a sexual transmitted disease[21].

Risk factors for antimicrobial resistance

The emergence of bacteria resistant to antimicrobial agents is an emerging global problem, resulting in limited options for oral treatment, and consequently an increasing number of hospital admissions for intravenous treatment[22,23]. The most important risk factors for acquisition of antimicrobial resistance are use of antibiotics and admission to health care facilities[23]. However, multidrug resistant organisms are also often observed in patients without previous hospital contacts or use of antibiotics. Therefore, we need a broader approach to study community-acquired risk factors for antimicrobial resistance to combat infections caused by these multidrug resistant organisms. International travel is considered to be an important risk factor for acquisition of multidrug-resistant bacteria[24-34].

Of all gastrointestinal infections caused by the usual bacterial pathogens (*Salmonella, Campylobacter, Shigella* and *Yersinia*) typhoidal *Salmonella* species are the only pathogens requiring antimicrobial treatment in immunocompetent patients. Therefore, studies on antimicrobial resistance, management and epidemiology are most clinically relevant for these species.

Enteric fever

Epidemiology

Enteric fever is the illness caused by *Salmonella* enterica serotype Typhi (S. Typhi), also called typhoid fever[5,6]. Enteric fever, can also be caused by *Salmonella* enterica serotype Paratyphi A, B and C, sometimes called paratyphoid fever[35,36]. The bacillus suspected to cause enteric fever was first describes by Karl Joseph Eberth in 1880[37]. Until the mid-twentieth century, enteric fever was endemic in developed countries such as the Netherlands[38]. Nowadays enteric fever is mainly a disease of developing countries and is occasionally diagnosed in ill returned travellers in developed countries[3,4]. Enteric fever has an estimated worldwide yearly incidence of 26.9 million episodes and the highest incidence in Southern Asia[35]. In contrast with other *Salmonella* species which have a strictly zoonotic reservoir, typhoidal *Salmonella* species only have human transmission. Transmission occurs by feco-oral route and therefore infections are mostly foodborne, due to poor sanitation and lack of clean water.

Clinical presentation

Enteric fever is a systemic febrile disease. The symptoms vary widely and therefore it is difficult to define a classical presentation of the disease. Abdominal complains are most frequently reported, such as abdominal pain accompanied by diarrhoea and (counterintuitively) often constipation[6,36].

Enteric fever has been associated with relative bradycardia (pulse-temperature dissociation), which may also be caused by other tropical infectious diseases such as malaria[39]. This might therefore not be a very useful feature for clinical use. Typical laboratory abnormalities in typhoid fever are decreased or normal leukocyte count with aneosinophilia, moderate thrombocytopenia, increased C-reactive protein, increased liver enzymes. Newer diagnostics such as procalcitonin have not been shown to have additional value compared to these traditional laboratory values in the diagnosis of enteric fever[40].

Risk factors

There is conflicting evidence regarding the risk of enteric fever in HIV-positive patients. Patient who are infected with HIV might have an increased risk of infection with typhoidal *Salmonella*[41]. However, a more recent publication observed a protective effect on acquiring a typhoidal *Salmonella* infection[42,43]. This in great contrast to non-typhoidal *Salmonella* infections which are the most important cause of bacteraemia in HIV positive patients in sub-Saharan Africa[19]. Another possible risk factor for typhoidal *Salmonella* infections is proton pump inhibitor therapy. Use of PPIs probably leads to an increased risk of enteric infections, because diminishing gastric acid leads to increased survival of *Salmonella* species. This has only been demonstrated for non-typhoidal *Salmonella* species, but based on the biological effect this will probably hold for typhoidal *Salmonella* species as well[44,45].

Diagnostics and treatment

To confirm the diagnosis of enteric fever, a positive blood culture is considered as the gold standard[46,47]. Typhoidal *Salmonella* species can be isolated from stool, urine or cerebral fluid as well. The Widal test is an old serologic assay for detecting IgM and IgG to the antigens of *Salmonella*. The test is unreliable but is still widely used in most developing countries because of its low cost[46,47]. Further, it is possible to detect typhoidal *Salmonella* species using other diagnostics, such as polymerase chain reaction (PCR) or Maldi-Tof[48-50]. A new serological assays (TPTest) is currently under research, but not yet used in clinical practice in the Netherlands[51].

As a result of the wide presence of multidrug-resistant (MDR) *Salmonella* strains (defined as resistance against chloramphenicol, amoxicillin and trimethoprim) in the 80s and 90s, infections caused by typhoidal *Salmonella* strains are now treated with fluoroquinolones, third generation cephalosporins or azithromycin[6,52]. Treatment has been complicated by the emergence of fluoroquinolone resistance in Asian countries[6,52]. Because of cases of treatment failure in systemic infections caused by *Salmonella* spp with low-level ciprofloxacin resistance (MIC 0.125–1.0 mg/L), it is now stated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) that all *Salmonella* isolates with an MIC >0.06 mg/L should be considered ciprofloxacin resistant and should be treated otherwise[53]. In the majority of the cases enteric fever is acquired in Asia, where

third generation cephalosporines and azithromycin are the only treatment options left. Typhoidal *Salmonella* strains expressing extended-spectrum beta-lactamases have been sporadically described from Asian countries[54-55]. Cases of azithromycin resistance in typhoidal *Salmonella* infection are still very uncommon[56-57].

Aim of the thesis

The overall aim of this thesis was to study risk factors and treatment options in gastrointestinal infections, especially in typhoidal *Salmonella* isolates.

Primary research questions

1 To study risk factors of gastrointestinal infections and antimicrobial resistance
2 What are the most important mechanisms involved in antimicrobial resistance in typhoidal *Salmonella* isolates?
3 Can a new rapid diagnostic methodology be developed to identify antimicrobial resistance in typhoidal *Salmonella* isolates?

Outline of this thesis

To avoid treatment challenges it is best to prevent gastrointestinal infection. PPIs are among the most frequently prescribed drugs worldwide and in many cases the indication is doubtful. In **Chapter 2** we studied the association between proton pump inhibitor therapy and *Campylobacter*, (non-typhoidal) *Salmonella*, *Yersinia* or *Shigella* infection in community-dwelling non-hospitalized individuals in The Rotterdam Study, a population-based cohort study. Another possible cause of emergence of antimicrobial resistance is introduction of multi-drug resistant microorganisms in the community as a result of international travel. In **Chapter 3** a systematic review was performed to study the available evidence of the association of international travel and asymptomatic fecal carriage of multidrug-resistant *Enterobacteriaceae*.

Chapter 4 describes the different mechanisms involved in reduced susceptibility to ciprofloxacin of *S.* Typhi and *S.* Paratyphi A isolates from travellers to South-East Asia. In all of these cases, resistance to fluoroquinolone-based antibiotics is associated with genetic mutations in the quinolone resistance-determining region (QRDR) of the bacterial DNA gyrase gene, *gyrA*. Though these mutations are invariably detected by sequencing of the *gyrA* gene, phenotypically important QRDR mutations also result in amino acid substitutions in the GyrA protein of typhoidal *Salmonellae*, which could potentially be detected using high resolution mass spectrometry techniques. In **Chapter**

5 we demonstrate the development and evaluation of a liquid chromatography-mass spectrometry (LC-MS) methodology for the detection of amino acid substitutions in the GyrA protein of the 23 typhoidal *Salmonella* isolates used in **Chapter 4**. In the same travellers of the previous two chapters we studied the impact of decreased ciprofloxacin susceptibility on the fate of travellers returning with enteric fever in **Chapter 6**. Possible alternative treatment options based on pharmacokinetic parameters of different fluoroquinolones were also determined in **Chapter 6**. In many countries azithromycin is the only antibiotic left for oral treatment of enteric fever. In **Chapter 7** we analysed trends in susceptibility to azithromycin among typhoidal *Salmonella* isolates in isolates already possessing increased MICs for ciprofloxacin in an epidemiological study, using all available typhoidal *Salmonella* isolates collected in the Netherlands during 1999-2012. Resistance to azithromycin is still very rare in gastrointestinal pathogens. **Chapter 8**, a case report of *Shigella flexneri* infection with treatment failure due to azithromycin resistance, acts as an example of a future problem, namely occurrence of antimicrobial resistance to this "last resort" antimicrobial drug.

Chapter 9 outlines and reflects on the main findings of the thesis. Limitations and methodological considerations will be discussed as well as clinical implications and future research.

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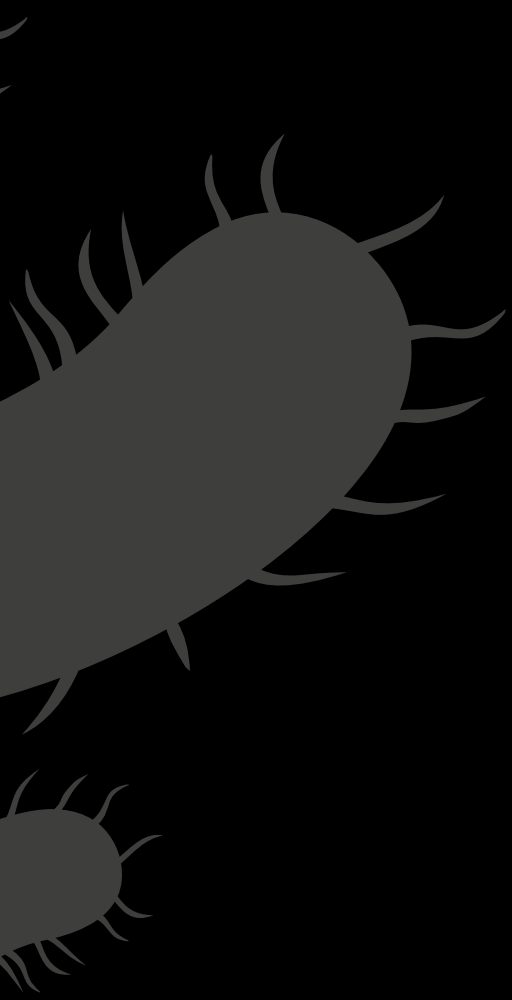
PART 1

Risk factors for gastroenteritis and antimicrobial resistance



CHAPTER 2

Proton pump inhibitors and gastroenteritis

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Robert-Jan Hassing

Annelies Verbon

Herman de Visser

Albert Hofman

Bruno H. Stricker

Abstract

BACKGROUND An association between proton pump inhibitor (PPI) therapy and bacterial gastroenteritis has been suggested as well as contradicted. The aim of this study was to examine the association between the use of PPIs and occurrence of bacterial gastroenteritis in the prospective Rotterdam Study.

METHODS The Rotterdam Study is a population-based cohort study among 14 926 subjects aged 45 years and older with up to 24 years of follow-up. Analyses were performed with a generalized estimating equations method in participants who handed-in a diagnostic stool sample. Furthermore, a nested case-control analysis was performed using the total cohort as a reference group.

RESULTS A bacterial microorganism was isolated in 125 samples, whereas 1174 samples were culture negative. In the generalized estimating equations analysis, we found that participants with a bacterial gastroenteritis were more likely than controls to be current users of PPIs (adjusted OR, 1.94; 95% CI 1.15-3.25). Different sensitivity analyses did not change this result. A considerably higher effect was observed (adjusted OR 6.14; 95% CI 3.81-9.91), using the total cohort as a reference in a nested case-control analysis.

CONCLUSIONS Current PPI therapy is associated with an increased risk of bacterial gastroenteritis. However, by reducing the risk of selection and information bias in our study design, we demonstrated that the effect is lower than previously assumed.

Introduction

Proton pump inhibitors (PPIs) are among the most frequently prescribed drugs worldwide, but seem to be associated with an increased risk of bacterial gastroenteritis[1-9]. As a result warnings are introduced for people using PPIs, such as avoiding raw meat consumption and antibiotic treatment on demand for travels to the tropics, to prevent food borne infections. This cohort study was designed to asses if the magnitude of this risk, warrants preventive recommendations.

Common indications for PPIs are dyspepsia, peptic ulcer disease, reflux esophagitis, and Barrett's oesophagus[10]. PPIs reduce gastric acid production by up to 99% by irreversible blocking of H+/K+ ATPase of parietal cells in the stomach[11]. They have a maximal effect within 4 days and the effect may persist up to three days after stopping use[12]. Associations between PPIs and infectious adverse events such as pneumonia, Clostridium difficile-associated diarrhoea and bacterial gastroenteritis have often been described[1-9,13-17]. An increased risk of gastroenteritis might be explained by the strong reduction in gastric acid resulting in increased susceptibility to bacterial infections. Exogenous bacteria are usually destroyed in the stomach when the pH is less than 3.0. For species such as *Vibrio cholerae* and *Campylobacter jejuni* it has been shown in vitro that they are very sensitive to pH[18]. However, *Salmonella* species have been found to respond to low pH by developing adaptive mechanisms that allow survival in acid environments[19]. Furthermore, PPIs change the gut flora, which provides a homeostatic protection against ingested pathogens[20,21]. PPIs also reduce the antibacterial activity of neutrophils which may facilitate *Salmonella* and *Campylobacter* infection[22,23]. Several case-control studies have shown an increased risk of acquiring gastrointestinal infections caused by *Campylobacter* or *Salmonella* species in patients using PPIs[1-8]. In these case-control studies, a relatively high adjusted odds ratio (aOR) or relative risk was observed, ranging from 2.9 to 11.7. In one nested case-control study, in which participants with a gastroenteritis prior to first PPI prescription were excluded, a considerably lower effect was observed (aOR 1.6)[9]. It has even been stated that there is no evidence that PPIs are associated with gastrointestinal infections based on outcomes adjusted for pre-treatment susceptibility to bacterial gastrointestinal infections and time-dependent confounding factors[24], which observation suggests that previous case-control studies have suffered from selection or information bias. Therefore, we designed a nested case control study within The Rotterdam Cohort, a prospective cohort study, to examine the association between the use of PPIs and occurrence of bacterial gastroenteritis. To minimize the risk of information bias we used participants with negative stool samples as a control group. To test the hypothesis that an incorrect control group will influence the study results we also analysed the association using the total cohort as a control group.

Materials and methods

Study population

The study was performed in The Rotterdam Study, a prospective population-based cohort study in 14,926 people aged ≥45 years, from one district (Ommoord) in the city of Rotterdam, the Netherlands[25]. In short, from 1990 through 1993, 7983 participants were included (cohort I). In 2000 an additional 3011 participants who had become 55 years old or older or who had moved into the district, were enrolled (cohort II). In 2006 another 3932 participants, aged 45 years and older were included (cohort III). Follow-up examinations are conducted every four to five years. Participants are continuously monitored through linkage of records from general practitioners.

The Rotterdam Study was approved by the medical ethics committee according to the Wet Bevolkingsonderzoek ERGO (Population Study Act Rotterdam Study) executed by the Ministry of Health, Welfare and Sports of the Netherlands. All study participants provided written informed consent.

Definition of outcome

A case was defined as a community-dwelling non-hospitalized individual with a positive stool sample for *Campylobacter, Salmonella, Yersinia* or *Shigella* species. A control was defined as an individual with a negative stool sample. Stool sample results were obtained from Star Medisch Diagnostisch Centrum (Star-MDC), a centre for medical diagnostics for outpatients in the city of Rotterdam. The majority of all laboratory tests, including microbiology tests, of patients from general practitioners within the Ommoord district of Rotterdam are performed at Star-MDC. Of all participants of The Rotterdam Study, of whom informed consent was obtained for requesting medical information, positive and negative microbiology tests between 1999 and April 2013 were obtained. Stool samples were selected and samples in which parasites were isolated were excluded. Detection of bacterial enteric pathogens in stool samples at Star-MDC is performed by Multiplex polymerase chain reaction (PCR), followed by culture and microscopy in case of a positive result. Until December 2010, when PCR was introduced at Star-MDC, detection of bacterial enteric pathogens was performed by conventional culture and microscopy only.

Assessment of exposure and covariables

Participants were considered as current user of PPI if the calendar date of the stool sample fell within a prescription episode of a PPI. Prescription episodes were calculated by dividing the total number of supplied pills by the recommended daily number. Ad-

ditional covariables assessed were age, sex, cohort, calendar date (year), BMI, household status, past use of proton pump inhibitors, current or past use of H2-receptor antagonists, current use of chronic medication (antidiabetic medication, antihypertensive medication, or statins), intestinal anti-inflammatory agents, corticosteroids, immunosuppressant medication, meat consumption, red meat consumption, chicken consumption, egg consumption and alcohol consumption (for all dietary variables: yes/no and gram/days).

BMI and household status was obtained from baseline characteristics of The Rotterdam Study. Medication use was obtained through automated linkage with pharmacy filled prescription data, available from January 1st, 1991 until April 2013. Dietary data were available from one week food consumption questionnaires obtained at the first visit of cohort I and cohort III and at the third visit of cohort II. Multiple imputation (10x) of missing dietary data and BMI was performed using all covariables.

Statistical analyses

Model design

To assess the association between current use of PPIs and gastrointestinal infections generalized estimating equations (GEE) was used to adjust for correlation between repeated measurements in the same participants, using a varying between-measurement time window[26,27]. The working correlation matrix in which the model had the lowest quasi likelihood under independence model criterion was selected. The model was adjusted for age and sex and additionally for covariables changing the point estimate (β) of current PPI use by more than 10% or if considered clinically relevant.

Sensitivity analyses

Different sensitivity analyses were performed. To exclude confounding by indication or protopathic bias we recoded PPI use started within 14 days before a positive stool sample as non-use. To take into account that in many occasions PPIs are not used on a daily basis, and therefore the actual period of exposure will probably exceed the period calculated based on the pharmacy data, we also included use during the past 14 days and 30 days as potentially exposed cases in sensitivity analyses. To exclude confounding by contra-indication we censored every participant at the first case in a sensitivity analysis. Participants receiving medication from other sources than the pharmacy, such as nursing home residents, might introduce information bias. Therefore we did a sensitivity analysis excluding participants without pharmacy prescriptions during the last 90 days because nursing home residents do not obtain medication through a community

pharmacy. Furthermore, we performed a sensitivity analysis for *Campylobacter* species and *Campylobacter* or *Salmonella* species only. Different analyses were stratified on sex.

Additional analysis

We also analysed the exposure of PPIs in a nested case-control analysis to assess the association between PPIs and gastrointestinal infections in the total population of The Rotterdam Study. The use of PPIs was used as a time-varying determinant of exposure as previously described[28]. To use the total population of The Rotterdam Study every case (participant with a positive stool sample) was matched to every other participant alive and eligible at the same calendar date (day). Each time a case was identified, exposure in this case was compared with exposure in the other participants (cases might become controls, and controls might become cases). The model was adjusted for age and sex and additionally for covariables used in the final model.

A two-sided p-value below 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Somers, NY, USA).

Results

During the study period, 1329 stool cultures of participants of The Rotterdam Study were identified at Star-MDC. We excluded 30 stool samples because of isolation of a parasite, resulting in 1299 stool samples for the study. A bacterial microorganism was isolated in 125 samples, whereas 1174 samples were culture negative. In total 105 (84.0%) *Campylobacter* species, 16 (12.8%) *Salmonella* species, 3 (2.4%) Yersinia species and 1 (0.8%) *Shigella* sonnei were isolated. All 125 positive stool samples were collected from 118 different participants and all 1174 negative stool samples from 903 different participants. The percentage of missing data of BMI was 7.6% and of dietary data 28.6%. Characteristics of cases and controls are shown in **table 1**.

Model design

For the generalized estimating equations, the independent working correlation structure had the best fit. Designing the final model, past use of H2-receptor antagonists increased the point estimate (β) of current PPI use, adjusted for age and sex, by more than 10%, whereas current use of chronic medication, all decreased the effect by more than 10%. We included age, sex, cohort, calendar date, past use of proton pump inhibitors, past use of H2-receptor antagonists, and current use of chronic medication in the final model (model 2). In the final model we found that participants with a bacterial gastroenteritis were more likely than control participants to be current users of PPIs, with an aOR of 1.94 (95% CI 1.15-3.25) [**table 2**].

Table 1 *Baseline characteristics of participants with stool sample*

	Participants with positive stool sample	Participants with negative stool sample
Total	118*	903
Age - yr (SD)	65.1 ±10.3	68.1 ±12.8
Male sex – no. (%)	49 (41.5)	301 (33.3)
Cohort (%)		
– I	34 (28.8)	325 (36.0)
– II	35 (29.7)	227 (25.1)
– III	49 (41.5)	351 (38.9)
Household alone – no. (%)	20 (16.9)	229 (25.4)
BMI‡ (SD)	25.2 ±4.2	24.5 ±4.3
Use of proton pump inhibitors – no. (%)	43 (36.4)	242 (26.8)
– Current use for 14 days or more	32 (27.1)	152 (16.8)
– Including use during past 14 days	50 (42.4)	269 (29.8)
– Including use during past 30 days	55 (46.6)	288 (31.9)
– >1 Defined Daily Dose/day	13 (30.2)	76 (31.4)
– Past use only	33 (28.0)	289 (32.0)
Medication use – no. (%)		
– H2-receptor antagonists	3 (2.5)	22 (2.4)
– H2-receptor antagonists – past use	37 (31.4)	312 (34.6)
– Antidiabetic medication	12 (10.2)	93 (10.3)
– Antihypertensive medication	50 (42.4)	316 (35.0)
– Statins	27 (22.9)	151 (16.7)
– Antidiabetic, antihypertensive medication or statins	55 (46.6)	376 (41.6)
– Intestinal anti-inflammatory agents	1 (0.8)	4 (0.4)
– Corticosteroids	3 (2.5)	15 (1.7)
– Immunosuppressant medication	0	6 (0.7)
Dietary – no. (%)		
– No meat consumer†	2 (2.4)	6 (0.7)
– No red meat consumerΠ	2 (2.4)	11 (1.2)
– No chicken consumerΠ	15 (18.3)	105 (11.6)
– No egg consumerΠ	5 (6.1)	48 (5.3)
– Alcohol$	72 (61.0)	558 (61.8)
– Dietary – gram/days (SD)		
– Meat†	110.9 ±60.4	103.3 ±54.4
– Red meatΠ	91.7 ±55.0	85.5 ±50.0
– ChickenΠ	17.1 ±18.1	16.4 ±16.5
– EggsΠ	15.0 ±11.1	15.7 ±12.9
– Alcohol$	13.0 ±15.7	13.4 ±15.2

* out of total 125 positive isolates
† Patients with positive stool sample N = 82, negative stool sample N = 644
‡ Patients with positive stool sample N = 111, negative stool sample N = 839
Π Patients with positive stool sample N = 82, negative stool sample N = 646
$ Patients with positive stool sample N = 93, negative stool sample N = 712

Table 2 *Association of use of proton pump inhibitors with bacterial gastrointestinal infections Generalized estimating equations method , negative stool cultures as control group*

	Number of cases	Number in cohort	Use of PPI (%)	Cases N = 125 OR (95% CI)	P value
Current use of proton pump inhibitor:					
Univariate analysis	125	1299	375 (28.9)	1.50 (1.01 ; 2.23)	0.047
Adjusted analysis – model 1	125	1299	375 (28.9)	1.62 (1.09 ; 2.43)	0.018
Adjusted analysis – model 2	125	1299	375 (28.9)	1.94 (1.15 ; 3.25)	0.013
Sensitivity analyses – model 2:					
Only including current use for 14 days or more	125	1299	242 (18.2)	1.99 (1.19 : 3.35)	0.009
Including use during past 14 days	125	1299	422 (32.5)	2.14 (1.35 : 3.38)	0.001
Including use during past 30 days	125	1299	453 (34.9)	2.28 (1.46 ; 3.55)	<0.001
Censored at first case	118	1246	353 (28.3)	2.02 (1.19 ; 3.42)	0.009
Excluding no medication for >90 days	124	1223	375 (30.7)	1.78 (1.05 ; 3.01)	0.032
Campylobacter only	105	1279	368 (28.8)	1.93 (1.11 ; 3.36)	0.019
Campylobacter and Salmonella	121	1295	375 (29.0)	2.05 (1.20 ; 3.49)	0.008
Male only	53	436	131 (30.0)	3.28 (1.44 ; 7.49)	0.005
Female only	72	863	244 (28.3)	1.31 (0.66 ; 2.60)	0.45

Model 1: adjusted for sex, age

Model 2: adjusted for sex, age, cohort, calendar date, past use of proton pump inhibitors, current use of chronic medication, past use of H2-receptor antagonists

Sensitivity analyses

Different sensitivity analyses did not result in a significant change of the effect [**table 2**]. A sensitivity analysis including use during the past 14 days and 30 days resulted in aORs of 2.14 (95% CI 1.35-3.38) and 2.28 (95% CI 1.46-3.55), respectively. Excluding current use of PPIs for 14 days or more, to exclude confounding by indication or protopathic bias, resulted in an aOR of 1.99 (95% CI 1.19-3.35). Censoring at the first case, to exclude confounding by contra-indication, resulted in an aOR of 2.02 (95% CI 1.19-3.42) and excluding participants without prescriptions during the last 90 days in an aOR of 1.78 (95% CI 1.05-3.01). An aOR of 1.93 (95% CI 1.11-3.36) and 2.05 (95% CI 1.20-3.49) was observed in sensitivity analyses for including only *Campylobacter* and *Campylobacter* or *Salmonella*, respectively (table 2). After stratifying on sex a difference was observed between male (aOR 3.28; 95% CI 1.44-7.49) and female (aOR 1.31; 95% CI 0.66 ; 2.60) participants [**table 2**].

Additional analysis

In a matched case-control analysis, adjusting for the same covariables of the final model, but using all other participants of The Rotterdam Study as control group, a consider-

ably higher aOR of 6.14 (95% CI 3.81-9.91) was observed compared to the GEE analysis using negative stool samples as control group [**table 3**].

Table 3 *Association of use of proton pump inhibitors with bacterial gastrointestinal infections.Nested case-control analysis – all other participants of The Rotterdam Study as control group*

			Total cohort N = 12515		
	Number of cases	**Number in cohort**	**Use of PPI (%)**	**Infections N = 125 OR (95% CI)**	**P value**
Current use of proton pump inhibitor					
Univariate analysis	125	12515	11.7*	3.35 (2.31;4.87)	<0.001
Adjusted analysis – model1	125	12515	11.7*	4.03 (2.77;5.87)	<0.001
Adjusted analysis – model2	125	12515	11.7*	6.14 (3.81;9.91)	<0.001
Male only – model2	53	5113	10.2†	11.07(5.51;22.24)	<0.001
Female only – model2	72	7402	12.7‡	3.83 (1.96;7.47)	<0.001

Model 1: adjusted for sex, age
Model 2: adjusted for sex, age, cohort, calendar date, past use of proton pump inhibitors, current use of chronic medication, past use of H2-receptor antagonists
* percentage over 125 strata
† percentage over 53 strata
‡ percentage over 72 strata

Discussion

PPIs have been associated with an increased risk of bacterial gastroenteritis in previous studies[1-9]. In this population based cohort study we found that current PPI therapy was associated with a strongly increased risk of bacterial gastroenteritis, with an aOR of more than six. However, this risk decreased to 1.94 after restriction to the subgroup with stool samples. Therefore, we suspect that information bias inflated the risks in other population based studies which have shown an association between PPI therapy and an increased risk of *Campylobacter* and *Salmonella* infections before. Although, people using PPIs still should be careful consuming food which could be contaminated, such as beef, poultry or processed food, the risk of gastroenteritis is probably less than previously assumed.

