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SUPPLEMENT ARTICLE

A Multicountry Molecular Analysis of *Salmonella enterica* Serovar Typhi With Reduced Susceptibility to Ciprofloxacin in Sub-Saharan Africa

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Background. *Salmonella enterica* serovar Typhi is a predominant cause of bloodstream infections in sub-Saharan Africa (SSA). Increasing numbers of *S.* Typhi with resistance to ciprofloxacin have been reported from different parts of the world. However, data from SSA are limited. In this study, we aimed to measure the ciprofloxacin susceptibility of *S.* Typhi isolated from patients with febrile illness in SSA.

Methods. Febrile patients from 9 sites within 6 countries in SSA with a body temperature of ≥38.0°C were enrolled in this study. Blood samples were obtained for bacterial culture, and *Salmonella* isolates were identified biochemically and confirmed by multiplex polymerase chain reaction (PCR). Antimicrobial susceptibility of all *Salmonella* isolates was performed by disk diffusion test, and minimum inhibitory concentrations (MICs) against ciprofloxacin were measured by Etest. All *Salmonella* isolates with reduced susceptibility to ciprofloxacin (MIC > 0.06 µg/mL) were screened for mutations in quinolone resistance-determining regions in target genes, and the presence of plasmid-mediated quinolone resistance (PMQR) genes was assessed by PCR.

Results. A total of 8161 blood cultures were performed, and 100 (1.2%) *S.* Typhi, 2 (<0.1%) *Salmonella enterica* serovar Paratyphi A, and 27 (0.3%) nontyphoid *Salmonella* (NTS) were isolated. Multidrug-resistant *S.* Typhi were isolated in Kenya (79% [n = 38]) and Tanzania (89% [n = 8]) only. Reduced ciprofloxacin-susceptible (22% [n = 11]) *S.* Typhi were isolated only in Kenya. Among those 11 isolates, all had a Glu133Gly mutation in the *gyrA* gene combined with either a *gyrA* (Ser83Phe) or *gyrB* mutation (Ser464Phe). One *Salmonella* Paratyphi A isolate with reduced susceptibility to ciprofloxacin was found in Senegal, with 1 mutation in *gyrA* (Ser83Phe) and a second mutation in *parC* (Ser57Phe). Mutations in the *parE* gene and PMQR genes were not detected in any isolate.

Conclusions. *Salmonella* Typhi with reduced susceptibility to ciprofloxacin was not distributed homogenously throughout SSA. Its prevalence was very high in Kenya, and was not observed in other study countries. Continuous monitoring of antimicrobial susceptibility is required to follow the potential spread of antimicrobial-resistant isolates throughout SSA.

Keywords. sub-Saharan Africa; ciprofloxacin; *S.* Typhi; susceptible.

Globally, there were 22 million cases of *Salmonella enterica* serovar Typhi in 2000 [1], and a disease burden of 12 million disability-adjusted life-years was estimated in 2010 [2]. After Southeast Asia, sub-Saharan Africa is the region that is most predominantly affected by *S.* Typhi infections, where the organism represents one of the leading causes of bloodstream infections [1, 3]. Treatment of *S.* Typhi infections can be challenging, with the increase of resistance to the former first-line drugs ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole (SXT). Resistance against all these 3 antimicrobials has been spreading since the 1990s in parts of Asia and Africa and is defined as multidrug-resistant (MDR) *S.* Typhi [4]. The emergence of MDR strains led the World Health Organization in 2003 to change recommendations for the first-line treatment for MDR strains of *S.* Typhi to ciprofloxacin or cefixime [4].

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However, recently high proportions of isolates with reduced susceptibility to ciprofloxacin are increasingly reported in *S.* Typhi, particularly from Cambodia (90%), Iraq (81%), Egypt (36%), and Kenya (13%) [5, 6]. The reported treatment failures of ciprofloxacin in *S.* Typhi infections [7, 8] led the Clinical and Laboratory Standards Institute (CLSI, 2012) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST, version 4, 2014) to lower the ciprofloxacin susceptibility breakpoint to a minimum inhibitory concentration (MIC) of ≤0.06 μg/mL [9].

The aim of this study was to investigate the ciprofloxacin susceptibility of *S.* Typhi in Burkina Faso, Guinea-Bissau, Kenya, Madagascar, Senegal, and Tanzania and to identify chromosomal mutations and/or plasmid-mediated quinolone resistance genes (PMQRs) associated with ciprofloxacin susceptibility.

METHODS

Study Design and Study Sites

This study was nested within the multicenter, multicountry Typhoid Fever Surveillance in Africa Program (TSAP) study. This substudy was conducted at 9 different healthcare facilities [10]. These healthcare facilities were located in Nikoko and Polesgo in Burkina Faso, Bandim in Guinea-Bissau, Kibera in Kenya, Imerintsiatosika and Isotry in Madagascar, Pikine in Senegal, and Moshi in Tanzania. The healthcare facilities in Madagascar and the Polesgo healthcare center in Burkina Faso had no inpatient facility; thus, recruitment was restricted to the outpatient department (Table 1). The other health posts enrolled patients from both inpatient and outpatient departments. Patients with fever ≥38.0°C who attended those healthcare facilities were eligible for study recruitment. The patients were enrolled in this study if written informed consent was obtained and blood culture was performed.

Bacterial Isolation and Identification at Site

Blood samples from the patients were obtained via venipuncture, inoculated into blood culture bottles