One of the strengths of our study is that we tried to use a comparable control group by only using participants with negative stool samples. In most of the previous population based studies the magnitude of the effect may have been overestimated as a result of the use of incomparable control groups. Typically, case control studies regarding use of PPIs may suffer from a “healthy control” bias. We further tried to avoid healthy control

bias by correcting for the use of chronic medication. Some of the previous studies used a random sample of population registries, or volunteering friends or relatives of the cases as control group[2,4-6]. These participants are probably healthier and will therefore differ considerably from the cases. But also commonly used strategies, such as using matched controls, obtained from general practitioner databases, will not prevent information bias[1]. Studies considering self-limiting diseases such as gastroenteritis, might select a "help seeking" and therefore biased population. As a consequence, people using a PPI will be more inclined to consult medical help in case of gastroenteritis compared to randomly selected controls, even if they are matched.

Residual confounding might also have influenced previous study results, because dietary pattern, which has been shown to be the most important risk factors for *Campylobacter* and *Salmonella* infections has not been included in these studies[29,30]. An association with dietary data was not observed in our study. Unfortunately, the number of missing dietary data in our study was rather high with as a consequence a large number of imputed data.

Observational studies may always suffer from bias and residual confounding. We had no data on other important risk factors for bacterial gastroenteritis, such as foreign travelling, eating in a restaurant, or contact with animals[29,30].

We believe we used a comparable control group using participants with negative stool samples. However, results from a test-negative study design may also underestimate the risk, since a number of the controls with negative stool cultures may be false-negatives. They can be false-negative either because of low diagnostic sensitivity against the four bacterial species (e.g., too little material received, long transportation time, stool collected many days after onset of gastroenteritis) or because the patient suffered from other causes of bacterial gastroenteritis (diarrheagenic *Escherichia coli* or *Clostridium difficile*).

Furthermore, in elderly gastric acid secretion is impaired compared to younger aged individuals[31]. If gastric acid secretion is already impaired, PPIs will have a smaller effect. Therefore, the smaller risk estimate in our study might be explained by the fact that our population mainly consisted of elderly, in which gastric acid secretion is already impaired.

Because *Salmonella* species are less susceptible to pH, the association between PPI therapy and gastroenteritis might be smaller for *Salmonella* species[19]. Unfortunately, however, the number of *Salmonella* infections in this study was too small to draw conclusions on this association.

During the study period the method of detection of enteric pathogens changed by the introduction of PCR, resulting in an increased sensitivity. To correct for an increased risk of false negative stool samples in the earlier years of the study, we included calendar date and cohort in the assessment of model design.

Although male gender has been shown to be an independent risk factor for bacterial gastroenteritis, it has not been shown before that the association between PPI therapy and bacterial gastroenteritis is much higher for males[32]. We were unable to explain this result by other covariables. Possibly there are unmeasured (hygiene related) behavioural aspects to explain this difference. In experimental studies a gender difference was observed between male and female neutrophils. Male neutrophils show higher responsiveness to stimulation with lipopolysaccharide and interferon-Ɣ[33]. Neutrophils play an important role in *Salmonella* and *Campylobacter* infections[22,23]. The harmful effect of PPIs may therefore be greater for male than for female subjects. Of course, studies are needed to test this rather speculative hypothesis.

In conclusion, current PPI therapy was associated with an increased risk of bacterial gastroenteritis. We demonstrated that the effect is lower than previously assumed, by reducing the risk of information bias in our study design.

Potential conflicts of interest

All authors report no potential conflicts.

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CHAPTER 3

International travel and acquisition of multidrug-resistant *Enterobacteriaceae*: a systematic review

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Robert-Jan Hassing

Jelmer Alsma

Maris S. Arcilla

Perry J. van Genderen

Bruno H. Stricker

Annelies Verbon

Abstract

International travel is considered to be an important risk factor for acquisition of multi-drug-resistant *Enterobacteriaceae* (MRE). The aim of this systematic review was to determine the effect of international travel on the risk of post-travel faecal carriage of MRE. Secondary outcomes were risk factors for acquisition of MRE. A systematic search for relevant literature in seven international databases was conducted according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Articles needed to report on (I) foreign travel, (II) screening in asymptomatic participants, (III) antimicrobial susceptibility data and (IV) faecal *Enterobacteriaceae* carriage. Two researchers independently screened the abstracts, assessed the full article texts for eligibility and selected or rejected them for inclusion in the systematic review. In case of disagreement, a third researcher decided on inclusion. Eleven studies were identified. In all studies, a high prevalence (>20%) of carriage of MRE after international travel was found. The highest prevalence was observed in travellers returning from Southern Asia. Foreign travel was associated with an increased risk of carriage of MRE. Further research is needed to assess if this leads to an increase in the number of infections with MRE. Systematic review registration number: PROSPERO CRD42015024973.

Introduction

Rationale

Worldwide, the number of international travellers has grown from 25 million in 1950 to 1087 million in 2013[1]. According to the World Tourism Organization, this number is expected to increase by an average of 3.3% a year[1]. Of the international travellers visiting the developing countries, 22-64% have self-reported health problems, and about 8% require medical care during or after travel[2,3]. Healthy travellers may be exposed to a broad range of microorganisms while travelling, including drug-resistant *Enterobacteriaceae*, which may subsequently be introduced into their home country[4,5].

Enterobacteriaceae are Gram-negative bacteria that are part of the human body's normal commensal flora, called microbiota. *Enterobacteriaceae*, such as *Escherichia coli* and *Klebsiella* species, are capable of causing both healthcare-associated, and community-acquired infections[6]. Multidrug-resistant *Enterobacteriaceae* (MRE), including extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* (ESBL-E) and plasmid-mediated Amp-C producing *Enterobacteriaceae* (pAmp C-E) are emerging worldwide[7]. Cases of carbapenemase-producing Enterobacteriaceae (CPE) are also reported more frequently[8].

Since 2003, community carriage rates of MRE have increased dramatically in various regions, such as South-East Asia, the Western Pacific and the Eastern Mediterranean[7]. During visits to such areas, travellers might acquire MRE and become asymptomatic carriers of MRE. In their home country, they may cause spread in the community and contribute to worldwide emerging antimicrobial resistance[6,9,10]. Acquired MRE in the digestive tract are considered apathogenic, however carriage of such *Enterobacteriaceae* have resulted in clinically relevant infections[8]. International travel has been reported as a risk factor for urinary tract infections caused by ESBL-E[11,12]. The question arises if these observations warrant clinicians being aware for MRE in recently returned otherwise healthy, international travellers who seek medical attention even for unrelated conditions.

Objectives

The aim of this systematic review was to determine the effect of international travel on the risk of acquisition of faecal carriage of MRE. A secondary objective was to determine risk factors for acquisition of drug resistance.

Methods

Protocol and registration

A specific protocol was designed and used to conduct the study. The study is registered in the International prospective register of systematic reviews (PROSPERO) under registration number CRD42015024973.

Search strategy and selection criteria

The systematic review was conducted according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[13]. The following databases were searched, attempting to identify all relevant studies: Embase, MEDLINE, Web of Science, Scopus, Cochrane Library, PubMed publisher and Google Scholar. The latest search was conducted on 17 August 2015.

The topic search terms used for searching the databases were "Gram negative bacteria", "Gram negative bacterial infections", "Enterobacteriaceae", "Escherichia", "Klebsiella", "Campylobacter", "Salmonella", "Shigella", "Yersinia", "travel", "traveller", "tourist", "tourism", "turista", "aviation", "air transport", "airport", "colonisation", "carriage", "carrier", "susceptibility" and "(multiple) drug resistance".

The queries differed per database searched and were developed with help of a biomedical information specialist (Supplement 1). Articles written in English, German, French and Dutch were included.

For inclusion the article needed to fulfil the following criteria: (1) it needed to be related to foreign travel (2), report on screening in asymptomatic participants (3), present antimicrobial susceptibility data and (4) report on faecal *Enterobacteriaceae* carriage. We used the following exclusion criteria: case reports, reviews, meta-analysis, veterinary medicine, in vitro studies, and studies regarding symptomatic patients. The reference list of reviews were screened to identify studies possibly missed by the search.

Two researchers (R.H. and J.A.) independently performed the screening of the abstracts. Any discordant result was discussed in consensus meetings. After screening the abstracts, the full text of the articles was assessed for eligibility by the same two researchers and selected or rejected for inclusion in the systematic review. In case of disagreement a third researcher (A.V.) decided on inclusion.

Data collection process

The following data (if available) were extracted from each article: year of publication, country of the study, study period, study design, microorganism studied, study population, study size, age, sex, sample time before and after travel, duration of travel, travelling in pairs or groups, symptoms during travel, countries visited, MRE prevalence before travel, MRE prevalence after travel, MRE resistance acquired during travel, resistance to other antibiotic drugs of acquired MRE, risk factors for acquisition (among which travel to predefined United Nations geographical region: southern Asia, Asia except southern Asia, Africa, South and Central America, North America, Europe and Oceania[14]), method of MRE susceptibility determination, phenotypic approaches, genotypic characterization of post-travel MRE isolates, molecular typing of post-travel MRE isolates, duration

of MRE colonisation and MRE transmission to household contacts. To obtain missing data, authors of the article were contacted.

Quality assessment

We assessed the methodological quality and the risk of bias in individual studies that may affect the cumulative evidence, using tools for assessing quality and susceptibility to bias in observational studies as recommended in the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement[15,16].

Data synthesis and analysis

As a result of the design of the studies (cohort studies) and the heterogeneity in patient populations (e.g. travellers, healthcare workers and healthcare students) a formal meta-analysis was not possible. Therefore, the study results were summarised to describe the main outcomes of interest. The principle summary measure was percentage of MRE acquisition during travel, defined as ESBL-E or pAmp C-E. Furthermore, risk factors for acquisition of drug resistance were assessed. If possible, percentages not presented in the articles were calculated from available data.

Results

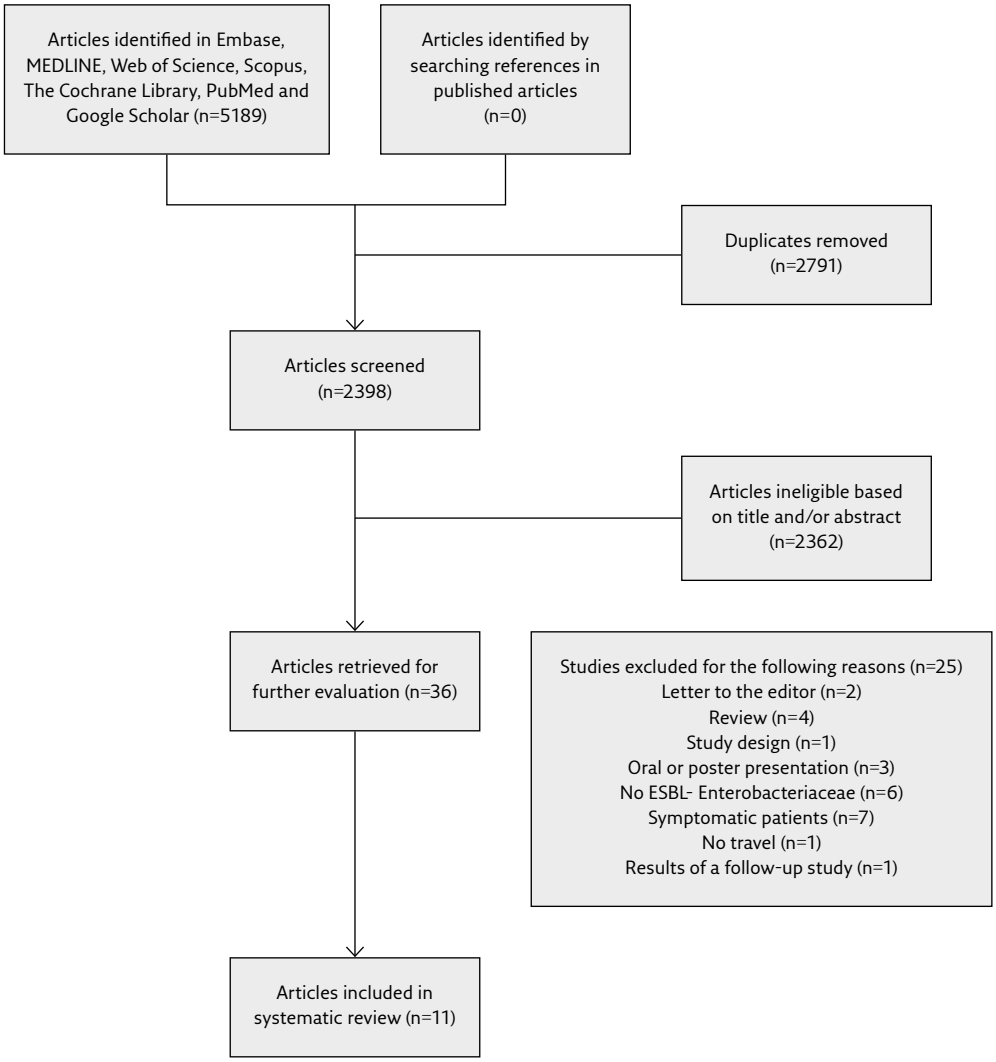
Study selection

A total of 2398 studies were identified through database searching after duplicates had been removed [**figure 1**]. After screening of titles and summaries, 36 articles were selected for full-text assessment. Eleven articles were included in qualitative synthesis of the systematic review [see **figure 1** for reasons for exclusion][17-27].

Study characteristics

Eleven prospective cohort studies, conducted in northern and western Europe, Australia and the United States (US) were included[17-27]. Characteristics of these studies are shown in **table 1**. Nine studies investigated travellers visiting a travel or vaccination clinic, one study hospital staff and contacts, and one study healthcare students working or studying abroad. The number of study participants ranged from 28 to 574. The median age of travellers in the individual studies varied between 25 and 66 years, with the youngest group being healthcare students. In all studies, the majority of travellers were female (range: 55-78%). The proportion of participants who were lost to follow up varied from 3.8% (4/106)[18] to 30% (12/40)[21]. The mean duration of travel was similar in all studies (14–21 days). In the study of Angelin et al. on healthcare students, median length of stay was 45 days (range: 13-365 days)[22]. In four studies, follow-up samples of MRE carriers were collected at six months after returning from travel, and in one of these studies, samples were collected monthly in the first three months with further follow-up until 12

Figure 1 *Flowchart for literature search on the acquisition of multidrug-resistant Enterobacteriaceae in international travel (n=4,989)*



months after return[25]. Ten studies used a phenotypic method for susceptibility testing, with genotypic confirmation of ESBL positivity by PCR[17-22,24-27]. One study used a PCR-based approach[23]. In one study, only isolated *E. coli* were included, whereas the other studies included all isolated *Enterobacteriaceae*, which mainly consisted of *E. coli*[17-27].

Table 1 *Characteristics of prospective cohort studies included for systematic review of the acquisition of multidrug-resistant Enterobacteriaceae in international travel (n=11)*

Study	Country	Study period	Population Characteristics	Study size[a]	Median Age in years (range or SD)	Proportion of women in %
Tängdén[17]	Sweden	November 2007 - 31 January 2009	Travel clinic	100	43 (2-84)	55
Kennedy[18]	Australia	January 2008 – April 2009	Hospital staff and contacts	102	45 (17-77)	62
Östholm-Balkhed[19]	Sweden	September 2008 - April 2009	Vaccination clinic	231	54 (18-76)	59
Kantele[20]	Finland	March 2009 - February 2010	Travel clinic	430	40 (0-77)	61
Weisenberg[21]	United States	July 2009 - February 2010	Travel clinic	28	66 (41-83)	68
Angelin[22]	Sweden	April 2010 - January 2014	Healthcare students	99	25 (20-15)	78
von Wintersdorff[23]	The Netherlands	November 2010 - August 2012	Travel clinic	122	43 (18-72)	58
Paltansing[24]	The Netherlands	March 2011 - September 2011	Travel clinic	370	33 (19-82)	63
Ruppé[25]	France	February 2012 - April 2013	Vaccination Centres	574	36 (SD 13)	61
Kuenzli[26]	Switzerland	December 2012 - October 2013	Travel clinic	170	41 (30-53)	56
Lübbert[27]	Germany	May 2013 – April 2014	Travel clinic	205	34 (3-76)	57

E.coli: Escherichia coli; MRE: multidrug-resistant *Enterobacteriaceae*; SD: standard deviation.

a Number of travellers who provided pre- and post-travel swab.

b Data of MRE-positive isolates newly acquired during travel.

c Data of MRE-positive isolates post-travel.

d Healthcare students, median duration of stay.

Identification of MRE-positive organisms in post-travel isolates	Sample method used	Sample time (range) before/ after travel	Mean duration of travel in days (range)	Total number of co-travellers participating in study	Follow-up of resistant isolates
Enterobacteriaceae 100% (24/24) *E.coli*	Stool	Unknown	14 (1-26)	23	6 months
E.coli	Rectal or perianal swab	Within 2 weeks before and after	21 (9-135)	Unknown	6 months
Enterobacteriaceae 90% (104/116) *E.coli*[b]	Stool sample	15 (1-114) days / 3 (0-191) days	16 (4-119)	Unknown	None
Enterobacteriaceae 97% (94/97) *E. coli*[b]	Stool sample	Before and first (or second) stool after	19 (4-133)	83	None
Enterobacteriaceae 100% (7/7) *E. coli*[b]	Stool sample	1 week before / 1 week after	16 (8-24)	Unknown	None
Enterobacteriaceae 100% (36/36)) *E. coli*[c]	Stool sample	Close to departure / 1 to 2 weeks after returning	45 (13-365)[d]	Unknown	None
Not done	Stool sample	Before and immediately after	21 (5-240)	Unknown	None
Enterobacteriaceae 92% (146/158) *E. coli*[c]	Rectal swab	Immediately before and after	21 (6-90)	None	6 months
Enterobacteriaceae 93% (491/526) *E. coli*[b]	Stool sample	Within 1 week before and after	20 (15-30)	None	12 months
Enterobacteriaceae 98% (157/161) *E. coli*[b]	Rectal swab	1 week before / directly after	18 (5-35)	Unknown	None
Enterobacteriaceae 92% (58/63) *E. coli*[b]	Stool sample	Before/ within 1 week after	21 (3-218)	22	6 months

Table 2 *Risk of multi-drug-resistant Enterobacteriaceae in travellers (n=11 studies)*

Study	Method of MRE determination	Phenotypic approaches	Results genotypic characterisation post-travel MRE isolates	Results molecular typing of post-travel MRE isolates
Tängdén[17]	Phenotypic approach with genotypic confirmation by PCR	Enrichment broth, selective media, AST: Etest, MRE confirmation: disc diffusion	TEM (n=11) , SHV (n=3), CTX-M group 1 (n=14) of which CTX-M-15 (n=13), CTX-M-1 (n=1), CTX-M group 4 (n=10) of which CTX-M-9 (n=3), CTX-M-14 (n=5), CTX-M-27 (n=2)[b]	No data
Kennedy[18]	Phenotypic approach with genotypic confirmation by PCR	Enrichment broth, selective media, AST: Vitek2, MRE confirmation: disc diffusion	TEM or SHV (n=4), CTX-M group 1 (n=12), CTX-M group 9 (n=6), and pAmp C genes (n=4)[d]	No data
Östholm-Balkhed[19]	Phenotypic approach with genotypic confirmation by PCR	Selective media, AST: Etest, MRE confirmation: Etest	TEM-19 (n=1), SHV (n=6), CTX-M-15-like (n=36), CTX-M-14-like (n=36), CTX-M-27-like (n=5), CTX-M-53-like (n=5), CTX-M-1/61 like (n=3), CTX-M-2 like (n=2), CTX-M-3-like (n=1), pAmpC genes (n=15), no genes detected (n=13)[b]	No data
Kantele[20]	Phenotypic approach with genotypic confirmation by PCR	Selective media, AST: Vitek2, MRE confirmation: disc diffusion	79% CTX-M-type (CTX-M-1 and CTX-M-9 most prevalent), other common strains TEM and OXA (data not published)[b]	No data
Weisenberg[21]	Phenotypic approach with genotypic confirmation by PCR	Selective media, AST: Vitek2, MRE confirmation: disc diffusion	SHV-12 (n=1), CTX-M-14 (n=3), CTX-M-15 (n=2), no gene detected (n=1)[b]	MLST typing 7 multidrug-resistant *E. coli* isolates: ST 39, 8 (n=2), 37, 399, 437, 83
Angelin[22]	Phenotypic approach for detection of ESBL, pAmp C and phenotypic approach with genotypic characterization for detection of OXA-48/OXA-181	Selective media, AST: disc diffusion, MRE confirmation: Etest (ESBL), disc diffusion (pAmpC)	No data	No data
von Wintersdorff[23]	Metagenomic approach (detection $bla_{CTX\text{-}M}$)	No data	blaCTX-M (n=41)[d]	No data

MRE prevalence pre-travel % (ratio)	MRE prevalence post-travel % (ratio)	New MRE acquisition during travel % (ratio)[a]	Persistent newly acquired MRE carriage 6 months after travel % (ratio)	Results univariate /multivariable risk factor analysis for MRE acquisition	MRE in non-travelling household contacts % (ratio)
1 (1/105)	No data	24 (24/100)	24 (5/21)	Gastroenteritis travel to India[c]	No data
2 (2/106)	22 (22/102)	21 (21/100)	6 (1/18)	Gastroenteritis Use of antibiotics travelling to Asia, South America and/or Middle East/Africa[e,c]	No data
2 (6/251)	31 (72/231)	30 (68/226)	No data	Age Diarrhoea or other gastrointestinal symptoms, travel to Asia, Africa (north of equator), Indian subcontinent[f]	No data
1 (5/430)	22 (93/430)	21 (90/430)	No data	Traveller's diarrhoea, age, use of antibiotics for traveller's diarrhoea[f]	No data
4 (1/28)	25 (7/28)	26 (7/27)	No data	No data	No data
7 (7/99)	36 (36/99)	35 (35/99)	No data	Travel to the South-East Asia region (India, Nepal, Vietnam, Indonesia, Sri Lanka), antibiotic treatment during travel[g]	No data
9 (11/122)	34 (41/122)	32 (36/111)	No data	Travel to Indian subcontinent[f]	No data

Table 2 *(sequel)*

Study	Method of MRE determination	Phenotypic approaches	Results genotypic characterisation post-travel MRE isolates	Results molecular typing of post-travel MRE isolates
Paltansing[24]	Phenotypic approach with genotypic characterisation by microarray	Enrichment broth, selective media, AST: Vitek2, MRE confirmation: disc diffusion	SHV (n=1), CTX-M group 1 (n=110) of which CTX-M-1-like (n=4), CTX-M-3-like (n=1), CTX-M-15-like (n=85), CTX-M-32-like (n=20), CTX-M-group 9 (n=42), CTX-M-group 2 (n=2), CTX-M-group 8/25 (n=1), pAmpC genes (n=3)[d]	MLST typing: 146 multidrug-resistant *E. coli* isolates: most prevalent ST 38 (n=17), ST10 (n=10), ST131 (n=9)
Ruppé[25 h]	Phenotypic approach with genotypic confirmation by PCR	Enrichment broth, selective media, AST: disc diffusion	Predominant CTX-M-type (95.4%) among which CTX-M-group 1 predominated (83.7% of all CTX-M), OXA-181 (N=2), NDM-1 (n=1)[b]	No data
Kuenzli[26]	Phenotypic approach with genotypic screening by microarray and confirmation by PCR/DNA sequence analysis	Enrichment broth, selective media, AST: Vitek2, MIC for meropenem and ertapenem: Etest, MRE confirmation: disc diffusion, modified Hodge test	TEM-1-like (n=33), SHV238S/240K (n=7), SHV238S (n=1), SHV-5/12-like (n=1), SHV-2/3-like (n=1), CTX-M-15-like (n=48), CTX-M group 9 (n=1), CTX-M group 1 (n=24), predominant ESBL gene was CTX-M-15 (80 representative E. coli isolates analysed), NDM-1 (n=1)[b]	80 representative *E. coli* isolates analysed by rep-PCR: not clonally related. MLST performed on 34 randomly selected *E. coli* isolates: only 3 pandemic strains found (ST131 n=2; ST648 n=1)
Lübbert[27]	Phenotypic approach with genotypic confirmation by PCR	Selective media,AST: microbroth dilution method, MRE confirmation:E-test	SHV-12 (n=1), CTX-M group 1 (n=37), of which CTX-M-15 (N=33), CTX-M-55 (n=4), CTX-M group 9 (n=19) of which CTX-M-14 (n=9), CTX-M-27 (n=1), CTX-M-65 (n=1)[b]	

AST: antibiotic susceptibility testing; bla: beta-lactamase; CTX-M: cefotaximase; *E. coli: Escherichia coli*; ESBL: extended-spectrum beta-lactamase; KPC: Klebsiella pneumoniae carbapenemase; MLST: Multilocus sequence typing; MRE multidrug-resistant *Enterobacteriaceae*; NDM: New Delhi metallo-beta-lactamase; OXA: oxacillinase; pAmp C: plasmid-borne AMpC; PCR: polymerase chain reaction; PFGE: pulsed-field gel electrophoresis; rep-PCR: repetitive extragenic palindromic PCR; SHV: Sulphydryl variable; TEM: Temoniera.

MRE prevalence pre-travel % (ratio)	MRE prevalence post-travel % (ratio)	New MRE acquisition during travel % (ratio)[a]	Persistent newly acquired MRE carriage 6 months after travel % (ratio)	Results univariate /multivariable risk factor analysis for MRE acquisition	MRE in non-travelling household contacts % (ratio)
9 (32/370)	36 (133/370)	33 (113/338)	17 (19/113)	Travel to South or East Asia[f]	18 (2/11)
12 (81/700)	No data	51 (292/574)	After 1 month 34 (83/245), after 2 months 19 (45/236), after 3 months 10 (24/233), after 6 months 5 (11/230), after 12 months 2 (5/227)	Travel to Asia or sub-Saharan Africa, beta-lactam use during travel, diarrhoea during travel, type of travel[f]	No data
3 (5/175)	No data	70 (118/170)	No data	Travel to India, Bhutan, or Nepal, visiting friends and relatives, consumption of ice cream and pastry, Length of stay[f]	No data
7 (14/205)	31 (63/205)	30 (58/191)	9 (3/35)	Travel to India or South-East Asia, Gastroenteritis[c]	No data

a Percentage of MRE- positive post-travel samples in those travellers whose pre-travel sample was MRE-negative.
b Acquired genes detected in post-travel MRE isolates.
c Univariate statistics.
d Prevalent genes detected in post-travel MRE isolates.
e Risk factors for resistance to gentamicin, ciprofloxacin and/or third generation cephalosporins.
f Multivariable logistic regression analysis; participants ESBL-positive before travel were excluded.
g Binary regression analysis.
h Carbapenemase-positive isolates were included in the definition MRE.

Table 3 *Proportion of travellers who acquired multidrug-resistant Enterobacteriaceae, by travel destination (n=11 studies)*

Study	Southern Asia % (ratio)	Asia except southern Asia % (ratio)	Northern Africa % (ratio)	Sub-Saharan Africa % (ratio)
Tängdén[17 a,b]	78 (7/9)	29 (10/34)	33 (4/12)	4 (1/23)
Kennedy[18 a,c]	57 (8/14)	25 (21/85)	33 (1/3)	0 (0/2)
Östholm-Balkhed[19 a,b]	71 (10/14)	43 (26/60)	57 (17/30)	21 (15/71)
Kantele[20 b,d]	46 (28/61)	32 (37/116)	67 (2/3)	12 (23/193)
Weisenberg[21 b]	29 (2/7)	25 (1/4)	33 (1/3)	13 (1/8)
Angelin[22]	63 (25/40)	67 (6/9)	-	10 (4/40)
von Wintersdorff[23 c]	58 (18/31)	20 (6/29)	31 (5/16)	29 (5/17)
Paltansing[24 b,e]	72 (18/25)	41 (62/161)	40 (4/10)	24 (20/82)
Ruppé[25 f]	88 (53/60)	66 (61/93)	No data	49 (89/182)
Kuenzli[26 b]	69 (118/170)	No data	No data	No data
Lübbert[27 a,b]	72 (13/18)[g]	33 (24/73)[g]	No data	24 (19/78)

MRE: multidrug-resistant Enterobacteriaceae.

a Travellers visiting more than one region are categorised in all the visited geographical regions.

b Study reports data on MRE acquisition in travellers.

c Study reports data on MRE prevalence in travellers.

d Travellers visiting more than one region are categorised in the geographical region with the longest stay for this study.

e One traveller who visited Iran is categorised in Asia instead of Southern Asia.

f 42 travellers visited more than one country in Asia and may be represented in more than one column in the Table; 28 of them acquired MRE.

g Exact numbers unpublished.

Southern Asia: Afghanistan, Bangladesh, Bhutan, India, Islamic Republic of Iran, Maldives, Nepal, Pakistan, Sri Lanka.
Asia (without southern Asia) Armenia, Azerbaijan, Bahrain, Brunei, Cambodia, China, Cyprus, Darussalam, Democratic People's Republic of Korea, Georgia, Hong Kong, Indonesia, Iraq, Israel, Jordan, Japan, Kazakhstan, Kuwait, Kyrgyzstan, Laos, Lebanon, Mongolia, Malaysia, Myanmar, North Korea, Oman, Philippines, Qatar, Republic of Korea, Saudi Arabia, South Korea, Singapore, Palestine, Syrian, Tajikistan, Thailand, Timor-Leste, Turkey, Turkmenistan, United Arab Emirates, Uzbekistan, Viet Nam, Yemen.
Northern Africa: Algeria, Egypt, Libya, Morocco, Sudan, Tunisia, Western Sahara.

South and Central America % (ratio)	North America % (ratio)	Europe % (ratio)	Oceania % (ratio)
0 (0/7)	0 (0/2)	13 (2/16)	No data
20 (1/5)	20 (2/10)	14 (3/21)	0 (0/2)
16 (5/31)	0 (0/15)	0 (0/15)	No data
0 (0/40)	0 (0/2)	0 (0/15)	No data
33 (2/6)	No data	No data	No data
0 (0/5)	0 (0/4)	No data	No data
0 (0/10)	No data	17 (1/6)	No data
15 (9/60)	No data	No data	No data
31 (48/155)	No data	No data	0 (0/2)
No data	No data	No data	No data
8 (6/78)	0 (0/2)	20 (2/10)	No data

Sub-Saharan Africa: Angola, Benin, Botswana, Burkina Faso, Burundi, Cameroon, Cape Verde, Central African Republic, Chad, Comoros, Congo (Brazzaville), Côte d'Ivoire, Democratic Republic of the Congo, Djibouti, Equatorial Guinea, Eritrea, Ethiopia, Gabon, Ghana, Guinea, Guinea-Bissau, Kenya, Lesotho, Liberia, Madagascar, Malawi, Mali, Mauritania, Mauritius, Mozambique, Namibia, Niger, Nigeria, Réunion, Rwanda, Sao Tomé and Principe, Senegal, Seychelles, Sierra Leone, Somalia, South Africa, Sudan, Swaziland, Tanzania, The Gambia, Togo, Uganda, Western Sahara, Zambia, Zimbabwe.
South and Central America: Anguilla, Antigua and Barbuda, Argentina, Aruba, Bahamas, Barbados, Belize, Bolivia, Bonaire, Sint Eustatius and Saba, Brazil, British Virgin Islands, Cayman Islands, Chile, Colombia, Costa Rica, Cuba, Curaçao, Dominica, Dominican Republic, Ecuador, El Salvador, Falkland Islands, French Guiana, Grenada, Guadeloupe, Guatemala, Guyana, Haiti, Honduras, Jamaica, Martinique, Mexico, Montserrat, Nicaragua, Panama, Paraguay, Peru, Puerto, Rico, Saint Kitts and Nevis, Saint Lucia, Saint Martin, Saint Vincent and the Grenadines, Saint-Barthélemy, Sint Maarten, Suriname, Trinidad and Tobago, Turks and Caicos Islands, US Virgin Islands, Uruguay, Venezuela
North America: Bermuda, Canada, Greenland, Saint Pierre and Miquelon, United States.
Europe: Åland Islands, Albania, Andorra, Austria, Belarus, Belgium, Bosnia and Herzegovina, Bulgaria, Channel Islands, Croatia, Czech Republic, Denmark, Estonia, Faeroe Islands, Finland, the former Yugoslav Republic of Macedonia, France, Germany, Gibraltar, Greece, the Holy See, Hungary, Iceland, Ireland, Isle of Man, Italy, Latvia, Liechtenstein, Lithuania, Luxembourg, Malta, Monaco, Montenegro, Netherlands, Norway, Poland, Portugal, Moldova, Romania, Russia, San Marino, Serbia, Slovakia, Slovenia, Spain, Svalbard and Jan Mayen, Sweden, Switzerland, Ukraine, United Kingdom.
Oceania: American Samoa, Australia, Cook Islands, Fiji, French Polynesia, Guam, Kiribati, Marshall Islands, Micronesia, Nauru, New Caledonia, New Zealand, Niue, Norfolk Island, Northern Mariana Islands, Palau, Papua New Guinea, Pitcairn Islands, Samoa, Solomon Islands, Tokelau, Tonga, Tuvalu, Vanuatu, Wallis and Futuna.

Acquisition of multidrug-resistant Enterobacteriaceae

Faecal carriage of MRE varied from 1 to 12% before travel and acquisition of MRE from 21% to 51% [**table 2**][17-21, 23-27].

In the study of Kuenzli et al. on travellers to the Indian subcontinent only, a much higher MRE acquisition rate of 69% was demonstrated[26]. The risk of acquisition of MRE varied with geographical region [**table 3**][17-21,23-27]. Travel to southern Asia posed the highest risk (range: 29–88%), followed by other Asian countries (18–67%) and Northern Africa (range: 31-57%). Acquisition of MRE after travelling to sub-Saharan Africa (range: 0–49%) or South and Central America (range: 0–33%) was less frequent, and three studies did not observe any acquisition MRE after travel to South or Central America [**table 3**]. Acquisition of MRE after travel to North America, Europe and Oceania was rare. Results of the genotypic characterisation of MRE isolated after travel are presented in **table 2**, the majority of the genes belonged to the CTX-M type.

Risk factor for acquisition of multidrug-resistant Enterobacteriaceae

Besides travel destinations, other risk factors for acquiring MRE were age, use of antibiotics during travel (beta-lactam use) and gastroenteritis or other gastro-intestinal symptoms [**table 2**]. The study of Kantele et al., designed to study these risk factors as primary outcome, showed that travel diarrhoea (adjusted odds ratio (AOR) 31.0; 95% confidence interval (CI): 2.7-358.1)) and antibiotic therapy for travel diarrhoea (AOR = 3.0; 95% CI: 1.4-6.7) proved to be the most important risk factors for acquiring MRE (20). In the study of Kuenzli et al. in which only travellers to southern Asia were included, risk factors for MRE acquisition were length of stay, visit to family or friend and consumption of ice cream or pastry [**table 2**][26]. Angelin et al. found a significant association for travel to the South-East Asia region (OR = 30; 95% CI: 6.3-147.2), and antibiotic treatment during travel (OR = 5; 95% CI: 1.1-26.2), but found no association with travellers' diarrhoea or patient-related healthcare work[22].

Resistance of multidrug-resistant Enterobacteriaceae to other antibiotic drugs

Resistance of post-travel MRE isolates to various antibiotics was determined in nine studies [**table 4**][17-19,21-24,26, 27]. In the study of Wintersdorff et al., a PCR-based approach was used, therefore it was not possible to determine which microorganism carried the resistance genes[23]. The resistance data to other antibiotic drugs in the study by Kennedy et al. were not part of the publication, but were provided on request[18]. Antimicrobial resistance was high for ciprofloxacin, varying from 31% to 57%, and for cotrimoxazole, varying from 49% to 86%[17-19,21-24,26,27]. Aminoglycoside resistance was high for gentamicin (range: 17-50%) and tobramycin (range: 18-59%) and low for amikacin (range: 2-5%)[17-19,21-24,26,27]. Carbapenemase-producing *Enterobacteriaceae* were observed in four travellers

who had all visited India (in the study by Ruppé et al., two OXA-181 and one New Delhi metallo-beta-lactamase 1 (NDM-1), and in the study of Kuenzli et al., one NDM-1, but this strain was not included in the resistance results)[25,26]. Resistance to nitrofurantoin, colistin and fosfomycin was only analysed in some of the studies [**table 4**][18,19,21-23,26].

Duration of multidrug-resistant Enterobacteriaceae carriage after return, risk factors for a long duration and rate of infection after travel

Five studies analysed MRE carriage six months after travel, and the persistence rate of acquired MRE after six months was 6-24% of travellers [**table 2**][17,18,24,25,27]. Ruppé et al. analysed MRE carriage one, two, three, six and twelve months after travel, showing persistence of carriage of an acquired MRE in 34, 19, 10, 5 and 2%, respectively[25]. Travellers to Asia showed longer carriage of MRE compared with other travel destinations. Carriage of multidrug-resistant *E. coli* had a lower risk for prolonged carriage than other multidrug-resistant species. No other risk factors were found for prolonged carriage of MRE. Eight travellers in this study reported an episode of urinary tract infection after their return, but no microbiological data were availabl[25]. In the study of Tängdén et al., five of 21 travellers remained carriers of MRE after six months. However, none of these participants reported clinical infections[17]. In the study of Kennedy et al., one person developed a urinary tract infection with a travel-related organism[18]. Kantele et al. performed a one-year laboratory-based follow-up and did not find any clinical samples with MRE[20].

Rate of transmission to household members

Only one study screened household contacts for MRE after return of the index traveller. Household contacts were defined as persons who shared the same household with a participant on a regular basis. Two of 11 contacts were found MRE-positive[24]. Both carried a different ESBL-producing *E. coli* based on multilocus sequence typing (MLST) than the associated traveller.

Limitations of the studies

The quality of the studies and the susceptibility of bias between the studies were assessed. In all but one study, participants constituted a non-random sample of the general travelling population[17-21,23-27]. However, Angelin et al. studied healthcare students working or studying abroad[22]. Studies were performed on three different continents. Travel destinations and travel behaviour may differ considerably between different nationalities and age groups. Including co-travellers, as done in all studies except Paltansing et al. and Ruppé et al., can result in similar travel behaviour and therefore, similar risk factors. Overall, the main outcome was not influenced by recall or interviewer bias. For other outcomes such as risk factors, the risk of recall bias or interviewer bias was low because of the use of self-administered questionnaires.

Table 4 *Antibiotic drug resistance of newly acquired multidrug-resistant Enterobacteriaceae in travellers (n=11 studies)*

Study	Ciprofloxacin % (ratio)	Cotrimoxazole % (ratio)	Gentamicin % (ratio)	Amikacin % (ratio)
Tängdén[17 a]	50[b]	79 (19/24)	45[b]	No data
Kennedy[18 c]	55 (12/22)	No data	50 (11/22)	No data
Östholm-Balkhed[19 a]	31 (36/116)	70 (81/116)	41 (48/116)	2 (2/116)
Kantele[20]	No data	No data	No data	No data
Weisenberg[21 a]	43 (3/7)[d]	86 (6/7)	43 (3/7)	No data
Angelin[22]	57 (28/49)	75b	30b	No data
von Wintersdorff[23 e]	37 (45/122) *qnrB* 56 (68/122) *qnrS*	No data	71 (86/122) *aac(6')-aph(2'')*	71 (86/122) *aac(6')-aph(2'')*
Paltansing[24 f]	36	67	35	No data
Ruppé[25]	No data	No data	No data	No data
Kuenzli[26 a]	41 (64/157)	49 (77/157)	No data	5 (7/157)
Lübbert[27 a]	43 (25/58)	83 (48/58)	17 (10/58)	2[b]

bla: beta-lactamase; CPE: carbapenemase-producing Enterobacteriaceae; ESBL: extended-spectrum beta-lactamase.

a Resistance among acquired ESBL-positive isolates detected in pot-travel samples

b Data extracted from bar chart, exact numbers of data unpublished.

c Resistance among prevalent ESBL-positive isolates detected in post-travel samples.

Every study had participants lost to follow-up for post-travel stool samples and follow-up stool samples. Asymptomatic faecal carriage of MRE is probably not related to loss to follow up, therefore, the risk of information bias is small. Ruppé et al. calculated post-travel MRE carriage as those travellers with persisting MRE carriage divided by all travellers with MRE acquisition plus all travellers without MRE post-travel[25]. However, travellers without MRE were not included in the follow-up. As a result, local MRE acqui-

Tobramycin % (ratio)	Carbapenem % (ratio)	Nitrofurantoin % (ratio)	Colistin % (ratio)	Fosfomycin % (ratio)
38[b]	0[b]	0[b]	No data	8[b]
59 (13/22)	No data	No data	No data	No data
46 (53/116)	0 (0/116)	7 (8/116)	No data	3 (3/116)
No data	No data	No data	No data	No data
No data	0 (0/7)	No data	No data	No data
No data	0 (0/49)	2b	No data	No data
71 (86/122) *aac(6')-aph(2'')*	0 (0/122) bla_{NDM}	No data	No data	No data
37	0	29	0	No data
No data	0.6 (3/526)[g]	No data	No data	No data
18 (28/157)	0 (0/157)	2 (3/157)	0 (0/157)	0.6 (1/157)
22[b]	0[b]	No data	0[b]	16[b]

d Percentage of susceptibility to levofloxacin.
e Prevalent resistance genes in faecal samples post-travel.
f Resistance among prevalent ESBL-positive isolates detected in pre- and post-travel samples.
g Three acquired CPE detected in post-travel samples.

sition was not included in the calculated post-travel MRE carriage prevalence. Therefore the true prevalence can be assumed to be higher.

In five studies, travellers visited multiple regions or even continents during their trip[17-20,27]. In these travellers, it was not possible to attribute MRE prevalence or MRE acquisition to a certain geographical region. However, travellers in these studies were included

in the MRE prevalence or MRE acquisition rates of more than one geographical regions, which may have introduced information bias.

Seven studies used stool samples for detection of MRE[17,19-21,23,25,27] and three studies used rectal or perianal swabs for detection of MRE[18,24,26]. This might have influenced detection of MRE carriage.

Discussion

In this systematic review we found a high prevalence of faecal carriage of MRE after international travel. The highest prevalence of MRE was observed in isolates from travellers returning from southern Asia, with up to 88% acquisition of MRE. In addition to the antibiotics not effective against MRE, an alarmingly high prevalence of resistance to other commonly used antibiotics such as cotrimoxazole (49-86%), ciprofloxacin (31-57%) and aminoglycosides (gentamicin 17-71%) was observed in ESBL-positive isolates in travellers in all studies[17-27].

Returning international travellers with MRE may introduce these microorganisms in their home countries. This may cause community-onset infections with MRE in patients without obvious risk factors transmitted by healthy carriers through food or person-to-person contact[9]. Infections caused by MRE are associated with poorer outcome and a higher overall mortality rate than infections caused by susceptible bacteria[28]. In this review, all studies showed an increased prevalence of faecal carriage of ESBL after international travel. It is not possible to evaluate the proportion of travellers who will develop infection with these resistant bacteria. However, studies have demonstrated that international travel is a risk factor associated with developing an infection with an MRE[11,12,29].

Many countries have infection prevention and control guidelines to detect and treat multidrug-resistant organisms (MDROs) including MRE[30]. In countries with low prevalence of MRE, infection prevention and control guidelines mainly include strategies for early identification and isolation strategies for patients recently hospitalised in foreign hospitals[30,31]. Patients with a recent history of travel to MRE-endemic areas but not admitted to healthcare facilities abroad are not normally considered at risk for carriage of MDROs. However, in hospitalised patients with a recent history of travel, increased rates of carriage of MRE have been observed[10,29,30]. Physicians should be aware of the risk that patients with recent travel to areas with high faecal carriage of MRE, as presented in this review, may introduce MRE to the hospital. Routine screening for MRE seems indicated in such patients. Furthermore, empiric antibiotic therapy may fail when an infection

by MRE is not taken into account. Therefore, careful recording of travel history needs to be incorporated in each patient evaluation. As shown in this review, there is also an increased risk of resistance against other antibiotics in travellers with MRE carriage. It is likely that this is caused by multiple genes, each encoding for resistance to different classes of antibiotics, which are often found on the same bacterial mobile genetic element (e.g. a plasmid)[32]. As a result, other antibiotics, such as aminoglycosides, will also fail in many MRE-positive patients.

Of the MDROs, emergence of CPE is most worrisome because of the limited treatment options for these infections. NDM-1-producing Enterobacteriaceae have been found in environmental samples in endemic regions[33]. CPE (NDM-1) in patients from the United Kingdom with a recent history of travelling or medical tourism to India are already an important public health problem[8]. Case reports have also demonstrated acquisition of CPE in travellers without contact with medical healthcare facilities[34,35]. In this review, four travellers from India were carrying a carbapenemase-producing *E. coli*[25,26]. Preliminary results of the Carriage Of Multiresistant Bacteria After Travel (COMBAT) study, a large-scale multicentre longitudinal cohort study conducted in the Netherlands among 2001 travellers, show acquisition of CPE in four travellers[36].

There are, besides the destination of travel, additional risk factors for acquisition of MRE during travel. Antibiotic therapy was found to increase the risk[20,22]. In five studies, traveller's diarrhoea or gastroenteritis were associated with an increased risk of MRE acquisition during travel[17-20,25]. Also, in one study, meticulous hand hygiene or strict consumption of bottled water did not lower the risk of acquiring MRE[22]. Therefore, it is not clear whether hygiene-related travel advice will decrease faecal carriage of MRE. Surprisingly, healthcare-related activities did not pose an increased risk of acquiring MRE in one study[22].

MRE and CPE could also be carried by food. International spread of these bacteria by food supply has been reported[37]. In this review, only one study showed that food consumption (ice cream and pastry) was associated with MRE carriage in travellers to southern Asia, whereas most of the studies did not focus on dietary patterns during travel.

One of the limitations of this review is the recruitment of travellers from travel clinics only, resulting in inclusion of very few travellers with European destinations. European countries such as Greece and Cyprus also are endemic for MRE and popular travel destinations[34]. In addition, travellers visiting their country of origin, especially Morocco and Turkey usually do not ask for a pre-travel consultation, although these countries are endemic for MRE and CPE[34]. It is not clear whether not including these patients may have led to an under- or overestimation of MRE acquisition.

Another limitation is the lack of sufficient data regarding the duration of carriage and the transmission among non-travelling household members. The study by Ruppé et al. suggests that three months after return, MRE carriage is comparable with the baseline prevalence before travelling. However, the study did not include baseline prevalence in the follow-up. The COMBAT study will address some of these questions[38].

Conclusion

International travel is a major risk factor for acquisition of MRE. This risk is particularly high after travelling to (southern) Asia and in persons with travel-related diarrhoea and antibiotic use. Carriage of MRE-positive isolates is also associated with a high risk of resistance to ciprofloxacin, cotrimoxazole and aminoglycosides. Further research is needed to assess duration of carriage, spread to household contacts and whether introduction of MRE results in an increase of MRE infections. Our results, combined with the worldwide emergence of CPE, further stress the importance of infection prevention and control guidelines.

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Supplement I

EMBASE.COM ('Gram negative bacterium'/exp OR 'Gram negative infection'/de OR Enterobacteriaceae/de OR Escherichia/exp OR Klebsiella/exp OR Salmonella/exp OR Shigella/exp OR Yersinia/exp OR 'Enterobacteriaceae infection'/exp OR ('Gram negative' OR Enterobacteri* OR (Enter* NEXT/1 bacteria*) OR Enterobacter* OR Escherichia* OR 'e coli' OR Klebsiella* OR Salmonell* OR Shigell* OR Yersinia*):ab,ti) AND (travel/de OR 'traveller diarrhea'/de OR aviation/exp OR (travel* OR touris* OR turista OR aviation OR 'air transport' OR airport*):ab,ti) AND ('antibiotic resistance'/exp OR 'multidrug resistance'/de OR 'drug resistance'/de OR 'antibiotic sensitivity'/de OR 'bacterial colonization'/exp OR 'bacterium carrier'/de OR (resistan* OR coloni* OR ((antibiotic* OR antimicrob*) NEAR/3 sensitivit*) OR susceptib* OR carriage* OR carrier*):ab,ti) NOT ([animals]/lim NOT [humans]/lim)

MEDLINE (OVIDSP) (exp "Gram-Negative Bacteria"/ OR exp "Gram-Negative Bacterial Infections"/ OR Enterobacteriaceae/ OR exp Escherichia/ OR exp Klebsiella/ OR exp Salmonella/ OR exp Shigella/ OR exp Yersinia/ OR exp "Enterobacteriaceae infections"/ OR ("Gram negative" OR Enterobacteri* OR (Enter* ADJ bacteria*) OR Enterobacter* OR Escherichia* OR "e coli" OR Klebsiella* OR Salmonell* OR Shigell* OR Yersinia*).ab,ti.) AND (travel/ OR "Travel Medicine"/ OR exp aviation/ OR (travel* OR touris* OR turista OR aviation OR "air transport" OR airport*).ab,ti.) AND (exp "Drug Resistance, Microbial"/ OR exp "Drug Resistance, Multiple"/ OR "drug resistance"/ OR "bacterium carrier"/ OR (resistan* OR coloni* OR ((antibiotic* OR antimicrob*) ADJ3 sensitivit*) OR susceptib* OR carriage* OR carrier*). ab,ti.) NOT (exp animals/ NOT humans/)

COCHRANE (('Gram negative' OR Enterobacteri* OR (Enter* NEXT/1 bacteria*) OR Enterobacter* OR Escherichia* OR 'e coli' OR Klebsiella* OR Salmonell* OR Shigell* OR Yersinia*):ab,ti) AND ((travel* OR touris* OR turista OR aviation OR 'air transport' OR airport*):ab,ti) AND ((resistan* OR coloni* OR ((antibiotic* OR antimicrob*) NEAR/3 sensitivit*) OR susceptib* OR carriage* OR carrier*):ab,ti)

WEB-OF-SCIENCE TS=(((("Gram negative" OR Enterobacteri* OR (Enter* NEAR/1 bacteria*) OR Enterobacter* OR Escherichia* OR "e coli" OR Klebsiella* OR Salmonell* OR Shigell* OR Yersinia*)) AND ((travel* OR touris* OR turista OR aviation OR "air transport" OR airport*)) AND ((resistan* OR coloni* OR ((antibiotic* OR antimicrob*) NEAR/3 sensitivit*) OR susceptib* OR carriage* OR carrier*)))

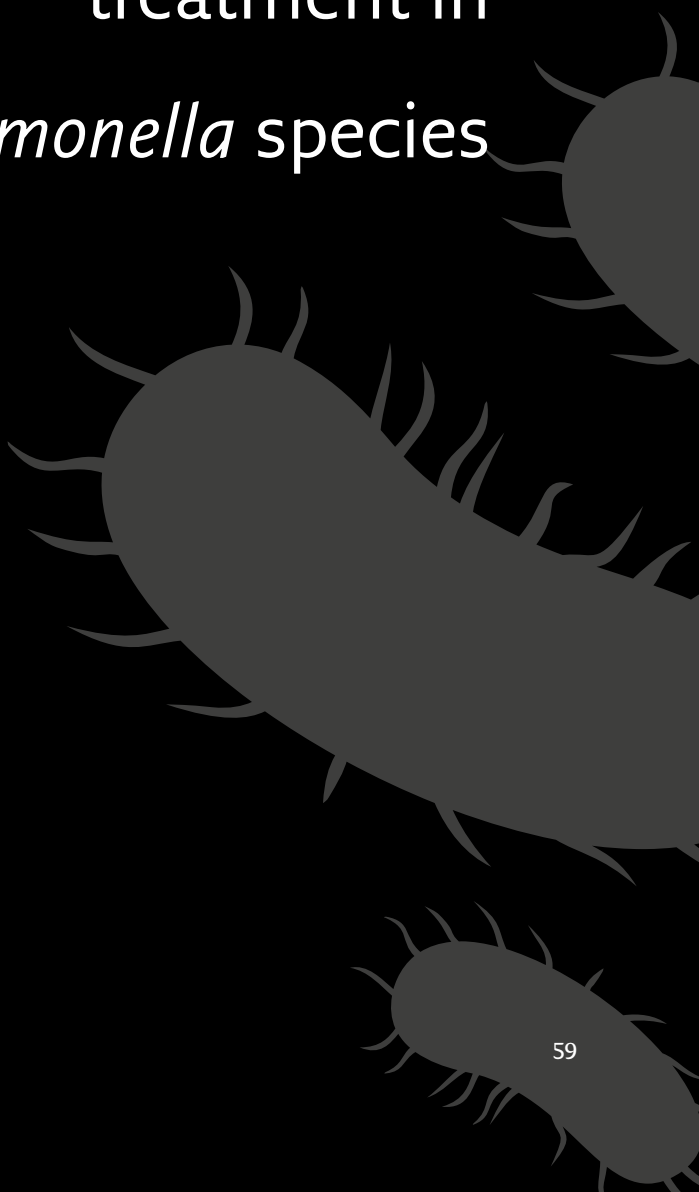
SCOPUS TITLE-ABS-KEY((("Gram negative" OR Enterobacteri* OR (Enter* W/1 bacteria*) OR Enterobacter* OR Escherichia* OR "e coli" OR Klebsiella* OR Salmonell* OR Shigell* OR Yersinia*)) AND ((travel* OR touris* OR turista OR aviation OR "air transport" OR airport*)) AND ((resistan* OR coloni* OR ((antibiotic* OR antimicrob*) W/3 sensitivit*) OR susceptib* OR carriage* OR carrier*)))

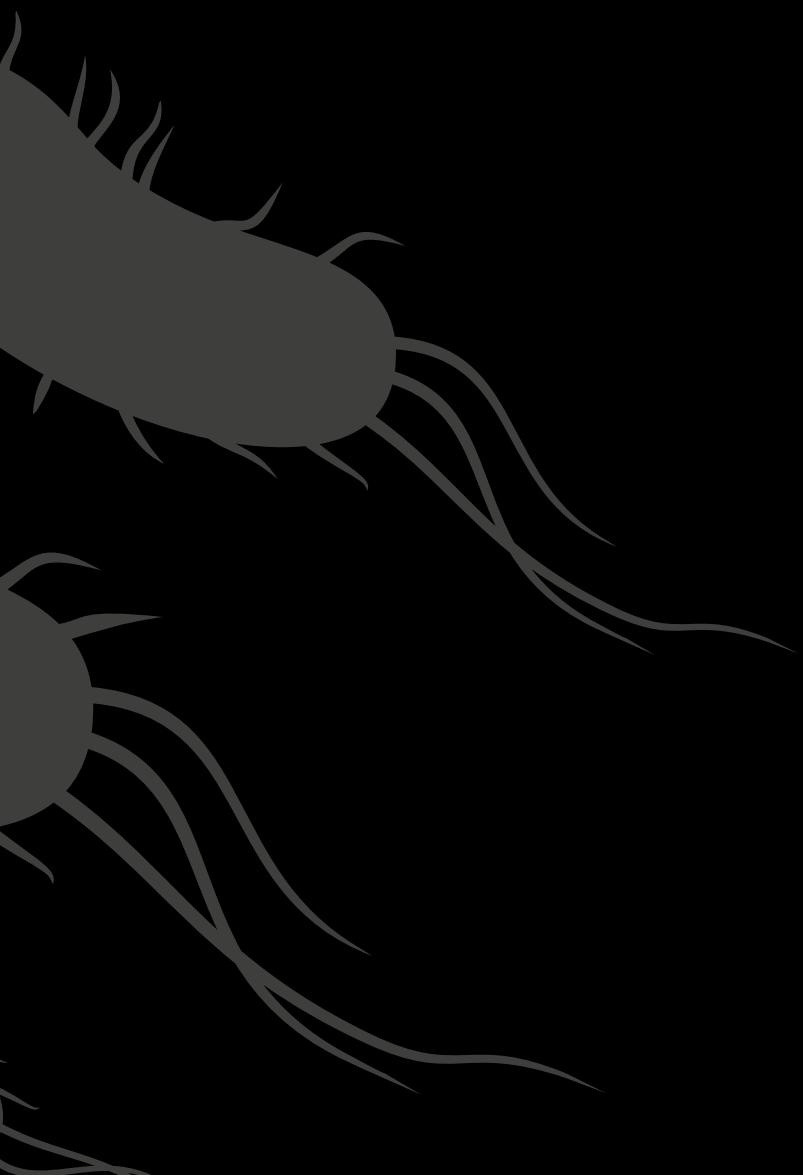
PUBMED PUBLISHER ((Gram negative[tiab] OR Enterobacteri*[tiab] OR Entero bacteria*[tiab] OR Enteric bacteria*[tiab] OR Enterobacter*[tiab] OR Escherichia*[tiab] OR e coli[tiab] OR Klebsiella*[tiab] OR Salmonell*[tiab] OR Shigell*[tiab] OR Yersinia*[tiab])) AND ((travel*[tiab] OR touris*[tiab] OR turista[tiab] OR aviation[tiab] OR air transport*[tiab] OR airport*[tiab])) AND ((resistan*[tiab] OR coloni*[tiab] OR ((antibiotic*[tiab] OR antimicrob*[tiab]) AND sensitivit*[tiab]) OR susceptib*[tiab] OR carriage*[tiab] OR carrier*[tiab])) AND publisher[sb]

GOOGLE SCHOLAR "Gram negative"|Enterobacteriaceae|Escherichia|Klebsiella|Salmonella|Shigella|Yersinia travel|traveller|tourist|tourism resistance|resistant|colonization|colonisation|susceptibility|carriage|carrier

PART 2

Antimicrobial resistance and treatment in *Salmonella* species





CHAPTER 4

Analysis of mechanisms involved in reduced susceptibility to ciprofloxacin of *Salmonella* Typhi and Paratyphi A isolates from travellers to South-East Asia

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Robert-Jan Hassing

Godfred A. Menezes

Wilfred van Pelt

Pieter L. Petit

Perry J. van Genderen

Wil H. Goessens

Abstract

Owing to multidrug resistance, quinolones and third-generation cephalosporins are currently used as key antibiotics to combat *Salmonella* organisms. Therapy failure due to reduced ciprofloxacin susceptibility has been reported in endemic areas, but also in imported disease. Different bacterial resistance mechanisms may result in reduced ciprofloxacin susceptibility. In this study, the presence and expression of different resistance mechanisms resulting in reduced minimum inhibitory concentrations (MICs) for ciprofloxacin were evaluated in 23 blood-culture-derived *Salmonella enterica* serotypes Typhi and Paratyphi A organisms from ill-returned travellers to Asia. The presence of mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene as well as an activated efflux pump and plasmid-mediated quinolone resistance genes was determined. Resistance selection during therapy and the clonal relatedness of all isolates were established. Efflux pump inhibition did not appear to affect the MICs of ciprofloxacin and activity of the efflux pump appeared to be specific for nalidixic acid. Repeated exposure of the isolates to ciprofloxacin did not result in a significant increase in the MICs for ciprofloxacin. Repetitive sequence-based polymerase chain reaction (rep-PCR) profiles identified five different genotypes, but no correlation with resistance was observed. However, a significant relation was found with geographic region; reduced ciprofloxacin susceptibility was only found in travellers returning from India and Pakistan. All isolates with reduced ciprofloxacin susceptibility had a mutation at position 83 in the QRDR region of the *gyrA* gene. Plasmid-mediated quinolone resistance was not found. These findings confirm that the reduced ciprofloxacin MIC in *S.* Typhi and *S.* Paratyphi A is solely due to an amino acid substitution in the QRDR 'cluster' of the *gyrA* gene.

Introduction

Resistance of *Salmonella* spp. to ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole has been described during the last 20 years. Owing to this multidrug-resistance, quinolones and third-generation cephalosporins are currently in use as key antibiotics to combat these multiresistant organisms[1]. As a consequence of the use of quinolones and cephalosporins, there have been reports describing clinical treatment failure following administration of ciprofloxacin to patients with typhoid fever owing to reduced ciprofloxacin susceptibility (defined as minimum inhibitory concentration (MIC) of 0.125-1 µg/mL). This reduced susceptibility is found not only in endemic areas but also in developed countries such as the USA and UK, reflecting a global problem[2,3].

Bacterial resistance to quinolones in *Salmonella* spp. may result from different resistance mechanisms. Quinolone resistance is usually associated with mutations in the target site (DNA gyrase), most commonly in the quinolone resistance-determining region (QRDR) of the GyrA subunit, conferring resistance to single-step mutants[4]. On the other hand, reduced susceptibility to quinolones is mostly due to mechanisms such as active efflux via efflux pumps as well as alterations in the expression of outer membrane proteins[5]. Plasmid-mediated quinolone resistance has also emerged in non-typhoidal *Salmonella enterica* strains resulting in reduced ciprofloxacin susceptibility; this mechanism has not yet been observed in typhoidal *Salmonella* spp. Outer membrane protein-deficient *Salmonella* spp. have only been described incidentally, resulting in increased MICs (>200 µg/mL) for chloramphenicol[6].

Owing to increased travel to endemic areas as well as migrant workers originating from endemic countries, The Netherlands is also confronted with patients not responding to empirical therapy with ciprofloxacin. In this retrospective study, *S. enterica* serotypes Typhi and Paratyphi A isolates were systematically characterised with respect to the presence and expression of different mechanisms of resistance causing reduced susceptibility to fluoroquinolones in 23 consecutive patients (travellers and migrant workers) diagnosed at the Institute for Tropical Diseases of Harbour Hospital-Rotterdam (The Netherlands) with blood culture-derived *S.* Typhi or *S.* Paratyphi A infection.

Materials and methods

Bacterial isolates

In total, 23 *Salmonella* isolates (*S.* Typhi from 11 individuals and *S.* Paratyphi A from 12 individuals) were collected from consecutive patients attending the Institute for Tropical Diseases during the period January 2002 to August 2008. All isolates derived from

positive blood cultures. Isolates were initially identified biochemically, followed by confirmation using specific antisera. Serotype determination was performed at the National Institute for Public Health and the Environment (RIVM) (Bilthoven, The Netherlands).

Antimicrobial susceptibility

Susceptibility of the isolates was determined using the VITEK-2 system (bioMérieux sa, Marcy L'Etoile, France). MICs for nalidixic acid and ciprofloxacin were determined by the microbroth dilution technique. All tests were performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines where applicable. For in vitro detection of efflux pump expression, a two-step dilution series of the antibiotics nalidixic acid, ciprofloxacin and chloramphenicol was made to detect the MIC in the absence and presence of the efflux pump inhibitor (EPI) phenylalanine β-naphthylamide (PAβN) (20 µg/mL). A reduction of the initial MIC by >3-step difference upon exposure to EPI is an indication for the presence of an activated efflux pump[7].

Polymerase chain reaction (PCR) screening and sequence analysis

The QRDR of *gyrA* was amplified by PCR using the primers described by Kariuki et al.[8]. Products resulting from amplifications were subjected to sequencing using a 3100 ABI Prism Genetic Analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Analysis and comparisons of nucleotide and amino acid sequence data were carried out using MegAlign software (DNAStar Inc., Madison, WI) and programs available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Presence of the *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib-cr* genes was determined by PCR[9,10]. The presence of integrons was determined using the method described by Lévesque et al[11].

Molecular typing

Clonal relatedness of all isolates was established using the repetitive sequence-based PCR (rep-PCR) DiversiLab® Microbial Typing System (bioMérieux) according to the manufacturer's instructions. Isolates with >95% identical profiles were considered to be closely related.

Results and discussion

During the last decade, treatment failures with ciprofloxacin have been increasingly reported. These failures have been associated with infection with *S.* Typhi and *S.* Paratyphi A strains that are resistant to nalidixic acid with decreased susceptibility to ciprofloxacin[2,3,12]. Studies describing mechanisms of fluoroquinolone resistance demonstrate only one or two specific mechanisms. However, several mechanisms of resistance can result in reduced susceptibility to ciprofloxacin[4]. Moreover, a combination of resistance mech-

anisms could also lead to enhancement of each of the separate mechanisms resulting in increased MICs. As mentioned previously, studies describing a systematic analysis of fluoroquinolone resistance mechanisms in different *S.* Typhi and *S.* Paratyphi A isolates are rare[13]. Therefore, we have systematically evaluated the presence and expression of different mechanisms of resistance resulting in reduced MICs for ciprofloxacin in 23 *Salmonella* isolates.

Of the 23 isolates, 14 (60.9%) were resistant to nalidixic acid (NAL^R) and displayed reduced MICs for ciprofloxacin ranging from 0.25 µg/mL to 2 µg/mL, except for one isolate (6838) that had a ciprofloxacin MIC of 32 µg/mL. The remaining nine isolates (39.1%) were all nalidixic-acid-susceptible (NAL^S) and had low MICs for ciprofloxacin (≤0.03 µg/mL) [**Figure 1**]. One of the mechanisms resulting in reduced susceptibility to quinolones may be the presence of activated efflux pumps. To detect the presence of these pumps, a phenotypic method was applied by determining MICs for nalidixic acid and ciprofloxacin in the presence and absence of the EPI PAßN. In most isolates, moderate efflux activity was determined for nalidixic acid resulting in MIC reductions of 3-4 steps in the presence of the EPI. However, the activity of this particular efflux pump(s) does not appear to affect the MICs for ciprofloxacin, indicating that the activity of this pump is specific, at least for nalidixic acid. As this phenomenon is not exclusively found in NAL^R isolates, it is concluded that this specific efflux pump(s) is not causing the increased MICs for ciprofloxacin in the NAL^R isolates (data not shown). Patients infected with isolates with reduced susceptibility to fluoroquinolones react less favourably on the initiated therapy[12]. This might be due to the fact that the strains that are already resistant to nalidixic acid may require fewer exposures to fluoroquinolones to develop high-level resistance to ciprofloxacin than strains that are fully ciprofloxacin-susceptible[5].

To check for resistance selection during therapy, a selected number of isolates were exposed for two rounds to increasing concentrations of ciprofloxacin. However, this limited exposure did not result in a significant increase in the MICs of ciprofloxacin. Also, efflux pump activity did not change after these two rounds of selection/induction by repeated exposure to ciprofloxacin (data not shown). In fact, the same base level efflux pump activity was observed for chloramphenicol (efflux indicator antibiotic) as was already demonstrated for nalidixic acid. Thus, the impaired clinical response is not due to resistance selection during antibiotic treatment but may be due to unfavourable pharmacodynamic parameters resulting in impaired killing.

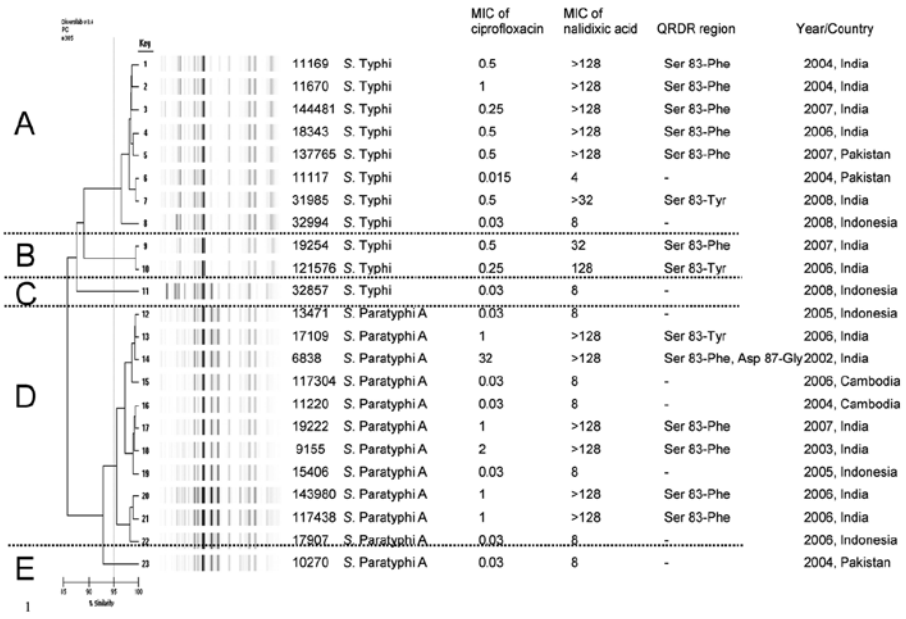
Resistance or reduced susceptibility for fluoroquinolones is due to the presence of active efflux pumps, impaired outer membrane permeability or gyrase mutations. As efflux does not explain the current findings, all isolates were subjected to sequencing of the *gyrA* gene. Mutations in the QRDR region of the *gyrA* gene resulting in amino acid substitutions are given in **Figure 1**. All NAL^S isolates show no mutations in the QRDR

region. The NAL^R isolates all had mutations at position 83 resulting in a substitution of serine to phenylalanine or tyrosine. Isolate 6838, with a ciprofloxacin MIC of 32 µg/mL, had a double mutation. In addition to the mutation at position 83, a substitution of asparagine to glycine at position 87 was also demonstrated. Mutations in *gyrA* outside of the QRDR were observed both in NAL^R and NAL^S isolates (data not shown). These data are in full agreement with the data of Nair et al.[14] who demonstrated that a single *gyrA* mutation at ser-83 alone resulted in resistance to nalidixic acid and reduced susceptibility to ciprofloxacin (MICs of 0.125-0.25 µg/mL). Therefore, it was concluded that Ser-83 is an important site for determining fluoroquinolone resistance within *S.* Typhi and *S.* Paratyphi A isolates. Complete resistance to ciprofloxacin (MIC >4 µg/mL) has been reported by Gaind et al.[13] and is due to a double mutation in the QRDR region.

In this investigation, the presence of plasmid-encoded qnr genes resulting in reduced susceptibility to quinolones in *Salmonella* spp. Was also studied[15]. Expression of these genes is phenotypically hard to detect as they have a moderate effect on ciprofloxacin MICs even in combination with the *aac(6')-Ib-cr* gene. In the 23 isolates studied, it was not possible to demonstrate the presence of plasmid-encoded *qnr* genes or *aac(6')-Ib-cr*, indicating that these qnr bearing plasmids are "host" specific. Therefore, these findings confirm other reports that the reduced MICs for ciprofloxacin in *S.* Typhi and *S.* Paratyphi A are solely due to the amino acid substitution in the QRDR "cluster". Besides reduced susceptibility to quinolones, two *S.* Typhi isolates demonstrated resistance to ampicillin, chloramphenicol, tetracycline and trimethoprim. One *S.* Paratyphi A isolate was resistant to ampicillin, chloramphenicol and tetracycline. In the two trimethoprim-resistant *S.* Typhi isolates an 800-bp integron cassette was demonstrated, indicating that the gene involved is probably the *dfrA7* gene encoding for a dihydrofolate reductase[16]. Integrons have been demonstrated in non-typhoidal *Salmonella* but only occasionally in *S.* Typhi[17].

To study the genetic relationship between the NAL^R and NAL^S clusters, all isolates were analysed using a commercial rep-PCR system. Results of the rep-PCR [**Figure 1**] showed three different clusters of 8, 2, 11 as well as 2 unique isolates. In the clusters A and D consisting of 8 and 11 isolates, respectively, susceptibility to the quinolones was randomly distributed, thereby demonstrating that resistance to nalidixic acid, i.e. reduced ciprofloxacin susceptibility, is not linked to a distinct cluster. Isolates belonging to cluster A were all *S.* Typhi isolates, whereas cluster D comprised *S.* Paratyphi A isolates. The only consistent correlation found related to the country of acquisition of the infection, i.e. the resistance to nalidixic acid and reduced susceptibility to ciprofloxacin was only found in travellers to India and Pakistan, but not in travellers to Indonesia and Cambodia. However, these geographical differences are not always that clear, as NAL^R isolates have also been reported in Vietnam[2]. However, based on these data it seems at least indicated to treat patients returning from India with an alternative drug instead of ciprofloxacin.

Figure 1 *Genotyping of 23 isolates by repetive sequence-based polymerase chain reaction (rep-PCR) (DiversiLab®). MIC, minimum inhibitory concentration; QRDR, quinolone resistance-determining region.*



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CHAPTER 5

Detection of amino-acid substitutions in the GyrA protein of fluoroquinolone resistant typhoidal *Salmonella* isolates using high-resolution mass spectrometry.

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Robert-Jan Hassing

Wil H. Goessens

Lona Zeneyedpour

Sadaf Sultan

Jeroen J. van Kampen

Annelies Verbon

Perry J. van Genderen

John P. Hays

Theo M. Luider

Lennard J. Dekker

Abstract

OBJECTIVES Infections with typhoidal *Salmonella* that are resistant to fluoroquinolone antibiotics have become very common in several Asian countries. In the majority of these cases, resistance to fluoroquinolone-based antibiotics is associated with genetic mutations in the quinolone resistance-determining region (QRDR) of the bacterial DNA gyrase gene, *gyrA*. The objective of the present paper is to detect these amino acid substitutions by high resolution mass spectrometry instead of sequencing of the *gyrA* gene.

METHODS A liquid chromatography-mass spectrometry (LC-MS) methodology was developed and evaluated for the detection of amino acid substitutions in the GyrA protein of 23 typhoidal *Salmonella* isolates. These isolates included typhoidal Salmonellae that possessed different antibiotic sensitivities to fluoroquinolone antibiotics.

RESULTS The LC-MS methodology correctly identified peptide sequences associated with phenotypic QRDR mutations of the GyrA protein in all of the 23 phenotypically diverse typhoidal *Salmonella* isolates tested.

CONCLUSIONS A reliable and rapid LC-MS methodology has been developed, which is able to identify *gyrA* QRDR mutations that are involved in the development of fluoroquinolone resistance in typhoidal *Salmonella* species. Further, this 'proof of principle' study, indicates the potential usefulness of LC-MS in future detection of antibiotic resistance.

Introduction

Infections with typhoidal *Salmonella* that are resistant to fluoroquinolone antibiotics (especially ciprofloxacin) have become very common in several Asian countries[1-3]. For example, a very recent phylogeographical analysis showed a single multidrug resistant lineage, H58, to be responsible for the emergence of these resistant isolates throughout Asia and Africa[4]. In the majority of cases, fluoroquinolone resistance in typhoidal *Salmonella* spp. has been associated with a *gyrA* mutation in the quinolone resistance-determining region (QRDR), resulting in a single amino acid substitution in the GyrA protein[5-6]. Expression of this mutated *gyrA* gene (at Ser-83) results in low-level resistance to fluoroquinolone antibiotics, as well as resistance to nalidixic acid (a closely related 'quinolone' antibiotic). Importantly, a double mutation in the *gyrA* gene (at Ser-83 and Asp-87) results in a double amino acid mutation in the gyrase protein, which has been associated with high-level resistance to fluoroquinolone antibiotics[5-6].

Susceptibility testing of micro-organisms is still performed in a classic fashion by determining the minimal inhibitory concentration after 18h exposure to different concentrations of antibiotic. Further, due to new mechanisms of resistance not only classic susceptibility testing is performed but extended by phenotypic screenings assay sometimes in combination with PCR to detect mechanisms of resistance. Due to the delay in obtaining susceptibility results and the increase in mechanisms of resistance there is a need for alternative techniques which are quicker with the same and perhaps better accuracy than current techniques. Currently, the use of mass spectrometry in medical microbiology laboratories is limited and mainly applied for microbial identification purposes, for example via matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis[7]. However, the range of mass spectrometry-related applications in the field of microbiological diagnostics is steadily increasing, with current emphasis being related to the detection of antibiotic resistance in clinically relevant bacteria[8]. Further, in contrast to MALDI-TOF MS, more advanced techniques such as liquid chromatography-mass spectrometry (LC-MS), can be used to identify large numbers of peptides in complex samples, and could potentially be used to identify non-synonymous amino-acid substitutions in protein sequences that are associated with the development of antimicrobial resistant phenotypes.

In the current study, a targeted LC-MS method for the detection of GyrA amino acid substitutions in typhoidal *Salmonella* isolates on a high resolution mass spectrometer using PRM (parallel reaction monitoring) was developed and evaluated. Recently this approach has been applied by Lesur and co-workers to identify mutations in oncoproteins in eukaryotic cancer cell lines[9]. Mass spectrometry has to our knowledge not been applied to identify mutations in proteins of bacteria before. This 'proof of principle' methodology demonstrates the feasibility of applying LC-MS-based techniques to the diagnosis of antimicrobial resistance in bacteria, as well as pointing to the possible use of LC-MS in future medical microbiology applications.

Materials and methods

Bacterial isolates and antimicrobial susceptibility

An initial set of *Salmonella* isolates (Set 1) was chosen for use in the development of the LC-MS methodology. This set comprised four *Salmonella enterica* serotype Paratyphi A (S. Paratyphi A) isolates selected from a collection of 23 genetically well-characterized blood culture-derived typhoidal *Salmonella* isolates that had been obtained from patients during the period January 2002 to August 2008 obtained from patients attending the Harbour Hospital and Institute for Tropical Diseases, Rotterdam, The Netherlands.

Set 1 isolates comprised: 1) one fluoroquinolone susceptible *S.* Paratyphi A isolate (13471) - which was selected as a wild type isolate; 2) two low-level ciprofloxacin resistant *S.* Paratyphi A isolates (17109 and 19222); and 3) one *S.* Paratyphi A isolate (6836) possessing high-level ciprofloxacin resistance.

For method validation a second set of isolates (Set 2) was used, which contained the remaining 19 typhoidal *Salmonella* isolates mentioned above, as well as the four isolates from Set 1. Therefore, Set 2 contained eleven *Salmonella enterica* serotype Typhi (S. Typhi) and twelve *S.* Paratyphi A isolates. The results of ciprofloxacin and nalidixic acid susceptibility testing for all 23 isolates was previously determined in a separate study[5]. According to EUCAST guidelines (http://www.eucast.org/clinical_breakpoints/), the isolates were defined as: 1) ciprofloxacin 'susceptible' if their ciprofloxacin MIC was ≤ 0.06 µg/ml; 2) 'low-level ciprofloxacin resistant' if their MIC was 0.125-1.0 µg/ml and 3) 'high-level resistant' if their MIC was >1.0 µg/ml.

Analysis

Sample pre-treatment for LC-MS method

Bacterial isolates were grown in 50 ml Tryptic Soy broth (Becton Dickinson, Sparks, Maryland, USA) and incubated overnight at 370C. The next morning, 1 ml of bacterial culture was pelleted by centrifugation (16,000 g, 2 min), washed 3 times using 1 ml of Phophate Buffered Saline (PBS). Subsequently, 50 µl of suspension was precipitated using 950 µl acetone. Pellets were dissolved in SDS-PAGE sample buffer and analyzed on a 7.5% SDS-PAGE gel for the non-targeted method or dissolved in 100 ul Rapigest 0.2% in 50 mM NH_4HCO_3 for the target-method (PRM). The SDS-PAGE non-targeted procedure was implemented in order to increase the DNA gyrase protein concentration, which was required for proper method development. Both 'in-gel' and 'in-solution' digestion procedures were performed as previously described10. In addition, a digestion using immobilized trypsin (Flash digest (Perfinity, West Lafayette, USA) was used. Precipitated protein pellets of the bacteria culture were dissolved in 100 ul digestion buffer (Perfinity, West Lafayette, USA) vortexed and incubated on a thermo mixer 1000 rpm at 70 °C for 4 hour. Subsequent samples we diluted in water 100 fold and acidified using TFA (pH < 2).

Non-targeted gel-based LC-MS analysis

Five gel bands of each sample were excised at the approximate molecular weight for GyrA (116 kDa). In order to determine if GyrA mutations could be detected, the gel bands were digested with trypsin and analyzed by nanoLC LTQ-Orbitrap in a data dependent acquisition mode as described previously[10]. Five microliters of digest from the gel bands, were injected onto a nanoLC system (nano-LC Ultimate 3000; Thermo Fisher Scientific, Sunnyvale, CA, USA). After pre-concentration and washing of the sample on a C18 trap column (1 mm × 300 µm id), peptides were separated on a C18 PepMap column (250 mm × 75 µm internal diameter) (Thermo Fisher Scientific) using a linear 90 min gradient (4–40% acetonitrile/H20; 0.1% formic acid) at a flow rate of 250 nL/min. The separation of the peptides was monitored by a UV detector (absorption at 214 nm). The nano-LC was coupled to the nanospray source of the LTQ-Orbitrap (Orbitrap XL, Thermo Fisher Scientific).

Data-analysis of non-targeted MS measurements

Based on the data obtained, the band with the highest sequence coverage for GyrA was selected from all four isolates and the data files from these 4 GyrA bands were re-analyzed using Mascot, and a separate GyrA database, which contained the different known mutational variants of the GyrA protein[11,12].

Sequence mutations in the QRDR of the *gyrA* gene of all *Salmonella* isolates tested had been previously determined using polymerase chain reaction (PCR) amplification and gene sequencing analysis[4].

Targeted MS analyses

The targeted analysis only includes the GyrA peptides of interest this in contrast to the non-targeted analysis in which peptides were selected for MS/MS acquisition based on their presence in the MS spectrum[13]. Two microliters of digested *Salmonella* protein extract were injected onto a nanoLC system (nano-LC Ultimate 3000 RSLC; Thermo Fisher Scientific, Bremen). After pre-concentration and washing of the sample on a C18 trap column (1mm × 300 µm id), peptides were separated on a C18 PepMap column (250 mm × 75 µm internal diameter) (Thermo Fisher Scientific) using a linear 30 min gradient (4–38% ACN/H2;0.1% formic acid) at a flow rate of 250 nL/min. The separation of the peptides was monitored by a UV detector (absorption at 214 nm). MS analysis was performed on a Q-Exactive Plus (Thermo Fisher Scientific) mass spectrometer equipped with a nano-spray ion source. A targeted MS/MS method was developed for all peptides containing GyrA mutations. In the targeted method we included not only the four possible tryptic fragments containing phenotypic GyrA mutations, but also included two peptides within the GyrA protein sequence outside the QRDR region. These peptides should be present in all samples tested and reflect the total amount of GyrA in a sample. For this reason, these peptides were used as a sample control and could also potentially be used for normalization. The method allows quantitation if stable isotope labelled peptide standards are included, this quantification step could be included in this method. A

Table 1 *Peptide masses targeted during Q-Exactive Orbitrap measurements*

Mass [m/z]	CS [z]	Polarity	(N)CE	Peptide sequence
577.28127	3	Positive	27	YHPHGDSAVYDTIVR
865.41827	2	Positive	27	YHPHGDSAVYDTIVR
577.95824	3	Positive	27	YHPHGDFAVYGTIVR
866.43372	2	Positive	27	YHPHGDFAVYGTIVR
903.43392	2	Positive	27	YHPHGDYAVYDTIVR
602.62504	3	Positive	27	YHPHGDYAVYDTIVR
895.43646	2	Positive	27	YHPHGDFAVYDTIVR
597.29340	3	Positive	27	YHPHGDFAVYDTIVR
816.35721	2	Positive	27	ETVDFVDNYDGTEK
845.98358	2	Positive	27	GRPIVNLLPLEANER

quadrupole isolation window of 0.7 m/z units, an automatic gain control target of 2e5 ions, a maximum fill time of 250 ms and an Orbitrap resolving power of 35000 at 200 m/z were used. A normalized collision energy of 27 was used for each GyrA peptide precursor. All different mutational variants of the tryptic peptide in the QRDR region were targeted (for each peptide the 2 and 3+ charge states were included), including the two additional peptides that are present in all three variants of the GyrA protein tested. Additional information regarding the different Gyr A peptides targeted are shown in **table 1**.

Data analyses of targeted MS measurements

The three most intense fragment ions for each targeted mass were extracted using Skyline software for each of the typhoidal *Salmonella* isolates tested. If signals for all three fragments with the correct mass (difference <2 ppm) and matching retention times were observed, then an isolate was considered positive for the measured peptide and based on this information the mutational state of GyrA could be determined.

Results

LC-MS method development

In a first attempt to detect amino acid substitutions in GyrA, trypsin digested extracts were used for analysis. However, GyrA specific peptides were not detected using LC-MS, which was probably due to the peptide concentration being too low. Therefore, in order

to specifically enrich the concentration of GyrA proteins, the extracts were first separated by SDS-PAGE and the protein band of the expected molecular mass excised and extracted [**figure 1**] before LC-MS. The results of the SDS-PAGE analysis for each of the four Salmonella isolates present in Set 1 were used to optimize the LC-MS method. Individual mass spectrometry analysis of all 5 bands for each isolate (n=4) resulted in the identification of 673 proteins in total. Further, LC-MS sequence coverage for GyrA varied between 54 and 65% for isolates 13471, 17109, 19222 and 6836 [**table 2**]. Therefore, by adapting the pre-treatment steps, the LC-MS methodology correctly identified the protein sequences for GyrA gyrases containing the different mutations associated with different fluoroquinolone susceptibility phenotypes. All peptides containing these mutations were identified with high confidence and Mascot ion scores of 50 and higher (Mascot ion score of >50 relates to a p-value of <0.00001) were obtained. The results of GyrA peptide mutation analysis was confirmed by nucleotide sequencing of the QRDR-region [**table 2**].

LC-MS method validation

As the results of the LC-MS approach were in agreement with the sequencing results, the number of isolates was extended with an additional 19 *Salmonella* isolates containing *S.* Typhi and *S.* Paratyphi isolates, as well as the 4 isolates that were previously used for LC-MS method development in Set 1. This resulted in a sequence coverage for GyrA of between 44 and 68%. Previous sequence analysis of the QRDR of *gyrA* also showed that the LC-MS method correctly identified GyrA mutations in the GyrA protein [**table 3**] in all isolates.

Figure 1 *Five gel bands of approximately 116 kDa were excised from each of 4 Salmonella isolates. Each band is indicated by a box. The band contained in the bold box resulted in the highest sequence coverage for GyrA and was used in further GyrA mutation analysis.*

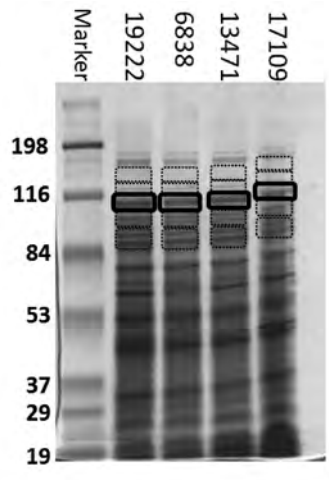

Table 2 *S. Paratyphi A isolates used for liquid chromatography mass spectrometry method development (Set 1).*

Isolate	Year / Country	MIC of Ciprofloxacin	MIC of Nalidixic acid	QRDR mutation	Identified peptide containing mutation sites	Sequence Coverage
13471 *S.* Paratyphi A	2005, Indonesia	0.03	8	-	YHPH GDSAVYDTIV R	54%
17109 *S.* Paratyphi A	2006, India	1	>128	Ser 83-Tyr	YHPH GD**Y**AVYDTIV R	64%
19222 *S.* Paratyphi A	2007, India	1	>128	Ser 83-Phe	YHPH GD**F**AVYDTIV R	62%
6838 *S.* Paratyphi A	2002, India	32	>128	Ser 83-Phe, Asp 87-Gly	YHPH GD**F**AVY**G**TIV R	65%

Underlined = mutation site
Bold = mutated amino acid

Targeted MS analyses

Enrichment by SDS-PAGE is labor intensive and increases the analysis time, considerably. Therefore, in order to further improve the sensitivity and obtain more quantitative results, analysis was performed using a Parallel Reaction Monitoring (PRM) method on a Q-Exactive Orbitrap instrument[14]. This PRM is a targeted mass spectrometry method that can be performed on the newest generations of high resolution Orbitrap equipment. This quite recently introduced PRM method has the advantage above the more regular applied selective reaction monitoring technique that the selectivity is much higher because of the high resolution. In this way false positive results can be almost eliminated which is of course crucial for this type of analyses. In addition, also the sensitivity of the PRM method is in comparison to the non-targeted method at least an order magnitude improved.

For this reason, the SDS-PAGE enrichment step could be omitted, reducing the sample preparation and analysis time to 15 hours [**table 4**], as compared with 48 hours. **Figure 2** shows the qualitative, integration of peak area for the different mutant peptides of GyrA. Further, the resulting integrated areas for each *Salmonella* isolate are displayed for the four peptides containing the specific amino acid substitutions and WT-variants within the QRDR region. All isolates were only positive for the peptide with the expected mutational profile, as earlier confirmed by the PCR/sequencing analyses. All samples were positive for the two control peptides from the non-mutated area of the GyrA protein.

To even further reduce analyses time an experiment was performed using immobilized trypsin. In this experiment we were able to reduce the overnight digestion to 4 hours without comprising the results (data not shown) reducing the analysis time to 5 hours.

Table 3 *S. Typhi A and S. Paratyphi A isolates used for liquid chromatography mass spectrometry method validation (Set 2).*

Isolate	Year / Country	MIC of Ciprofloxacin	MIC of Nalidixic acid
13471 *S.* Paratyphi A	2005, Indonesia	0.03	8
17109 *S.* Paratyphi A	2006, India	1	>128
19222 *S.* Paratyphi A	2007, India	1	>128
6838 *S.* Paratyphi A	2002, India	32	>128
11169 *S.* Typhi	2004, India	0.5	>128
11670 *S.* Typhi	2004, India	1	>128
144481 *S.* Typhi	2007, India	0.25	>128
18343 *S.* Typhi	2006, India	0.5	>128
137765 *S.* Typhi	2007, Pakistan	0.5	>128
11117 *S.* Typhi	2004, Pakistan	0.015	4
31985 *S.* Typhi	2008, India	0.5	>32
32994 *S.* Typhi	2008, Indonesia	0.03	8
19254 *S.* Typhi	2007, India	0.5	32
121576 *S.* Typhi	2006, India	0.25	128
32857 *S.* Typhi	2008, Indonesia	0.03	8
117304 *S.* Paratyphi A	2006, Cambodia	0.03	8
11220 *S.* Paratyphi A	2004, Cambodia	0.03	8
9155 *S.* Paratyphi A	2003, India	2	>128
15406 *S.* Paratyphi A	2005, Indonesia	0.03	8
143980 *S.* Paratyphi A	2006, India	1	>128
117438 *S.* Paratyphi A	2006, India	1	>128
17907 *S.* Paratyphi A	2006, Indonesia	0.03	8
10270 *S.* Paratyphi A	2004, Pakistan	0.03	8

Underlined = mutation site
Bold = mutated amino acid

QRDR mutation	Identified peptide containing mutation sites	Sequence Coverage
-	YHPH GDSAVYDTIV R	56%
Ser 83-Tyr	YHPH GD**Y**AVYDTIV R	65%
Ser 83-Phe	YHPH GD**F**AVYDTIV R	68%
Ser 83-Phe, Asp 87-Gly	YHPH GD**F**AVY**G**TIV R	65%
Ser 83-Phe	YHPH GD**F**AVYDTIV R	67%
Ser 83-Phe	YHPH GD**F**AVYDTIV R	57%
Ser 83-Phe	YHPH GD**F**AVYDTIV R	56%
Ser 83-Phe	YHPH GD**F**AVYDTIV R	58%
Ser 83-Phe	YHPH GD**F**AVYDTIV R	63%
-	YHPH GDSAVYDTIV R	64%
Ser 83-Tyr	YHPH GD**Y**AVYDTIV R	57%
-	YHPH GDSAVYDTIV R	58%
Ser 83-Phe	YHPH GD**F**AVYDTIV R	63%
Ser 83-Tyr	YHPH GD**Y**AVYDTIV R	44%
-	YHPH GDSAVYDTIV R	63%
-	YHPH GDSAVYDTIV R	60%
-	YHPH GDSAVYDTIV R	57%
Ser 83-Phe	YHPH GD**F**AVYDTIV R	61%
-	YHPH GDSAVYDTIV R	44%
Ser 83-Phe	YHPH GD**F**AVYDTIV R	62%
Ser 83-Phe	YHPH GD**F**AVYDTIV R	52%
-	YHPH GDSAVYDTIV R	50%
-	YHPH GDSAVYDTIV R	60%

Figure 2 *Bar graph showing the peak area for the different mutant peptides of GyrA. Extracted ion chromatographs were generated for the 3 most intense fragment ions for each peptide using Skyline open access software. The signals were manually verified for correct retention time (all fragments possessed the same retention time, a difference <0.2 min between samples and correct mass of fragment ions (<2 ppm). The resulting integrated areas are displayed for the four peptides from each Salmonella isolate. All isolates are only positive for the peptide with the expected mutant profile as obtained by gyrA gene PCR/sequencing analyses.*

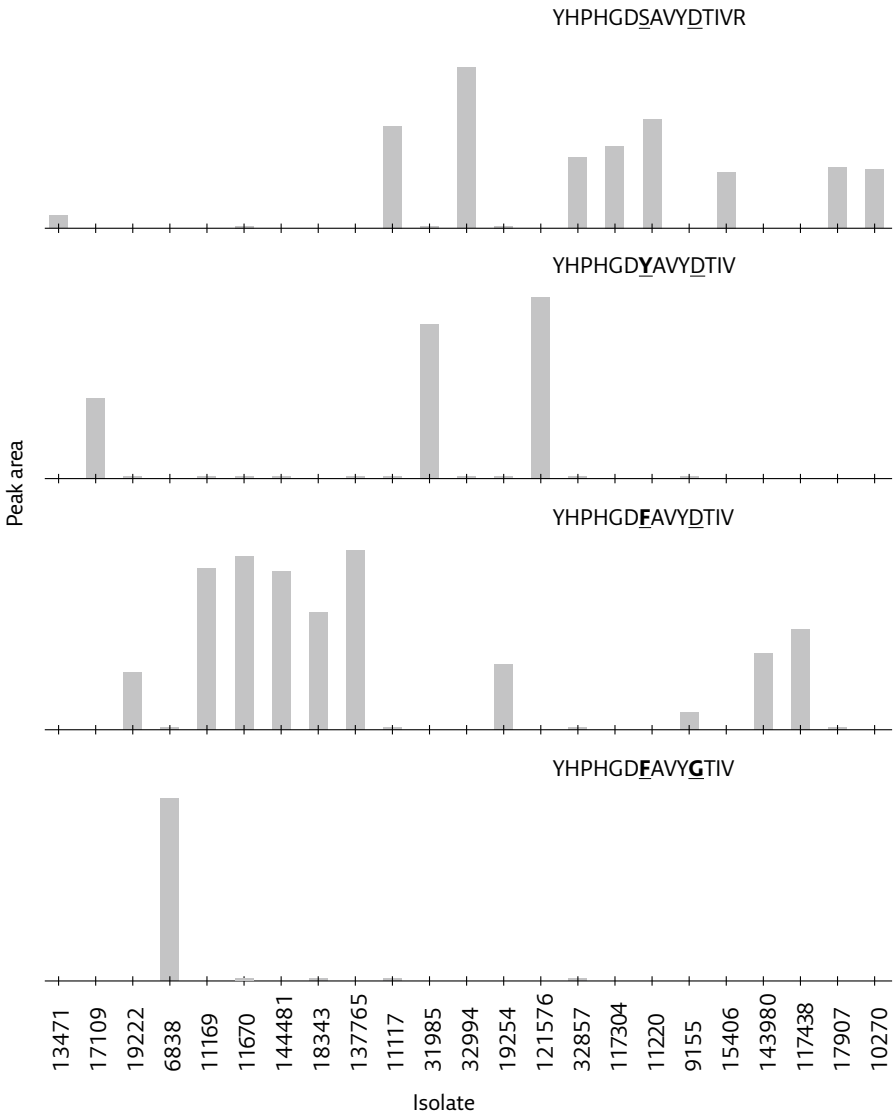

Table 4 *Estimated time required for the detection of fluoroquinolone resistance using currently available methods*

	Detection Method	Estimated time (hours)	Estimated costs (euro)	Comment
Phenotypic detection Susceptibility testing by minimal inhibitory concentration (MIC)	MIC determinations	18	100	information of mechanisms of resistance is not provided
Genotypic detection Gene sequences determined by nucleotide sequencing	Mutations in the QRDR of the gyrA gene	10-18	150	All mutations in the QRDR region will be detected
Mass spectrometry Detection of amino-acid substitutions	GyrA amino acid mutations	15	300	Up to ~50- 100 mutations can be followed
	GyrA amino acid mutations, and using immobilized enzymes for protein digestion	5	300	Process time will decrease considerably but cost will not decrease (same measurement time)

Discussion

In general, antimicrobial resistance is detected phenotypically, or genetically, by making use of biochemical susceptibility assays, or DNA gene detection/DNA sequencing by means of PCR. Using these methods, decreased susceptibility or resistance to antibiotics may be demonstrated for example, by detecting the presence of acquired genes encoding, specific antibiotic degrading enzymes e.g. β-lactamases or by detecting the presence of genetic mutations that render the target protein insensitive to a particular antibiotic e.g. DNA gyrases.

Further, mutations in bacterial antibiotic resistance genes may generate proteins containing non-synonymous sequences when compared to the protein translated from the original, non-mutated, gene. This occurs when DNA mutations lead to changes in triplet codon usage via mutated nucleotide sequences in transcribed messenger RNA. Importantly, high resolution mass spectrometry enables the detection and characterization of mutant proteins containing non-synonymous amino acid sequence substitutions.

In the present study we demonstrated that a combination of SDS-PAGE based enrichment of ~116 kDa proteins followed by LC-MS analysis enabled the identification of mutations in the QRDR region of GyrA. Frequently, the accuracy of detection of proteins

and peptides by LC-MS is influenced by the expression levels of the target protein/peptide and by the 'background noise' levels generated by non-target peptides present in the sample. In this publication, we show that the latter problem may be circumvented without compromising the sensitivity of the assay by the application of a 'targeted' LC-MS methodology in which prior enrichment using SDS-PAGE is replaced. Using this targeted LC-MS methodology we were able to detect not only the wild type QRDR region amino acid sequence in the GyrA protein (fluoroquinolone sensitive), but also QRDR amino acid substitutions associated with fluoroquinolone resistance. Further, using the targeted LC-MS methodology and using immobilized enzymes for protein digestion, allowed us to perform GyrA peptide analysis within a time-span of 5 hours after initial culture of the typhoidal Salmonella isolates being tested. This is much faster compared with other currently used fluoroquinolone susceptibility detection methods [**table 4**]. The use of immobilized trypsin has been extensively described in the scientific literature[15,16] and resulted in a significant reduction in digestion time to 3 hours, compared to the current approximate 12 hour (overnight) digestion period, decreasing the total time required for LC-MS analysis to 5 hours.

At the moment the use of LC-MS technique is limited to research laboratories. However, future advances in mass spectrometry technology, coupled to the development of a multiplex approach (where additional mutated proteins associated with antibiotic resistance can be detected) means that LC-MS could potentially become widely used in the microbiology clinical laboratory of the future.

The LC-MS methodology described in this publication shows that the detection and characterization of amino acid substitutions in the QRDR-region of GyrA is feasible using high-resolution mass spectrometry (LC-MS). Importantly, this publication shows that LC-MS may be used to detect non-synonymous amino acid substitutions that are phenotypically and clinically relevant — in this case, relevant for the detection and diagnosis of antibiotic resistance. However, in the present paper we only solved part of the puzzle. Fluoroquinolone resistance is not only the result of mutations in the gyrA protein but can also be due to mutations in the topoisomerase IV (parC) in combination with decreased permeability and/or activated efflux pumps. Also plasmid encoded mechanisms have been described like the Qnr proteins. These additional mechanisms of resistance are all related to the absence/presence of specific proteins. So in theory detectable by high resolution mass spectrometry. With the current technique in a non-targeted fashion we are already able to detect 60-70% of the proteins. By optimization of the sample pre-treatment protocol and the use of a specific universal antibiotic resistance mechanism database, the accuracy could be significantly further improved. In pilot experiments we already succeeded in the detection of several different β-lactamase enzymes as well as aminoglycoside modifying enzymes. However, before translation of these data can be used to reliably predict the presence of resistance mechanisms the obtained data should be normalized by including an internal reference. With normalized

results an algorithm could be developed to predict the presence of resistance mechanisms followed by deduction of the susceptibility to different antibiotics.

Future research based on our findings will show the usefulness of our LC-MS methodology for the detection of other clinically relevant antimicrobial resistance phenotypes, for example amino acid substitutions in Penicillin-Binding Proteins (PBPs), or amino acid substitutions in the active site of β-lactamase enzymes, possibly in a new multiplexed methodology. This could eventually lead to major changes in the way that antibiotic resistance is detected and diagnosed in the clinical microbiological laboratory.

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Competing interests

None declared

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CHAPTER 6

Decreased ciprofloxacin susceptibility in *Salmonella* Typhi and Paratyphi infections in ill-returned travellers: the impact on clinical outcome and future treatment options

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Robert-Jan Hassing

Wil H. Goessens

Dik J. Mevius

Wilfrid van Pelt

Johan W. Mouton

Annelies Verbon

Perry J. van Genderen

Abstract

The emergence of decreased ciprofloxacin susceptibility (DCS) in *Salmonella enterica* serovar Typhi and serovar Paratyphi A, B or C limits treatment options. We studied the impact of DCS isolates on the fate of travellers returning with enteric fever and possible alternative treatment options. We evaluated the clinical features, susceptibility data and efficacy of empirical treatment in patients with positive blood cultures of a DCS isolate compared to patients infected with a ciprofloxacin-susceptible (CS) isolate in the period from January 2002 to August 2008. In addition, the pharmacokinetic and pharmacodynamic parameters of ciprofloxacin, levofloxacin and gatifloxacin were determined to assess if increasing the dose would result in adequate unbound fraction of the drug 24-h area under the concentration-time curve/minimum inhibitory concentration ($fAUC_{0-24}$/MIC) ratio. Patients with DCS more often returned from the Indian subcontinent and had a longer fever clearance time and length of hospital stay compared to patients in whom the initial empirical therapy was adequate. The mean fAUC0-24/MIC was 41.3±18.8 in the patients with DCS and 585.4±219 in patients with a CS isolate. For DCS isolates, the mean $fAUC_{0-24}$/MIC for levofloxacin was 60.5±28.7 and for gatifloxacin, it was 97.9±28.0. Increasing the dose to an adequate $fAUC_{0-24}$/MIC ratio will lead to conceivably toxic drug levels in 50% of the patients treated with ciprofloxacin. Emerging DCS isolates has led to failure of empirical treatment in ill-returned travellers. We demonstrated that, in some cases, an adequate $fAUC_{0-24}$/MIC ratio could be achieved by increasing the dose of ciprofloxacin or by the use of alternative fluoroquinolones.

Introduction

Enteric fever (typhoid fever) is caused by *Salmonella enterica* serovar Typhi (*S.* Typhi) or serovar Paratyphi A, B, or C (*S.* Paratyphi)[1,2]. Although most of the burden of the disease occurs in developing countries with poor hygiene and sanitation, enteric fever is an imported disease in returning travellers, immigrants or migrant workers in developed countries[3-7]. Thirty years ago, many treatment options for enteric fever were available, such as ampicillin, chloramphenicol or trimethoprim/sulphamethoxazole. Due to the increased occurrence of multidrug resistant (MDR) *S.* Typhi, these agents were no longer applicable for empirical treatment and ciprofloxacin became the first-line drug of treatment. However, after this switch to ciprofloxacin, isolates with decreased ciprofloxacin susceptibility (DCS) or even resistance to ciprofloxacin became evident. Clinical failure due to reduced susceptibility to ciprofloxacin has been reported[8-11]. Therefore, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has issued a warning in their guidelines that ciprofloxacin susceptibility testing for *S.* Typhi isolates should be performed by quantitative susceptibility methods. Especially, organisms with a minimum inhibitory concentration (MIC) greater than 0.06 mg/L may result in failure, while, paradoxically, the breakpoint for susceptibility was set at 0.5 mg/L. Therefore, third-generation cephalosporins and azithromycin are now recommended as first-line treatment options for enteric fever patients from South Asia[3,12]. However, the first cases of resistance to third-generation cephalosporins due to the presence of extended-spectrum β-lactamases or plasmid encoded AmpC β-lactamases have already been reported[13-16].

In the Netherlands, about 30 cases of enteric fever are diagnosed every year[17]. Fluoroquinolones are still recommended by most authorities for the treatment of enteric fever. However, in this era of increasing ciprofloxacin resistance among *S.* Typhi and *S.* Paratyphi, it is not clear whether this still results in optimal empirical antibiotic therapy. Here, we evaluated clinical features, susceptibility data and efficacy of empirical treatment in enteric fever patients with positive blood cultures of a DCS isolates compared to patients infected with a ciprofloxacin-susceptible (CS) isolate. Additionally, pharmacokinetic and pharmacodynamic parameters (PK/PD) of several fluoroquinolones were determined to assess if increasing the dose would result in adequate unbound fraction of the drug 24-h area under the concentration-time curve/minimum inhibitory concentration ($fAUC_{0\text{-}24}$/MIC) ratio.

Materials and methods

Patient characteristics

All travellers diagnosed with blood-culture-confirmed S.Typhi and *S.* Paratyphi infections at the Harbour Hospital and Institute for Tropical Diseases, Rotterdam, the Neth-

erlands, in the period January 2002 to August 2008 were included in this retrospective study. These patients were grouped in DCS and CS strains and these groups were compared with respect to sex, age, country of acquisition, symptoms on admission, physical findings and laboratory findings on admission and clinical outcome.

Susceptibility testing

The susceptibility for ampicillin, chloramphenicol, ceftazidime, cefotaxime, tetracycline and trimethoprim/sulphamethoxazole were determined by the VITEK 2 system. The MICs for the fluoroquinolones ciprofloxacin, levofloxacin, moxifloxacin, ofloxacin and gatifloxacin were determined by E-test. EUCAST guidelines were applied for category interpretation for the different antibiotics (http://www.eucast.org/clinical_breakpoints/). The isolates were defined as DCS if the MIC for ciprofloxacin was 0.125–1.0 mg/l and CS if the MIC for ciprofloxacin was < 0.125 mg/l.

Pharmacokinetics

Further, 24-h area under the concentration-time curve ($AUC_{0\text{-}24}$), unbound fraction of the drug $AUC_{0\text{-}24}$ ($fAUC_{0\text{-}24}$) and $fAUC_{0\text{-}24}$/MIC ratios were calculated for individual doses of ciprofloxacin. The AUCo-24 of ciprofloxacin were based on the pharmacokinetic data from a previous clinical study[18]. Estimated $AUC_{0\text{-}24}$ for levofloxacin and gatifloxacin were calculated with mean $AUC_{0\text{-}2}4$ reported in the literature[19]. The $fAUC_{0\text{-}24}$ of ciprofloxacin, levofloxacin and gatifloxacin was calculated by correcting the $AUC_{0\text{-}24}$ for the fraction of drug bound to serum proteins, respectively 0.3, 0.3 and 0.2[19]. The $fAUC_{0\text{-}24}$/MIC was taken as target for optimal fluoroquinolone activity[20]. Based on an in vitro infection model to identify the efficacy of ciprofloxacin, levofloxacin and gatifloxacin against *S.* Typhi, an $fAUC_{0\text{-}24}$/MIC ratio of 105 is associated with a 90% maximal drug effect[19-21]. With this assumption, we calculated the individual dose per patient for ciprofloxacin, levofloxacin and gatifloxacin to fulfil the critical ratio of $fAUC_{0\text{-}24}$/MIC ratio of 105.

Data analysis

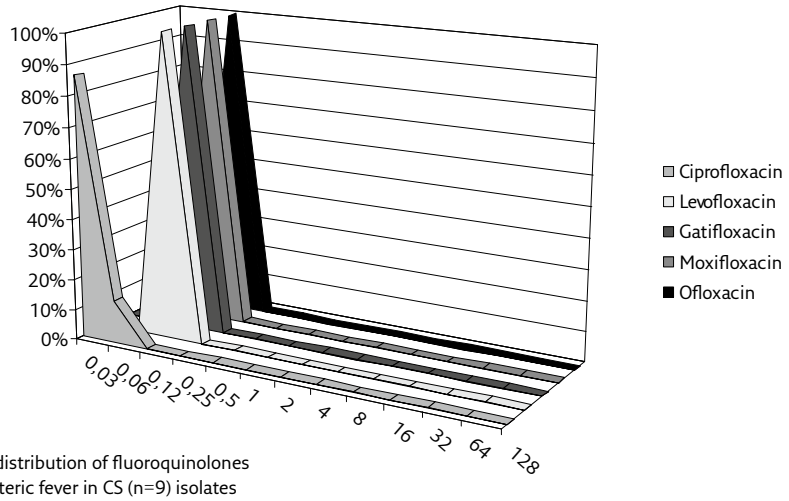
For categorical or dichotomized parameters, proportions between groups were compared using the Fisher's exact test. For continuous parameters with a normal distribution, *t-test* was used. Statistical significance was accepted at *p*-values <0,05. Data were analysed using SPSS 20.

Results

Susceptibility of different isolates

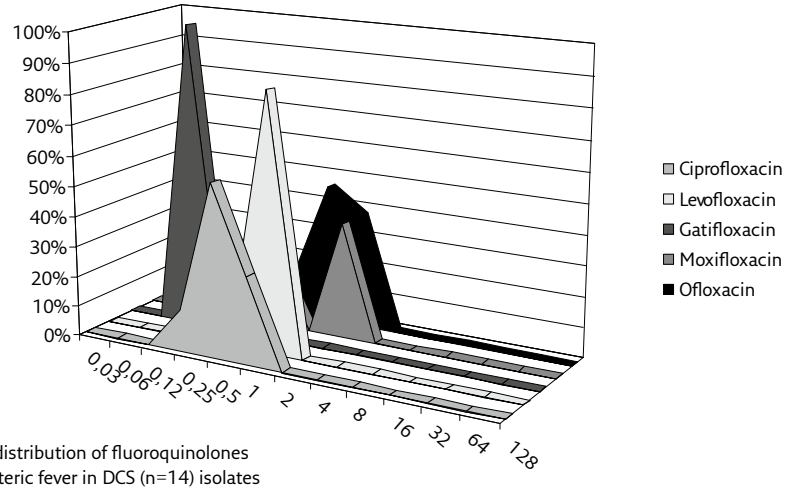
A total of 26 patients were identified, which were grouped in DCS and CS strains. DCS was found in 17 of 26 isolates (S. Typhi, *n*=9; *S.* Paratyphi A, *n*=8), whereas nine isolates were CS (*S.* Typhi, *n*=3; *S.* Paratyphi A, *n*=6). Only two MDR isolates were found, re-

Figure 1a *Minimum inhibitory concentration (MIC) distribution of fluoroquinolones in enteric fever in ciprofloxacin-susceptible (CS) (n=9) isolates.*



MIC distribution of fluoroquinolones
in enteric fever in CS (n=9) isolates

Figure 1b *MIC distribution of fluoroquinolones in enteric fever in decreased ciprofloxacin susceptibility (DCS) (n=14) isolates.*



MIC distribution of fluoroquinolones
in enteric fever in DCS (n=14) isolates

sistant to amoxicillin, chloramphenicol, trimethoprim/sulphamethoxazole and tetracycline. These two isolates belonged to the DCS group. None of the isolates was resistant to third-generation cephalosporins. The DCS isolates also showed increased MICs to alternative fluoroquinolones, such as levofloxacin, moxifloxacin or ofloxacin. The only fluoroquinolone that did not show increased MICs was gatifloxacin [**figure 1**].

Clinical features, treatment and outcome

The clinical data of patients included in the study are summarised in **table 1**. All DCS strains were acquired from the Indian subcontinent, whereas CS strains came from Southeast Asia in general. From 2002 to 2007, ciprofloxacin was the first-line treatment on admission in all patients who were later diagnosed with enteric fever. During these years, 13 patients with a DCS isolate were empirically treated with ciprofloxacin, from which only one patient became afebrile within 72 hours of treatment. After the demonstration of DCS or resistance or persisting fever, therapy was switched to intravenous ceftazidime (*n*=9) or trimethoprim/sulphamethoxazole (*n*=3). In the DCS group, one of the patients was treated with oral ciprofloxacin and one with oral azithromycin monotherapy without relapse. In the CS group, 6 of 9 patients were initially treated with oral ciprofloxacin, two received intravenous ceftazidime and one patient was treated with oral azithromycin, respectively. After the demonstration of ciprofloxacin susceptibility, follow-up treatment consisted of oral ciprofloxacin (*n*=7), oral azithromycin (*n*=1) and trimethoprim/sulphamethoxazole (*n*=1). There was one relapse of enteric fever in the DCS group (previous treatment consisted of ciprofloxacin for 3 days, followed with intravenous ceftazidime for 10 days) and one in the CS group (previously treated with ciprofloxacin and intravenous ceftazidime for 5 days, followed by trimethoprim/sulphamethoxazole for 9 days). In addition, DCS was associated with a suggested higher complication rate: an ileal perforation at day 3 after 2 days of ciprofloxacin monotherapy in one DCS patient and sudden deafness after ciprofloxacin and ceftazidime in another DCS patient, whereas no complications were observed in the CS group[22]. Patients with DCS isolates had significantly longer fever clearance times and were hospitalised longer as compared to CS patients. There were no case fatalities.

Pharmacokinetics

The mean $fAUC_{0\text{-}24}$ per group showed no significant differences between patients infected with a DCS or CS isolate, respectively, 25.7±7.4 and 30.0±6.3 (p=0.29). However, the pharmacodynamics index of efficacy for fluoroquinolones is foremost determined by the $fAUC_{0\text{-}24}$/MIC ratio. As MICs for ciprofloxacin differed considerably between both groups, the $fAUC_{0\text{-}24}$/MIC ratios also differed between both patient groups. The mean $fAUC_{0\text{-}24}$/MIC was 41.3±18.8 in DCS strains and 585.4±219.1 in CS strains (p=0.035). Thus,

Table 1 *Clinical features and laboratory parameters on admission of 26 patients with imported, blood-culture-proven enteric fever, grouped into decreased ciprofloxacin susceptible (DCS) and ciprofloxacin-susceptible (CS) isolates. Data are given as median (range).*

Parameter	DCS (n = 17)	CS (n=9)	*p*-Value
General features			
Serovar Typhi / Paratyphi A	9 / 8	3 / 6	0.21
Male / female	12 / 5	7 / 2	0.34
Age (yrs)	32 (18-62)	29 (9-67)	0.62
Country of acquisition			
Southeast Asia[a]/ Indian subcontinent[b]	0 / 17	7 / 2	**0,000055**
Symptoms on admission			
Anorexia	4/17	0 / 8	0.19
Abdominal pain	9/17	6 / 8	0.21
Vomiting	6/17	1 / 8	0.21
Diarrhoea	10/17	6 / 8	0.27
Intestinal bleeding	1/17	1 / 8	0.45
Headache	13/17	7 / 8	0.36
Cough	7/17	4 / 8	0.31
Jaundice	0/17	0 / 8	1.00
Physical findings on admission			
Pulse (beats/min)	99 (64-120)	82 (72-120)	0.11*
Temperature (ºC)	39,5 (36.9-40.5)	39,2 (36.8-40.0)	0.17*
Pulse/ temperature ratio	2,50 (1.64-3.37)	2,06 (1.69-2.42)	0.16
Maximal temperature (ºC)	40,1 (38.2-41.0)	39,8 (38.5-40.5)	0.13
Hepatomegaly	2/17	1 / 8	0.47
Splenomegaly	2/17	2 / 8	0.30
Laboratory findings on admission			
ESR (mm/h)	27 (5-68)	31 (17-40)	0.69
CRP (mg/L)	115 (25-194)	72 (14-168)	0.16
Haemoglobin (mmol/L)	8.5 (6.1-9.9)	8.4 (7.6-9.4)	0.96
Leukocytes ($\times 10^9$/L)	6.3 (3.0-11.9)	7.1 (4.1-9.6)	0.45
Platelets ($\times 10^9$/L)	168 (33-349)	179 (115-356)	0.21
Serum LDH (U/L)	1034 (500-2764)	605 (430-1429)	0.20**
Follow-up			
Fever clearance time (days)	7 (1-18)	4 (3-6)	0.041*†
Hospitalisation duration (days)	14 (1-24)	7 (1-20)	0.034*

* For serovar Typhi: DCS vs. CS (pulse), p=0.001; DCS vs. CS (temperature), p=0,031; DCS vs. CS (fever clearance time) P=0,0080; DCS vs CS (hospitalization duration) P=0,0076, DCS vs CS (pulse/ temperature ratio), p=0,0039

** For serovar Paratyphi A: DCS vs. CS (LDH), p=0,049

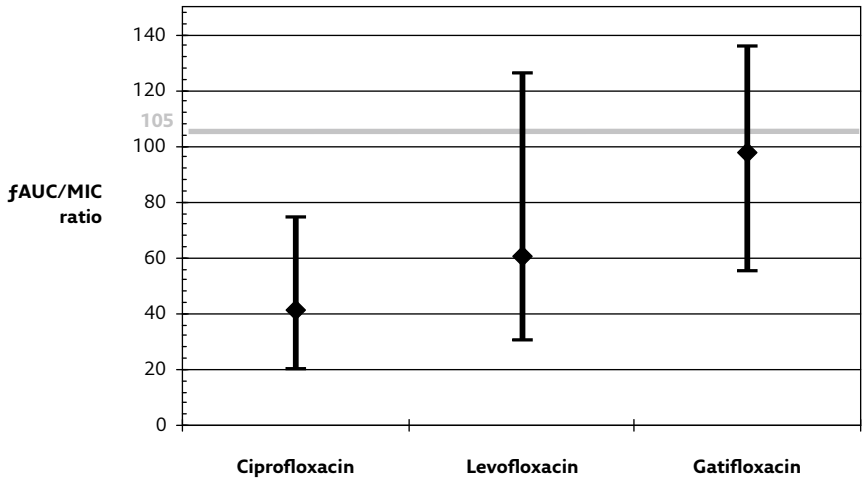
a = Indonesia, Vietnam, Malaysia, Cambodia, Thailand and Laos

b = India, Pakistan, Bangladesh, Nepal, Sri Lanka and Bhutan

all the patients infected with a CS isolate fulfilled the condition $fAUC_{0\text{-}24}$/MIC >105 and those with a DCS isolate fulfilled the condition $fAUC_{0\text{-}24}$/MIC <105. As the AUCs for ciprofloxacin are not significant different in both groups, the two options to improve the $fAUC_{0\text{-}24}$/MIC ratio are either to increase the dose or to apply agents with lower MICs. So, alternatively, we calculated the $fAUC_{0\text{-}24}$/MIC ratios for other fluoroquinolones as well. For DCS isolates, the mean $fAUC_{0\text{-}24}$/MIC for levofloxacin was 60.5±28.7 and for gatifloxacin, it was 97.9±28.0, with half of the isolates even fulfilling the critical ratio of $fAUC_{0\text{-}24}$/MIC >105 [**figure 2**].

The option would be to increase the dose to fulfil the critical ratio of 105. For every individual, we calculated the dose of ciprofloxacin, levofloxacin and gatifloxacin necessary to achieve this ratio. For our patients with DCS isolates, this would result in a dosage in the range of 1134-4240 mg/day for ciprofloxacin, 666–2206 mg/day for levofloxacin and 405–788 mg/day for gatifloxacin. Increasing the dose will lead to conceivably toxic drug levels in 50% of the patients treated with ciprofloxacin (>1500 mg/day), in 87.5% of the patients treated with levofloxacin (>1000 mg/day) and in only 25% of the patients in case of treatment with gatifloxacin (>600 mg/day).

Figure 2 *Calculated unbound fraction of the drug 24-h area under the concentration-time curve/minimum inhibitory concentration (fAUCo-24/MIC) ratios of ciprofloxacin and levofloxacin in DCS isolates (n=7) and the range in our population*



Calculated fAUC/MIC ratio's of ciprofloxacin, levofloxacin in DCS isolates (n=7) and the range in our population

Discussion

Emerging DCS isolates among enteric fever infections in countries of the Indian subcontinent and Southeast Asia limit treatment options. These infections are not only a problem in endemic regions, but may also cause clinical problems in, for instance, Western Europe due to travellers who return home ill with imported enteric fever. This problem is clearly exemplified in our study, where 17 of 26 (65%) travellers with enteric fever were infected with a DCS isolate.

We observed a prolonged fever clearance time and hospital stay for DCS, but this finding may relate to the use of ciprofloxacin as first-line treatment in the early years of the 21st century. From the year 2002 onwards, the global emergence of DCS isolates in enteric fever became apparent, also in imported cases, resulting in failure of empirical treatment with ciprofloxacin in 12 of 13 patients. Since 2007, patients from the Indian subcontinent are, therefore, treated upfront with third-generation cephalosporins.

The increase of DCS isolates and upcoming resistance to third-generation cephalosporins in enteric fever necessitates alternative therapies, because, as a result, empirical therapy may become ineffective. In case of ciprofloxacin, this is a dose-related effect, based on pharmacokinetic and pharmacodynamic principles. The $fAUC_{0\text{-}24}$/MIC ratio is used as a parameter to evaluate adequate therapy of ciprofloxacin in patients with a Gram-negative bacterial infection[20]. Increasing the dose of ciprofloxacin should theoretically result in a better cure rate in enteric fever treatment. Although three patients would have achieved an adequate $fAUC_{0\text{-}24}$/MIC ratio with 1500 mg of ciprofloxacin daily, our study also demonstrates that, for half of the patients with a DCS isolate, the ciprofloxacin dosage which should be administered will probably result in toxic levels[23]. Treatment with higher ciprofloxacin doses seems, therefore, not a feasible option.

In countries with a high burden of DCS, other fluoroquinolones have been studied as potential alternative treatment options. The best clinical evidence is available for gatifloxacin[24,25]. There are no firm clinical data about treatment with levofloxacin or moxifloxacin in DCS enteric fever. Based on PK/PD data, the administration of non-toxic doses of levofloxacin will likely result in adequate $fAUC_{0\text{-}24}$/MICs ratios, so this fluoroquinolone may be a possible option for future treatment. However, in the present study, we demonstrated that, theoretically, this could only be achieved in some of the DCS isolates. For gatifloxacin, the results looked more promising, but this fluoroquinolone was removed from market in 2006 because of its serious side effects. Since mechanisms of resistance to different fluoroquinolones are similar, the consequence of usage of higher doses of levofloxacin may be a rapid evolution in DCS isolates towards clinical resistance to all fluoroquinolones. Also, previous clinical studies with fluoroquinolones show that a sub-

stantial proportion of hospitalised patients do not reach target $fAUC_{0\text{-}24}$/MIC ratios with recommended doses[26,27]. Based on PK/PD principles only, one could argue that, for any Enterobacteriaceae organism, the clinical breakpoint should be 0.125 mg/L rather than 0.5 mg/L[19,28,29]. If pharmacokinetic variation in the population is taken into account, it would be even lower, probably 0.06 mg/L.

In areas with increased use of ciprofloxacin as a first-line drug of choice over more traditional antimicrobial agents for the treatment of enteric fever, the multidrug resistance against ampicillin, chloramphenicol and trimethoprim/sulphamethoxazole is decreasing[8]. We observed the same trend in our strain collection containing only two MDR isolates. Using these antibiotics in an individual is a treatment option when the susceptibility is proven. It should be borne in mind, however, that widespread use of these antibiotics as first-line treatment will likely result in fast re-emergence of resistance.

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CHAPTER 7

Salmonella subtypes with increased MICs for azithromycin in travelers returned to the Netherlands

EMERGING INFECTIOUS DISEASES 2014: 20; 705-8

Robert-Jan Hassing

Wil H. Goessens

Wilfrid van Pelt

Dik J. Mevius

Nicky Molhoek

Bruno H. Stricker

Annelies Verbon

Perry J. van Genderen

Abstract

Antimicrobial susceptibility was analyzed for 354 typhoidal *Salmonella* isolates collected during 1999-2012 in the Netherlands. In 16.1% of all isolates and in 23.8% of all isolates that showed increased MICs for ciprofloxacin, the MIC for azithromycin was increased. This resistance may complicate empirical treatment of enteric fever.

Enteric fever caused by *Salmonella enterica* serotypes Typhi and Paratyphi A, B and C is mainly a disease of the developing world, and it is occasionally diagnosed as an imported disease in countries where the disease is not endemic[1]. Its empirical treatment has been hampered by resistance to ampicillin, chloramphenicol, and trimethoprim and by decreased ciprofloxacin susceptibility (MIC for ciprofloxacin 0.125-1.0 µg/mL), and ciprofloxacin resistance (MIC for ciprofloxacin >1.0 µg/mL)[2,3]. As a consequence, third-generation cephalosporins are used as first-line drugs for intravenous treatment, and azithromycin is frequently used for empirical treatment of uncomplicated enteric fever.

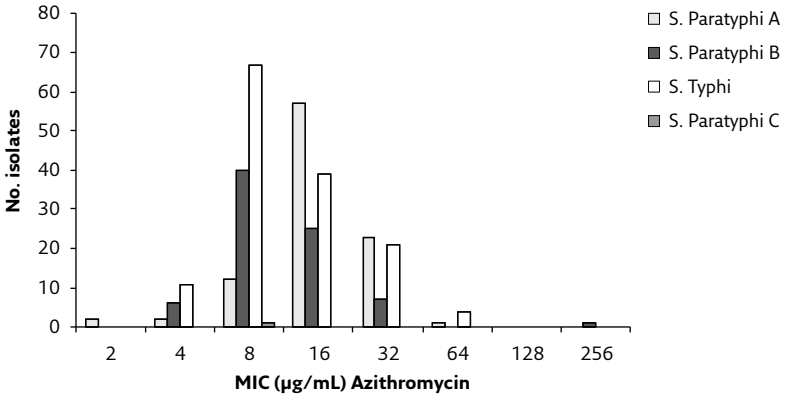
Although no clinical breakpoints are available to define azithromycin susceptibility or resistance, several clinical studies have demonstrated good efficacy of azithromycin for the treatment of uncomplicated enteric fever in clinical and in vitro studies[4-10]. Regarding MIC breakpoints, the European Committee on Antimicrobial Susceptibility Testing states that isolates with an MIC <16 µg/mL for azithromycin should be considered as wild-type organisms that are responsive to treatment[3]. The Clinical and Laboratory Standards Institute (www.clsi.org/) does not provide clinical breakpoints for macrolides for the group of *Enterobacteriaceae*. In previous studies of typhoidal *Salmonella* isolates, MICs for azithromycin ranged from 4 µg/mL to 64 µg/mL[8-13].

The first clinical case for which treatment of illness caused by typhoidal *Salmonella* spp. with azithromycin (MIC 256 µg/mL) failed was reported during 2010, evidenced by testing a *S.* enterica Paratyphi A isolate from Pakistan[14]. Further, in a study of isolates from blood samples collected in India during 2005-2008, MICs ≥16 µg/mL for azithromycin were observed in 34.7% (35/101 isolates) of the *Salmonella* isolates; clinical non-response was reported in 19 of 36 patients treated with azithromycin[13]. Whether this problem of increasing MICs for azithromycin is limited to India or is emerging globally is not clear. The objective of our study was to investigate azithromycin susceptibility and trends in antibacterial drug resistance over time in isolates collected during 1999-2012 in the Netherlands.

The Study

Enteric fever is a notifiable disease in the Netherlands. During January 1999-December 2012, a total of 354 isolates were submitted by microbiology laboratories to the Salmonella National and Community Reference Laboratory (www.eurlsalmonella.eu/): 177 (50%) *S. enterica* Typhi isolates, 98 (27.7%) *S. enterica* Paratyphi A isolates, 78 (22.0%) *S. enterica* Paratyphi B isolates, and 1 (0.3%) *S.* Paratyphi C isolate. There was no statistically significant difference in sex among patients whose tests showed *S. enterica* Typhi isolates (56.3% male, 43.7% female, p=0.18) and *S. enterica* Paratyphi isolates (51.6% male,

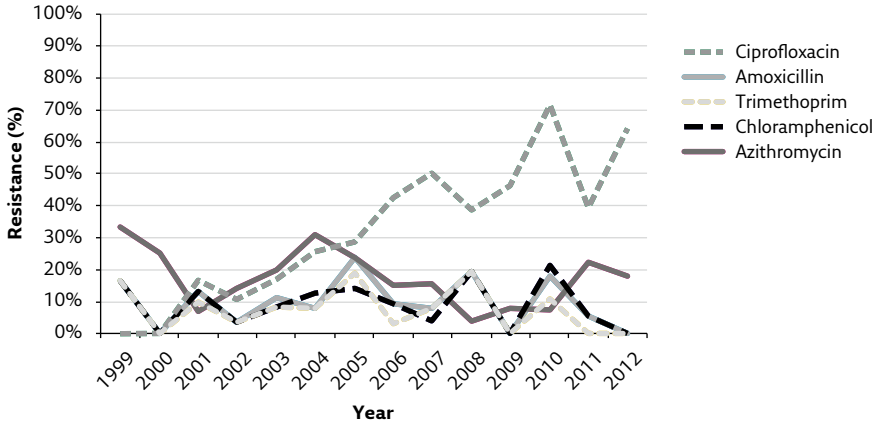
Figure 1 *MICs of azithromycin of 354 Salmonella enterica serotypes Typhi and Paratyphi A, B, and C isolates from samples collected from ill returned travelers in the Netherlands, 1999-2012. For wild type isolates, MIC ≤16 µg/mL.*



48.4% female, p=0.18). Patients ranged in age from 0 to 92 years. The median ages, 28.4 and 29.5 years, respectively (p=0.60), did not differ between patients in whose samples *S. enterica* Typhi and Paratyphi were isolated. Trends in cumulative 1-year incidence were determined by linear regression analysis; data were weighted on the number of isolates collected each year. The cumulative 1-year incidence of enteric fever was relatively stable during 1999-2012, with an average of 25 isolates (4-39/year) for that period (p=0.42).

All MICs were determined by using the broth microdilution method with Mueller-Hinton II cation–adjusted broth (Difco, Franklin Lakes, NJ, USA). We applied European Committee for Antimicrobial Susceptibility Testing guidelines for category interpretation for different antibacterial drugs[3]. Azithromycin MICs were 2–256 µg/mL among the 354 isolates and were increased in 57 (16.1%) of the isolates [**figure 1**]. The distribution of azithromycin MICs of *S. enterica* Typhi and Paratyphi A and B peaked at 8, 16 and 8 µg/mL, respectively [**figure 1**]. Trend analysis showed no increased MIC over time for all isolates (p=0.21) or for *S. enterica* Typhi (p=0.35) or Paratyphi (p=0.70). One Paratyphi A isolate, from a sample acquired in Malaysia in 2007, required an MIC of 256 µg/mL. Decreased susceptibility to ciprofloxacin was observed in 116 (32.8%) and ciprofloxacin resistance in 6 (1.7%) of the 354 isolates. Cumulative 1-year incidence of isolates with decreased susceptibility or resistance to ciprofloxacin increased significantly from 0% (0/12 isolates) in 1999 to 64.3% (18/28 isolates) in 2012 (p<0.001) [**figure 2**]. Among isolates with decreased susceptibility or resistance to ciprofloxacin, 23.8% (29/122 isolates) showed an increased MIC for azithromycin; MICs increased for 12.1% (28/232 isolates)

Figure 2 *Trends in antimicrobial resistance rates of enteric fever isolates of ill travelers returned to the Netherlands, 1999-2012. Trend analysis shows significant increase in decreased ciprofloxacin susceptibility or ciprofloxacin resistance (p<0.001).*



of the ciprofloxacin-susceptible isolates (p=0.004) [**figure 3**]. No significant increase in amoxicillin, trimethoprim and chloramphenicol resistance was observed (amoxicillin, p=0.97; trimethoprim, p=0.95; and chloramphenicol, p=0.99) [**figure 2**]. For all isolates, the MICs for erythromycin ranged from 64 to ≥512 µg/mL. Resistance to third-generation cephalosporins was not observed in the isolates.

Figure 3 *MICs of azithromycin in relation to ciprofloxacin susceptibility of 354 Salmonella enterica serotypes Typhi and Paratyphi isolates. Increased MICs for azithromycin (MIC>16 µg/mL) in isolates with decreased ciprofloxacin susceptibility or ciprofloxacin resistance (MIC≥0.125 µg/mL) versus ciprofloxacin-susceptible isolates (MIC<0.125 µg/mL) (p=0.004).*

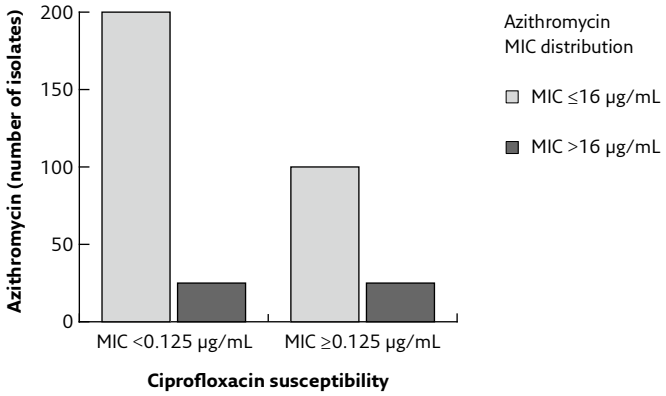


Table 1 *Isolates with elevated MICs for ciprofloxacin and isolates with an MIC for azithromycin above wild type (>16 µg/mL) in Salmonella enterica serotype Typhi and Paratyphi isolates collected in travelers returning to the Netherlands, 1999–2012.*

Region	No. (%) isolates	No. (%) isolates with elevated MICs		
		Azithromycin	Ciprofloxacin	Azithromycin and Ciprofloxacin
All	205	36 (17.6)	83 (40.5)	20 (9.8)
Asia	161 (78.5)	31 (19.3)	80 (49.7)	20 (12.4)
Southern Asia*	84 (40.0)	21 (25)	69 (82.1)	18 (21.4)
South-Eastern Asia†	44 (21.5)	5 (12.2)	3 (6.8)	1 (2.3)
Western Asia‡	27 (13.2)	1 (3.7)	6 (22.2)	1 (3.7)
Eastern Asia§	5 (2.4)	1 (20)	2 (40)	0
Unknown	1 (0.5)	0	0	0
Africa¶	35 (17.1)	5 (14.3)	1 (2.9)	0
Europe**	4 (2.0)	0	2 (50)	0
Latin America††	5 (2.4)	0	0	0

* India, Pakistan, Bangladesh, Nepal, Afghanistan, Nepal, Sri Lanka
† Indonesia, Cambodia, Malaysia, Thailand
‡ Turkey, Iraq, Syria
§ China
¶ Morocco, Ghana, Nigeria, Senegal, Tunisia, Malawi, Tanzania
** Gibraltar (Great Britain), Greece, Italy, Romania
†† Peru, Mexico, unknown

The origins of isolates for which the country of acquisition was known (205 of 354 strains) were distributed among geographical regions by using the United Nations geoscheme (http://unstats.un.org/unsd/methods/m49/m49regin.htm). Besides imported cases from countries in which enteric fever is highly endemic, such as India, Indonesia and Pakistan, rates of importation were high for travelers from Turkey and Morocco [**table 1**].

Percentages of elevated MICs for azithromycin were highest for isolates acquired in regions that had concurrent high proportions of isolates with decreased susceptibility or resistance to ciprofloxacin [**table 1**]. In isolates acquired in countries from Southern Asia, increased MICs for ciprofloxacin and increased MICs for azithromycin were observed in 21.4% (18/84 isolates) of the isolates.

Conclusions

We found high percentages of elevated azithromycin MICs in typhoidal *Salmonella* isolates collected in the Netherlands during 1999-2012. MICs >16 µg/mL for azithromycin

were found in 16.1% of all isolates and in 23.8% of isolates with elevated MICs for ciprofloxacin. This observation may be explained by increased use of azithromycin in countries from which samples yielded high rates of typhoidal *Salmonella* isolates with decreased susceptibility or resistance to ciprofloxacin. Moreover, our findings are aligned with an alarming report from India on increasing MICs for azithromycin[13]. Our study shows higher MICs than anticipated based on another Western case series[11], implying that these potentially resistant strains are not confined to India.

Besides treatment with third-generation cephalosporins, empirical treatment options may be scarce for patients with potential azithromycin-resistant Salmonella serotypes. Reuse of antibacterial drugs, such as ampicillin, chloramphenicol or trimethoprim may be a valuable treatment option upon proven susceptibility, but widespread use of these antibacterial drugs as first-line treatment will likely result in rapid reemergence of multidrug resistance and associated drug-related adverse effects. Further, increasing the dose of ciprofloxacin or using alternative fluoroquinolones had been suggested as an effective treatment in some cases[15]. This option will not be feasible for empirical treatment because it applies only in a minority of cases and may be associated with drug toxicity. The danger of losing azithromycin to antimicrobial resistance could be detrimental in countries faced with endemic or epidemic enteric fever and complicated by poverty; therefore, azithromycin should be used with care. The results of this study also implicate the importance of developing more effective vaccines as control measures for enteric fever. Future research is needed to evaluate clinically relevant breakpoints of azithromycin by analyzing the treatment outcome of azithromycin in relation to their MICs.

In conclusion, typhoidal *Salmonella* isolates in ill returned travelers from the Netherlands already show a high percentage of increased MICs for azithromycin. Because the highest proportions of increased MICs for azithromycin are found in isolates with increased MICs for ciprofloxacin and in regions where decreased susceptibility or resistance to ciprofloxacin is already widely prevalent among *S. enterica* Typhi and Paratyphi isolates, this resistance may further limit future treatment options for enteric fever.

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CHAPTER 8

Case of *Shigella flexneri* infection with treatment failure due to azithromycin resistance in a HIV positive patient

INFECTION 2014; 42: 789-90

Robert-Jan Hassing

Damian C. Melles

Bart J. Rijnders

Abstract

High rates of quinolone-resistant strains of *Shigella sonnei* in HIV-infected men who have sex with men (MSM) have recently been reported. Patients with a *Shigella* infection are often treated with azithromycin, although no clinical breakpoints are available. We would like to describe the first report of treatment failure, due to resistance to azithromycin (MIC >256 mg/L), in a HIV positive patient with a *Shigella flexneri* bacteremia.

Dear editor,

We read the paper by Hoffmann et al. on high rates of quinolone-resistant strains of *Shigella sonnei* in human immunodeficiency virus (HIV)-infected men who have sex with men (MSM) in Germany[1]. The authors report that, due to these high rates of resistant isolates, the empirical use of quinolones in HIV-infected patients presenting with *S. sonnei* infection is no longer recommended. We fully agree with this statement. Further, traditional antibiotics cannot be used as the first-line treatment, because multidrug resistance is already widely spread among *Shigella* isolates. As a consequence of the emergence of quinolone resistance in *Campylobacter* isolates, azithromycin is already widely used as empirical treatment of bacterial gastroenteritis. Patients with a gastroenteritis caused by a *Shigella* infection will, therefore, often be treated with azithromycin as first-line treatment. Although, no clinical breakpoints are available, cases of *Shigella* isolates with increased minimum inhitory concentrations (MICs) for azithromycin have already been documented in Asian countries[2]. Recently, the first observations of increased MICs for azithromycin in *Shigella* isolates have been reported in the United States[3]. Therefore, we would like to describe the first report of treatment failure in a HIV positive patient with a *Shigella* bacteremia which we treated with azithromycin.

Case report

A Dutch untreated HIV-positive man with a CD4 count of 650/ml was hospitalized in April 2012 with fever, abdominal pain, and bloody diarrhea. He had a sexual encounter with another man in Berlin, Germany 7 days earlier. As clusters of sexually transmitted *Shigella* infections among MSM have been reported previously, we suspected a *Shigella* infection. To cover *Campylobacter* and *Salmonella* as well, which are the most frequent causes of bacterial gastroenteritis in the Netherlands, we initiated therapy with azithromycin 500 mg per day. 48 h later, *Shigella flexneri* infection was confirmed in the blood and feces cultures. Because, at that time, the patient continued to have diarrhea, abdominal pain, and high fevers, we initiated intravenous therapy with ceftriaxone 2 g QD and azithromycin was discontinued. Antimicrobial susceptibility testing showed susceptibility to ciprofloxacin (MIC ≤0.25), ceftriaxone (MIC ≤1), and trimethoprim/sulphametoxazole (MIC ≤1 mg/L) but the isolates was resistant to azithromycin (MIC >256 mg/L). To confirm the increased azithromycin MIC, initially obtained by the Etest, the MIC was determined by the broth microdilution method with Mueller-Hinton II cation-adjusted broth (MIC=128 mg/L). Repeated blood cultures after treatment were negative. The patient became afebrile 48 h after the initiation of ceftriaxone and he fully recovered without complications.

Shigella infections among MSM were first reported in San Francisco in 1998[4] but later outbreaks were also reported in other cities, like Berlin, London, Amsterdam, Chicago, Montreal and Vancouver. In only one of these reports was susceptibility to azithromycin tested and the MIC was 8 to 16 mg/L[4]. Although no clinical breakpoints are available, on epidemiological grounds, it can be suggested that an MIC ≤16 mg/L is comparable to the MIC distribution in wild-type *Shigella* isolates and, therefore, expected to respond to treatment[5]. The frequent use of azithromycin for the treatment of sexually transmitted infections among MSM in Europe may have contributed to the azithromycin resistance in *S. flexneri* observed in our patient. Since July 2010, all *Shigella* isolates from the Erasmus Medical Center in Rotterdam have been tested for azithromycin susceptibility by the Etest method. All the other 15 consecutive isolates tested during this period showed an MIC ≤16 mg/L, compatible with the wild-type distribution. Although increased MICs in *Shigella* isolates are still rare, we advise that MICs for azithromycin should always be determined in patients with a *Shigella* infection. It should be encouraged to report MICs for azithromycin in surveillance studies in order to contribute to the establishment of clinical breakpoints and to monitor the emergence of resistance. Clinical outcome studies are required to establish the clinical breakpoints for azithromycin. In a study examining invasive cases of *Shigella* in South Africa, HIV-infected cases were 4.1 times more likely to die than HIV-uninfected cases[6]. Because the clinical course of a *Shigella* infection may progress faster in HIV-positive patients, a third-generation cephalosporin should be considered if the patients presents with severe sepsis and the clinical presentation suggests Shigella infection.

Acknowledgments

Denise Vermeulen-de Jongh is acknowledged for the susceptibility testing.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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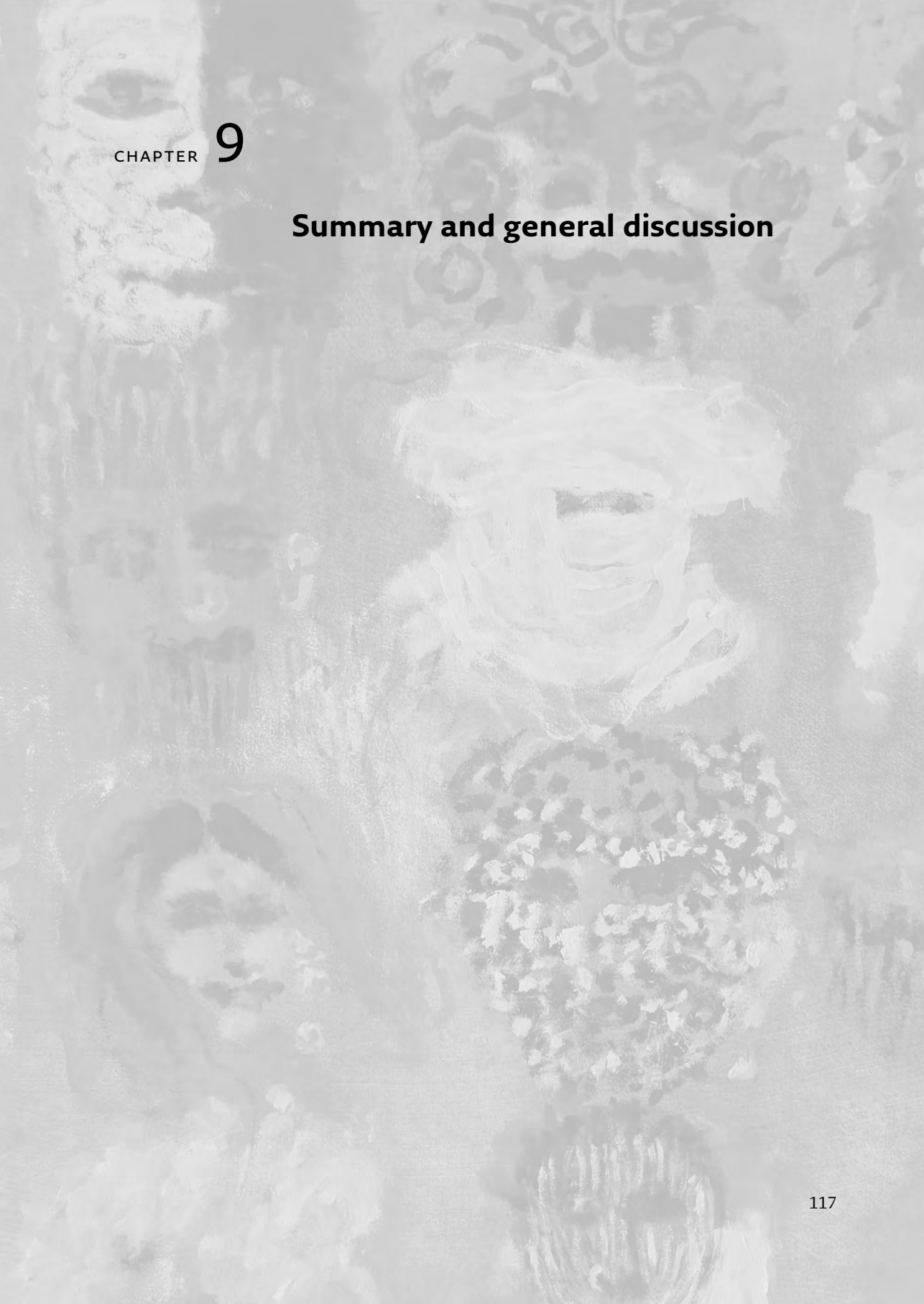
CHAPTER 9

Summary and general discussion



Introduction

In this thesis, the objective was to study management of antimicrobial resistance in gastrointestinal bacteria. This was performed by investigating risk factors, analysing mechanisms involved in antimicrobial resistance and developing new diagnostic tools to detect resistance in gastrointestinal bacteria. Of all gastrointestinal infections caused by the usual bacterial pathogens (*Salmonella, Campylobacter, Shigella* and *Yersinia*), typhoidal *Salmonella* species are the only pathogens requiring antimicrobial treatment in immunocompetent patients. Most of the studies in this thesis therefore focus on typhoidal *Salmonella* isolates.

Main findings

Risk factors for gastroenteritis and antimicrobial resistance

An association between proton pump inhibitor (PPI) therapy and bacterial gastroenteritis has been suggested as well as contradicted[1-10]. As a result, warnings are introduced for people using PPIs, such as avoiding raw meat consumption and antibiotic treatment on demand for travels to the tropics, to prevent food borne infections. The aim of the study in **Chapter 2** was to analyse this association in the prospective Rotterdam Study, a population-based cohort study among 14 926 subjects aged 45 years and older with up to 24 years of follow-up[11].

In this study, we found that participants with a bacterial gastroenteritis were more likely than controls to be current users of PPIs (adjusted OR, 1.94; 95% CI 1.15-3.25). A considerably higher effect was observed (adjusted OR 6.14; 95% CI 3.81-9.91), using the total cohort as a reference in a nested case-control analysis. We showed that current PPI therapy is associated with an increased risk of bacterial gastroenteritis. However, by reducing the risk of selection and information bias in our study design, we demonstrated that the effect is lower than previously assumed.

International travel is considered to be an important risk factor for acquisition of multiresistant *Enterobacteriaceae*[12-22]. The aim of the systematic review in **Chapter 3** was to determine the effect of international travel on the risk of post-travel faecal carriage of multidrug-resistant *Enterobacteriaceae* (MRE). A systematic search for relevant literature in seven international databases was conducted according to PRISMA guidelines. Eleven studies were identified. In this systematic review, we demonstrated that international travel is a major risk factor for acquisition of MRE. This risk is particularly high after travelling to (Southern) Asia and in persons with travel related diarrhoea and antibiotic therapy for travellers' diarrhoea. Further, high percentages of resistance to ciprofloxacin, co-trimoxazole and aminoglycosides were observed in travellers with MRE carriage after travel.

Antimicrobial resistance

Owing to multidrug resistance, and an increase in fluoroquinolones resistance, it becomes more problematic to combat *Salmonella* organisms[23]. Different bacterial resistance mechanisms may result in reduced ciprofloxacin susceptibility[24]. In the study in **Chapter 4**, the presence and expression of different resistance mechanisms resulting in reduced minimum inhibitory concentrations (MICs) for ciprofloxacin were evaluated in 23 blood-culture-derived *Salmonella enterica* serotypes Typhi and Paratyphi A organisms from ill-returned travellers to Asia. Reduced ciprofloxacin susceptibility was only found in travellers returning from India and Pakistan. All isolates with reduced ciprofloxacin susceptibility had a mutation at position 83 in the QRDR region of the *gyrA* gene. Efflux pump inhibition did not appear to affect the MICs of ciprofloxacin and activity of the efflux pump appeared to be specific for resistance to nalidixic acid. Repeated exposure of the isolates to ciprofloxacin did not result in a significant increase in the MICs for ciprofloxacin. Repetitive sequence-based polymerase chain reaction (rep-PCR) profiles identified five different genotypes, but no correlation with resistance was observed. Plasmid-mediated fluoroquinolone resistance was not found. This study shows that the reduced ciprofloxacin MIC in *S.* Typhi and *S.* Paratyphi A is solely due to an amino acid substitution in the QRDR 'cluster' of the *gyrA* gene.

Though these QRDR mutations are invariably detected by sequencing of the *gyrA* gene, phenotypically important QRDR mutations also result in amino acid substitutions in the GyrA protein of typhoidal *Salmonellae*, which could potentially be detected using high resolution mass spectrometry techniques[25,26]. In **Chapter 5** a liquid chromatography-mass spectrometry (LC-MS) methodology was developed and evaluated for the detection of amino acid substitutions in the GyrA protein of 23 typhoidal *Salmonella* isolates, with different antibiotic sensitivities to fluoroquinolone antibiotics. PCR sequencing of the *gyrA* gene was used to validate the results. The LC-MS methodology correctly identified peptide sequences associated with phenotypic QRDR mutations of the GyrA protein in all of the 23 phenotypically diverse typhoidal *Salmonella* isolates tested. A reliable and rapid LC-MS methodology has been developed, which is able to identify *gyrA* QRDR mutations that are involved in the development of fluoroquinolone resistance in typhoidal *Salmonella* species.

In **Chapter 6** we studied the impact of decreased ciprofloxacin susceptibility (DCS) isolates (due to changes in EUCAST expert rules currently defined as ciprofloxacin resistant isolates; http://www.eucast.org/expert_rules/) on the fate of travellers returning with a typhoidal *Salmonella* infection and possible alternative treatment options. We evaluated the clinical features, susceptibility data and efficacy of empirical treatment in patients with positive blood cultures of a DCS isolate compared to patients infected with a ciprofloxacin-susceptible (CS) isolate in the period from January 2002 to August 2008.

In addition, the pharmacokinetic and pharmacodynamic parameters of ciprofloxacin, levofloxacin and gatifloxacin were determined to assess if increasing the dose would result in adequate unbound fraction of the drug 24-h area under the concentration–time curve/minimum inhibitory concentration ($fAUC_{0-24}$/MIC) ratio. DCS was associated with a longer fever clearance time and length of hospital stay compared to patients in whom the initial empirical therapy was adequate. We demonstrated that, in some cases, an adequate $fAUC_{0-24}$/MIC ratio could be achieved by increasing the dose of ciprofloxacin or by the use of alternative fluoroquinolones.

Because of the emergence of ciprofloxacin resistance, azithromycin is now often used as first line treatment for enteric fever, although clinical breakpoints and epidemiologic data are lacking[27-34]. In **Chapter 7** we analysed the incidence and variation in antibiotic resistance over time of 354 typhoidal *Salmonella* isolates collected from January 1999 to December 2012 in The Netherlands. In 16.1% of all isolates the MIC for azithromycin was increased. A significantly higher proportion (23.8%) of isolates with increased MICs for azithromycin was found in isolates with decreased susceptibility or resistance to ciprofloxacin and in regions where decreased susceptibility or resistance to ciprofloxacin is already widely prevalent among typhoidal *Salmonella* isolates. This resistance may complicate future empirical treatment of enteric fever.

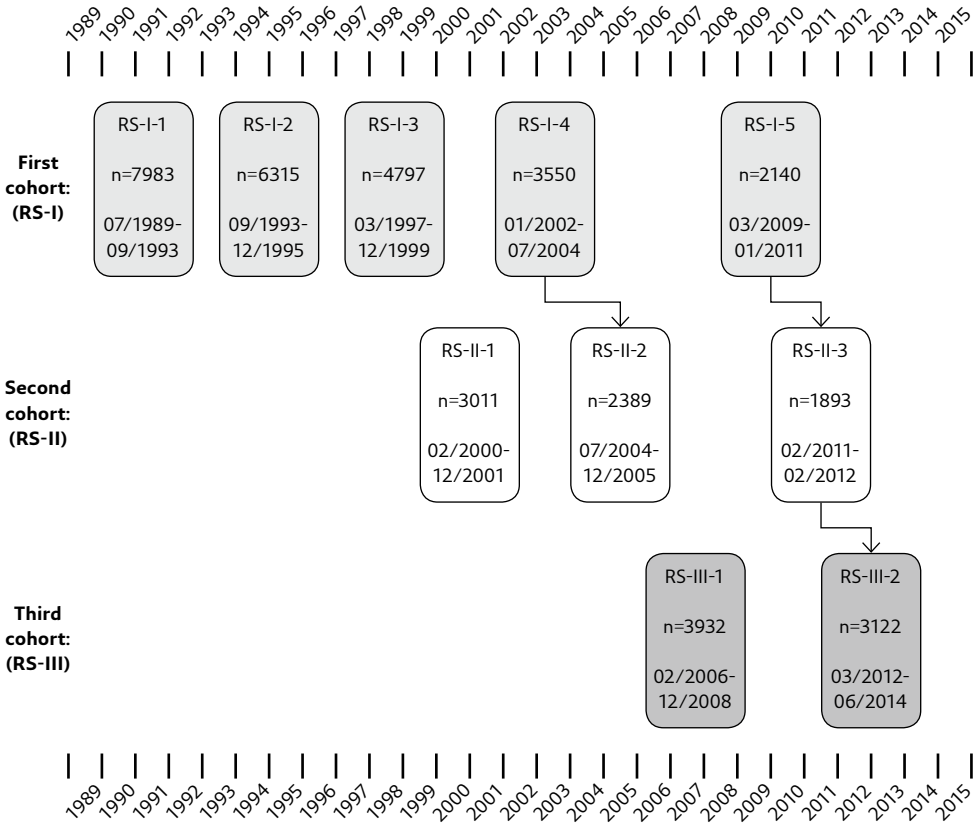
Fortunately, resistance to azithromycine and treatment failure in gastrointestinal pathogens is still very uncommon[35-38].

Methodological considerations

Study setting and design (The Rotterdam Study)

To study the association between PPIs and the bacterial gastroenteritis in **Chapter 2** The Rotterdam Study was used, a prospective population-based cohort study in 14,926 people aged ≥45 years, from one district (Ommoord) in the city of Rotterdam, the Netherlands11. In short, from 1990 through 1993, 7983 participants were included (cohort I). In 2000, an additional 3011 participants who had become 55 years old or older or who had moved into the district, were enrolled (cohort II). In 2006, another 3932 participants, aged 45 years and older were included (cohort III) [**figure 1**]. Follow-up examinations are conducted every four to five years. Participants are continuously monitored through linkage of records from general practitioners. Results of bacterial pathogens (*Salmonella, Campylobacter, Shigella* and *Yersinia* species) isolated from stool samples were obtained from Star Medisch Diagnostisch Centrum (Star-MDC), a centre for medical diagnostics for outpatients in the city of Rotterdam. The majority of all laboratory tests, including microbiology tests, of patients from general practitioners within the Ommoord district of Rotterdam are performed at Star-MDC. Of all participants of The Rotterdam Study, of

Figure 1 *Design of The Rotterdam Study*[11]



whom informed consent was obtained for requesting medical information, positive and negative microbiology tests between 1999 and April 2013 were obtained.

Randomized controlled trials are often considered as the golden standard to study efficacy and effectiveness, and common adverse reactions of drugs[39]. However, several adverse events, such as increased susceptibility to gastrointestinal infections, are relatively rare and therefore other observational study designs are more eligible to investigate this kind of research questions[39]. In general, to study a rare outcome (disease), a case control design is considered to be the most appropriate design but when a rare determinant (risk factor) is expected, a study design using a cohort control study is preferred[39]. PPIs are widely used worldwide, and also in our study population. Occurrence

of a bacterial gastroenteritis is relatively rare (11 cases per 1000 people per year) in terms of observational study design, which was also observed in our study[40]. Based on this information it is a rational approach of the authors of most of the previous studies to use a case control study design to address the association between PPI therapy and bacterial gastroenteritis. However, this study design is also prone to selection and information bias. Studies considering self-limiting diseases such as gastroenteritis, might select a "help seeking" and therefore biased and selected population. As a consequence, people using a PPI will be more inclined to consult medical help in case of gastroenteritis compared to randomly selected controls, even if they are matched in a case-control study. Recall bias, a well-known example of information bias, is an important problem in case control studies in which questionnaires are used to access risk factors. Further, there is an increased risk of residual confounding in case-control studies compared to prospective cohort studies, because relevant information is not systematically and retrospectively collected. For these reasons a prospective population-based cohort study, such as The Rotterdam Study, is probably the best study design to address our research question and minimize the risk of selection or information bias and residual confounding. Within The Rotterdam Study, we were able to investigate the association between PPI therapy and bacterial gastroenteritis in a nested case-control study design. Based on the previous arguments, the selection of a comparable control group failed in virtually all previous studies with a similar research question. As a result, most of these studies may have suffered from a "healthy control" bias, because of the use of an incomparable healthier control groups (using random samples from population registries, or volunteering friends or relatives of the cases as control group). We tried to avoid healthy control bias by only using participants with negative stool samples as control group and correcting for the use of chronic medication.

Our study may also have suffered from bias and residual confounding. We had no data on other important risk factors for bacterial gastroenteritis, such as foreign travelling, eating in a restaurant, or contact with animals. Probably the most important risk factor for bacterial gastroenteritis is the dietary pattern, because transmission of the pathogen goes by raw meat, especially chicken[41,42]. An association with dietary data was not observed in our study. Unfortunately, the number of missing dietary data in our study was rather high with as a consequence a large number of imputed data. Also, the dietary data in our study were collected during one week and do therefore not necessarily represent the dietary pattern at the time of infection.

A limitation of our study design, using negative test results, may be underestimation of the risk. A number of the controls with negative stool cultures may be false-negatives. They can be false-negative either because of low diagnostic sensitivity against the four bacterial species (e.g., too little material received, long transportation time, stool collected many days after onset of gastroenteritis) or because the patient suffered from other causes of bacterial gastroenteritis (diarrheagenic *Escherichia coli* or *Clostridium difficile*).

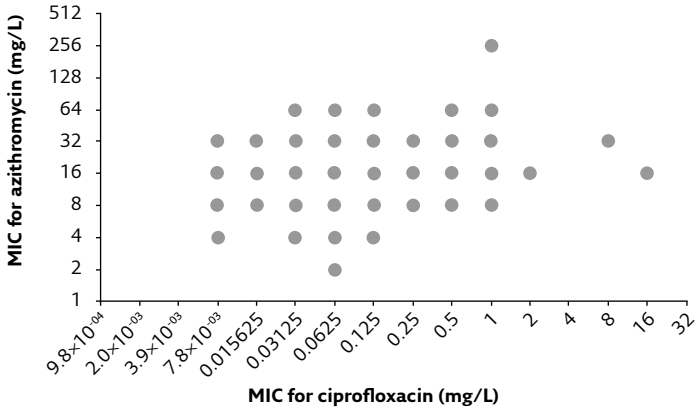
Clinical cohort

For **Chapter 4**, **5** and **6** a collection of 23 well-characterized blood culture-derived typhoidal *Salmonella* isolates was used that had been obtained from patients during the period January 2002 to August 2008 obtained from patients attending the Harbour Hospital and Institute for Tropical Diseases, Rotterdam, The Netherlands. All travellers diagnosed with blood-culture-confirmed *S.* Typhi and *S.* Paratyphi infections in this hospital were included and clinical symptoms, physical findings and laboratory findings were collected retrospectively. Serotype determination was performed at the Salmonella National and Community Reference Laboratory (www.eurlsalmonella.eu/) by the National Institute for Public Health and the Environment (RIVM), Bilthoven the Netherlands. All travellers did acquire the infection in Asia. Although the majority of endemic cases of typhoidal *Salmonella* infections worldwide are from Asian countries, it is still remarkable that this cohort of isolates does not include any isolate acquired in Africa[23]. This is probably because the number of Dutch travellers to Asia strongly exceeds the number of travellers to Africa. Also high risk countries for enteric fever, such as India, Nepal and Indonesia are very popular travel destinations for Dutch travellers. Another possible explanation might be that travellers to Africa are more aware of hygiene related health risks, compared to travellers to Asia. In Asia eating local food plays an important role during travel, whereas this is less the case in Africa. Furthermore, Asia is a more popular destination for 'backpackers' who are usually more exposed to local food and lesser hygiene. The fact that our cohort consists of solely Asian isolates also explains a relatively high percentage of resistant Salmonella isolates, because antimicrobial resistance in enteric fever is mainly a problem in Asian countries[23].

RIVM cohort

Enteric fever is a notifiable disease in the Netherlands (group B2). For **Chapter 7** we used all available typhoidal isolates detected in the Netherlands during January 1999-December 2012. During this period a total of 354 isolates were submitted by microbiology laboratories in the Netherlands to the EURL. It is remarkable that isolates sent to the EURL by local laboratories covered only 43% of the overall notifications to the RIVM, whereas this is expected to be 100% because a notifiable disease is required by law to be reported to government authorities. As a consequence, **Chapter 7** could have suffered from selection bias. The reason for not sending in the isolate, will probably be unawareness off the laboratory and will probably not relate to the determinant (country of acquisition) or outcome (antimicrobial resistance) studied in Chapter 3.4 Because all isolates were sent anonymously to the EURL, we only had information on gender, age and travel destination and a multivariate analysis was not performed. Therefore, there might always be a risk of residual confounding. It would have been interesting to include some additional covariates to this analysis, such as use of antimicrobial drugs, chronic diseases and use of proton pump inhibitors.

Figure 2 *MIC distribution for azithromycin of 354 S. Typhi and S. Paratyphi A, B and C isolates, collected from 1999–2012, from ill returned travellers in the Netherlands. For wild type isolates MIC ≤16 mg/L.*



In **Chapter 7** an association was observed between increased MICs for ciprofloxacin and increased MICs for azithromycin [**figure 2**]. In a recent phylogeographical analysis, a single multidrug resistant lineage, H58, proved to be responsible for the emergence of these resistant isolates throughout Asia and Africa[34]. The phylogenetic agreements of these isolates, which have not been analyzed in our study, probably explain this association.

Clinical implications

Use of PPIs and bacterial gastroenteritis

Although we found a lower association (odds ratio 1.9) between use of PPIs and bacterial gastroenteritis in **Chapter 2** compared to previous studies, this association was not rejected by our study. As a result, clinical doctors and patients must be aware of the risk and patients should take hygiene precautions into account, such as avoiding to eat undercooked eggs. On the other hand, the association concerns a relatively low risk and a relatively rare disease. Patients should therefore not be scared and be discouraged to participate in barbeques or to travel to countries with lower hygiene standards. For the same reason, prescribing prophylactic antibiotics to every traveller using a PPI should be discouraged. Antibiotic therapy for travellers' diarrhoea has even been shown to be one of the major risk factors for acquisition of MRE in **Chapter 3**

International travel and acquisition of MRE

In **Chapter 3** a very high prevalence of carriage of MRE after international travel was found. Routine screening for asymptomatic faecal carriage of MRE seems indicated in hospitalized patients with a recent travel history to high risk countries. It is still not clear what the duration of carriage is after travel. For infection prevention and control guidelines this information will be essential to create new screening strategies.

Diagnostic tools for detecting antimicrobial resistance in typhoidal Salmonella

Susceptibility testing of micro-organisms is still performed in a classic fashion by determining the Minimal Inhibitory Concentration by 18 h exposure to different concentrations of the antibiotic. Further, due to new mechanisms of resistance not only classic susceptibility testing is performed but extended by phenotypic screenings-assays sometimes in combination with PCRs to detect/confirm the presence of mechanisms of resistance. The reason for performing PCRs is that some mechanisms of resistance are not accurately detected by susceptibility testing due to the fact that systems are not able to detect small shifts in MICs. With the alternative technique i.e. whole genome sequencing, the genetic information is obtained but no information of expression levels of the genes is provided. Due to the delay in obtaining susceptibility results and the increase in mechanisms of resistance there is a need for alternative techniques which are quicker with the same and perhaps better accuracy than current techniques. In the study in **Chapter 5** we show a proof of principle that with high resolution MS we are able to demonstrate the presence of non-synonymous amino-acid substitutions in the gyrase enzyme in *Salmonella*. This methodology can also be used to detect different beta-lactamases and aminoglycoside modifying enzymes. By improving the sample pretreatment of samples in combination with the development of a database consisting out of adequate information of proteins involved in antibiotic resistance, this technique could become a quick and accurate alternative for current resistance determinations.

Treatment

In **Chapter 7** it is shown that third generation cephalosporin's or azithromycin are the only empiric antibiotic treatment options in travellers who acquired enteric fever in Southern Asia. Based on the results of **Chapter 3**, in a traveller who very recently returned from Southern Asia with a severe septicaemia, (combined) resistance to cephalosporin's, aminoglycosides and fluoroquinolones will probably be (much) higher than 10% and should be taken into account in the choice of empiric antibiotics. This advice seriously conflicts with Dutch guidelines being very restrictive with prescription of broad spectrum antibiotics, resulting in the lowest prevalence of antibiotic resistance worldwide. Therefore, more research is needed to assess if newly acquired MRE carriage also leads to an increase of infections with MRE.

Future treatment options

In **Chapter 6** we demonstrated that, in some cases, a patient infected with a fluoroquinolone resistant *Salmonella* isolate could potentially be treated with fluoroquinolones by increasing the dose of ciprofloxacin or by the use of alternative fluoroquinolones and achieving an adequate $fAUC_{0\text{-}24}$/MIC ratio. In our study, increasing the dose to an adequate $fAUC_{0\text{-}24}$/MIC ratio will lead to conceivably toxic drug levels in 50% of the patients treated with ciprofloxacin. If alternative treatment options are available, increasing the dose of ciprofloxacin or using alternative fluoroquinolones will therefore not be an advisable treatment option.

Typhoidal *Salmonella* infections should be treated with third-generation cephalosporin's or azithromycin empirically. Reuse of antibacterial drugs, such as ampicillin, chloramphenicol or trimethoprim may be a valuable treatment option upon proven susceptibility, but widespread use of these antibacterial drugs as first-line treatment will likely result in rapid reemergence of multidrug resistance and associated drug-related adverse effects.

Azithromycin in the treatment of Salmonella

Good efficacy of azithromycin in the treatment of *Salmonella* infections have been shown in clinical trials. Although cases with treatment failure with azithromycin are very uncommon, increased MICs of azithromycin have already been reported[35,36]. Even more worrisome, we demonstrated in **Chapter 7** an increased MIC for azithromycin in 23.8% of the isolates already possessing increased MICs for ciprofloxacin. This highly resistant *Salmonella* strain already emerged from Asian countries to several African countries[37]. The danger of losing azithromycin to antimicrobial resistance could be detrimental; therefore, azithromycin should be used with care.

Taken together, emergence of antimicrobial resistance in gastrointestinal bacteria should be combated by determining risk factors, analysing mechanisms involved in resistance and developing faster diagnostic tools to detect resistance.

Future research

Use of PPI and travellers' diarrhoea

It would be interesting to assess the association of PPI use and gastroenteritis in a prospective study design. In a normal setting this design would not be possible, because it concerns a relatively rare disease[38]. In a population of travellers to the tropics, the incidence of travellers' diarrhoea might be high enough. Travellers' diarrhoea also includes Escherichia coli related diarrhoea. There is no literature about an association between PPI use and travellers' diarrhoea, but diminishing gastric acid and changes of microbiota will probably lead to increased survival of diarrheagenic Escherichia coli

as well. Participants could be studied using questionnaires during travel about stool pattern. Detection bias might be a problem, because people using a PPI will always be warned for the risk of gastroenteritis, while visiting a travel clinic. Therefore the control group should also be warned for gastroenteritis in a routine way. Multivariate analysis should be performed, because there is still a high risk of bias and residual confounding.

International travel and infections with MRE and spread to the community

Two crucial questions still remain unanswered: does increased carriage of MRE results in infections with MRE and does increased carriage of MRE results in spread to the community? These questions can only be answered by including these analyses in one of the big travel studies, such as the French VOYAG-R study or the Dutch COMBAT study[20,39]. Follow up of participants should be performed, monitoring infections including antimicrobial susceptibility data. Household contacts should also be screened for carriage of MRE.

International travel and microbiota

There is increasing evidence that the microbiota of the gut plays an important role in many diseases. A normal microbiota protects the human body against pathogens [ref]. It is interesting to study if the gut microbiota 1. changes during international travel 2. Protects against bacterial gastroenteritis and/or travellers' diarrhoea during travel 3. Protects against newly acquisition of MRE?

High-resolution mass spectrometry

We developed and evaluated a "proof-of-principle" methodology using LC-MS for the detection of amino acid substitutions in the GyrA protein of typhoidal *Salmonella* isolates. Future research based on our findings can show the usefulness of our LC-MS methodology for the detection of other clinically relevant antimicrobial resistance phenotypes, for example amino acid substitutions in Penicillin-Binding Proteins (PBPs), or amino acid substitutions in the active site of β-lactamase enzymes, possibly in a new multiplexed methodology.

General prospects

Gastrointestinal infections usually not get as much attention as other infectious diseases like for example HIV, tuberculosis or *Staphylococcus aureus* infections. Maybe because serious gastrointestinal infections mainly occur in developing countries. Enteric fever is still a neglected disease, resulting in many deaths every year in these countries. Although enteric fever is a quite rare disease among travellers, still a few dozens of Dutch travellers acquire this potentially lethal disease every year. This number is not decreasing, although (partially covering) vaccination is available. It is therefore worrisome that

the indication of vaccination is now being restricted, driven by shortage of the vaccine as a result of manufactory problems. This thesis highlights some other aspects of enteric fever, which goes behind just incidence data. For example, we show that most cases of enteric fever in the Netherlands were acquired in Asian countries and these typhoidal *Salmonella* isolates were highly resistant to antibiotics. This fact should be taken into account when considering to vaccinate an individual. Vaccination indications for high risk Asian countries should therefore not be limited. Studies like ours should be used to convince policymakers about the severity of this disease. The vaccine for typhoid fever should be improved (to achieve a broader coverage), instead of reduced in production by pharmaceutical companies.

In the future different antimicrobial agents might be needed to combat antimicrobial resistance in gastrointestinal bacteria. Old antibiotics such as colistin and fosfomycin are used again as an ultimate refuge antimicrobial drug in the treatment of infections caused by multidrug resistant Gram-negative bacteria[43]. Worrisome, emergence of plasmid-mediated colistin resistance in *Enterobacteriaceae* have recently been observed in China[44]. Carriage of this colistin resistant *Enterobacteriaceae* have also been detected in healthy Dutch travellers[45]. These observation further stresses the importance to combine clinical-, epidemiological- and laboratory research, as demonstrated in this thesis.

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Samenvatting

List of publications

PhD portfolio

Dankwoord

About the author

In **Hoofdstuk 1** wordt een introductie gegeven over bacteriële gastro-intestinale infecties en antibiotica resistentie bij darmbacteriën. Bacteriële gastro-intestinale infecties zijn wereldwijd een veel voorkomend probleem. De meest voorkomende bacterie soorten die deze infecties veroorzaken zijn *Campylobacter, Salmonella, Shigella* en *Yersinia* soorten. Dit zijn allen Gram-negatieve bacteriën die behoren tot de familie van de *Enterobacteriaceae*. Binnen deze bacterie soorten nemen de tyfeuze *Salmonella* soorten, die het ziektebeeld buiktyfus veroorzaken, een bijzondere plek in. Gastro-intestinale infecties hebben, bij personen met een normale afweer, doorgaans een self-limiting beloop. Een buiktyfus infectie moet echter wel antibiotisch behandeld worden. Buiktyfus wordt in Nederland vooral als importziekte gezien bij reizigers. De afgelopen jaren wordt er een toename in antibiotica resistentie waargenomen bij tyfeuze *Salmonella* bacteriën. Dit proefschrift bestaat om die rede voornamelijk uit studies die betrekking hebben op tyfeuze Salmonella bacteriën. Naast het bestuderen van antibiotica resistentie mechanismen, hebben we in dit proefschrift ook studies uitgevoerd naar risicofactoren voor het krijgen van gastro-intestinale infecties en antibiotica resistentie.

Voorkomen is beter dan genezen. In **Hoofdstuk 2** wordt de associatie beschreven tussen het gebruik van maagzuurremmers en bacteriële gastro-enteritis. Deze studie is uitgevoerd binnen de Rotterdam Studie, een prospectieve cohortstudie in een algemene populatie van 14.926 mensen van 45 jaar en ouder. Wij laten zien dat bij gebruikers van maagzuurremmers het risico op bacteriële gastro-enteritis significant hoger is dan bij vergelijkbare mensen die geen maagzuurremmers gebruiken. Indien wij echter goed corrigeren voor informatie en selectie bias, blijkt dit risico een stuk minder hoog te zijn dan verwacht op basis van eerdere studies.

Internationaal reizen wordt gezien als risicofactor voor dragerschap van multiresistente *Enterobacteriaceae* in de darm, zoals *Escherichia coli* (*E. coli*). Dit zijn normaal gesproken geen pathogene bacteriën, maar onder bepaalde omstandigheden kunnen ze wel ernstige infecties kunnen veroorzaken, zoals urineweginfecties en sepsis. In **Hoofdstuk 3** laten wij in een systematisch review zien dat het percentage dragerschap in de darm van deze multiresistente bacteriën bij net teruggekeerde reizigers ernstig verhoogd is. Het hoogste percentage dragerschap werd gezien bij reizigers teruggekeerd uit (zuidelijk) Azië, en bij reizigers met gastro-enteritis en/of antibiotica gebruik tijdens de reis. Een andere zorgwekkende observatie in het systematisch review was dat deze bacteriën, naast resistentie voor 3^{e} generatie cefalosporines ook vaak resistent zijn voor fluoroquinolonen, cotrimoxazol en aminoglycosiden.

Tyfeuze *Salmonella* bacteriën zijn al ruim 20 jaar multiresistent voor antibiotica (gedefinieerd als resistent voor amoxicilline, chlooramfenicol en cotrimoxazol). Sinds ongeveer 10 jaar is er in diverse Aziatische landen een toename van ciprofloxacine resistentie, wat de behandeling van buiktyfus verder bemoeilijkt. In **Hoofdstuk 4** analyseren we de verschillende antibiotica resistentie mechanismen die een rol kunnen spelen in ciprofloxacine resistentie bij 23 tyfeuze *Salmonella* bacteriën geïsoleerd bij reizigers. In alle gevallen blijkt een bekende puntmutatie (positie 83) in het *gyrA* gen de oorzaak van ciprofloxacine resistentie. Er werden geen andere resistentie mechanisme gevonden die verantwoordelijk kunnen zijn voor ciprofloxacine resistentie.

Resistentie als gevolg van een gen mutatie kan worden gedetecteerd door middel van polymerasekettingreactie (PCR). Een gen mutatie leidt alleen tot antibiotica resistentie wanneer de mutatie ook verandering van het bijbehorende aminozuur tot gevolg heeft, resulterend in een ander eiwit. In het geval van ciprofloxacine resistentie bij *Salmonella* bacteriën is dat het *GyrA* eiwit. Eiwitten kunnen worden gedetecteerd door middel van massaspectrometrie. In **Hoofdstuk 5** tonen wij een studie waarin een methode met vloeistofchromatografie met massaspectrometer (LC-MS) wordt ontwikkeld en geëvalueerd om mutaties in het *GyrA* eiwit te detecteren in dezelfde 23 tyfeuze *Salmonella* bacteriën als gebruikt in Hoofdstuk 3.1. We laten zien dat het in onderzoekssituatie mogelijk is om met grote zekerheid en in korte tijd de mutaties te detecteren die tot ciprofloxacine resistentie leiden. Deze methode zou theoretisch ook toegepast kunnen worden voor antibiotica resistentie bij andere klassen antibiotica.

Farmacokinetiek is essentieel bij het bepalen van resistentie bij fluorochinolonen. In **Hoofdstuk 6** tonen wij klinische parameters, antibiotica gevoeligheid en werkzaamheid van empirische behandeling van patiënten met een bewezen buiktyfus infectie. In deze studie worden patiënten met een ciprofloxacine ongevoelige stam vergeleken met een gevoelige stam. Tevens wordt bij deze patiënten de farmacokinetiek (($fAUC_{0\text{-}24}$/MIC) ratio) getoond van verschillende fluorochinolonen. Verminderde ciprofloxacine gevoeligheid is geassocieerd met langere tijd tot koortsvrij zijn en een langere ziekenhuis opname duur. We laten zien dat in sommige gevallen van buiktyfus met een ciprofloxacine ongevoelige stam, toch een adequate behandeling zou kunnen worden bewerkstelligd door de dosering van ciprofloxacine te verhogen of door te behandelen met een ander fluorochinolon (levofloxacine of gatifloxacine).

Vanwege de forse toename van ciprofloxacine resistentie wordt azitromycine nu dikwijls gebruikt als eerstelijns behandeling voor ongecompliceerde buiktyfus infecties. In **Hoofdstuk7** laten wij zien dat ondanks dat azitromycine resistentie bij tyfeuze *Salmonella* bacteriën zeer zeldzaam is, de MIC (minimum inhibitory concentration) voor azitromycine al verhoogd is bij 16.1% van alle *Salmonella* stammen, geïsoleerd in Neder-

land tussen 1999 en 2012. De MIC voor azitromycine was significant vaker verhoogd bij isolaten die ook resistent zijn voor ciprofloxacine.

In **Hoofdstuk 9** wordt een overzicht gegeven van de belangrijkste bevindingen van dit proefschrift. Ook worden de methodologische overwegingen, klinische implicaties van de studies gegeven en wordt stilgestaan bij perspectieven voor de toekomst. Indien we nog meer antibiotica gaan verliezen aan resistentie moeten we misschien ook bij buiktyfus terug vallen op ouderwetse antibiotica. Chlooramfenicol zou hier een voorbeeld van kunnen zijn. Er wordt steeds meer bekend over de rol van het microbioom bij verschillende ziektebeelden. Mogelijk speelt het microbioom ook een belangrijke rol bij het krijgen van infecties en antibiotica resistentie. Er is echter meer onderzoek nodig om hier beter inzicht in te krijgen.

Vanwege de vele facetten die invloed hebben op antibiotica resistentie is het belangrijk om laboratorium-, klinisch- en epidemiologisch onderzoek te combineren om antibiotica resistentie te bestrijden.

This thesis is based on the following publications

Hassing RJ, Verbon A, de Visser H, Hofman A, Stricker BH. Proton pump inhibitors and gastroenteritis. *Eur J Epidemiol* 2016 Mar 10 [Epub ahead of print]

Hassing RJ, Alsma A, Arcilla MS, van Genderen PJ, Stricker BH, Verbon A. International travel and asymptomatic fecal carriage of multidrug-resistant *Enterobacteriaceae*: a systematic review. *Euro Surveill* 2015;20(47)

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Name PhD candidate: Robert-Jan Hassing

Erasmus MC department: Epidemiology and Internal Medicine

PhD period: 2013-2016

Supervisors: Prof.dr. B. H. Ch. Stricker and Prof.dr. A. Verbon

Co-supervisors: Dr. P. J. J. van Genderen and Dr. W. H. F. Goessens

PhD training

General courses

- Master of Science in Clinical Epidemiology, Netherlands Institute of Health Sciences, Rotterdam, the Netherlands (Graduated in 2014)

Specific courses

- Research integrity, Erasmus Medical Center, Rotterdam, the Netherlands (2014)
- Good Clinical Practice, Jeroen Bosch Ziekenhuis, Den Bosch, the Netherlands (2016)

Seminars and workshops

- Research seminars, Department of Epidemiology, Erasmus MC, the Netherlands (2013-2014)
- Research seminars, Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam, the Netherlands (2013-2014)

Presentations

- ICAAC 2013, Denver, United States. Antimicrobial resistance in ill returned travelers with typhoid fever in the Netherlands, 1999–2012. *Poster presentation* (2013)
- TCO travel symposium 2015, Arnhem, the Netherlands. Proton pump inhibitors and gastroenteritis. *Oral presentation* (2015)
- IDWeek 2015, San Diego, United States. Proton pump inhibitors and gastroenteritis. *Poster presentation* (2015)

Teaching activities

Lecturing

- Internal medicine practical sessions, 4th year medical students (2013–2014)
- Infectious diseases practical sessions, 4th year medical students (2013–2014)
- Infectious diseases in obstetrics practical sessions, obstetrics master student (2013–2014)

Supervising

- Bachelor thesis Aart van der Meijden: 'De rol van SOS respons in resistentie ontwikkeling bij Salmonella Typhi en Salmonella Paratyphi', HLO Utrecht (2013)
- Student research project of Edis Sevo: Association of proton pump inhibitors on bone density in the Rotterdam Study, Erasmus MC, Rotterdam, the Netherlands (2014)

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Familie. Lieve familie, als oudste uit een gezin met zes kinderen en twee artsen als ouders is er een hoop ambitieuze en emotionele dynamiek. Ik heb mijn best gedaan om een toekomst te zoeken buiten de geneeskunde, maar het plezier van mijn ouders in hun werk heeft mij toch doen besluiten dezelfde weg te kiezen. PAP, jouw goedwillende strategische adviezen hebben mij zeker geholpen tijdens mijn loopbaan. MAM, jij bent een echte internist die voor de kinderen heeft gekozen. Toen ik 11 was en een beroepskeuze in moest vullen bij de middelbare school was het 'internist net als mijn moeder', zonder te weten wat dat inhield. Maar als twee handen op één buik moest dat wel een goede keuze zijn. Nu vind ik het heel mooi dat ik jouw ambities als internist door kan zetten op mijn eigen manier. Bedankt voor jullie onvoorwaardelijke steun en vertrouwen. CARLIJNE, mijn paranimf, jij was de eerste die in de familie promoveerde. Dus toen moesten er meer gaan volgen. Als enige andere arts onder de kinderen hebben wij veel gemeenschappelijke ervaringen om over praten. Al blijft het wel Amsterdam tegen Rotterdam. ANNE-ROOS, jij hebt het andere gezinnetje in de familie met DAAN, VESPER en AXEL. Ondanks de afstand is het heel leuk dat onze kinderen al echte maatjes aan het worden zijn. RIANNE-FLEUR, jij kan met THOM ook al volledig door als een gezin. Voor ons altijd een warme plek om te bezoeken. Dan komen we toch weer een beetje thuis in Rotterdam. JORICK, mijn enige broertje, met jou was het leeftijdsverschil te groot om vroeger veel samen te doen. Het is daarom ook zo uniek dat we tijdens mijn laatste jaar in Rotterdam voor het eerst in hetzelfde voetbalteam hebben gespeeld. Het was voor mij een afbouwjaar en jouw fysieke hoogtepunt. JASMIJN, alias 'mini', door jouw enorme diepgang in de moleculaire microbiologie van gisten ben jij een gevreesde sparringpartner geworden. Mocht je na je studie doorgaan in de microbiologie dan denk ik dat ik het snel af ga leggen, maar dan kan ik je nog wel in de maling blijven nemen.

Tot slot. Lieve LAURA, met RUBEN en CARO hebben wij ons perfecte gezinnetje. Samen voor ons gezin zorgen, contact met de familie en allebei als arts werken is voor ons het belangrijkste in het leven. Dan blijft er soms weinig tijd over voor andere dingen. Omdat wij zo'n goed team samen vormen leidt dat eigenlijk nooit tot problemen. Deze rustige stabiele basis is noodzakelijk voor een proefschrift waar ook veel thuis aan is gewerkt. Veel dank daarvoor. Nu we onze liefde kunnen delen met onze prachtige kindjes maakt dat onze band alleen maar sterker. Op naar een mooie toekomst samen. Ik hou van jullie.

ABOUT THE AUTHOR

ROBERT-JAN HASSING was born on the 23rd of September 1980, in Utrecht, the Netherlands. He grew up in Venlo and graduated from Secondary School at Thomas College Venlo in 1999. That year he started the study Medicine at the Erasmus University of Rotterdam. His finalizing internships were Tropical Medicine in Newala District Hospital in Tanzania and Internal Medicine and Travel Medicine in the Havenziekenhuis Rotterdam, before he graduated in 2005. During his internships he generated a great interest for Internal Medicine and Infectious Diseases. After one year as a resident in Internal Medicine at the Havenziekenhuis, he continued his specialized training Internal Medicine and Infectious Diseases at the Havenziekenhuis (DR. P.J. WISMANS) and subsequently at the Erasmus MC Rotterdam (PROF.DR. J. L. C. M. VAN SAASE & DR. J. L. NOUWEN) from January 2007 until January 2013. In 2013 he worked in Suriname for four months, together with his wife, both as a resident. During his specialisation he participated in many studies concerning typhoidal *Salmonella* infections. From January 2013 until January 2015 he worked as a PhD candidate at the department of Epidemiology at the Erasmus MC, as a Pharmacovigilance Inspector at the Health Care Inspectorate in The Hague (PROF. DR. B. H. CH. STRICKER) and as a Infectious Diseases specialist at the Erasmus MC (PROF. DR. A. VERBON). In 2014 he obtained the Master of Science degree in Clinical Epidemiology. Since January 2015 he is a staff member of the department of Internal Medicine of Rijnstate Hospital in Arnhem. Robert-Jan is married to LAURA VERMEER and they live in Arnhem together with their beautiful children RUBEN and CARO.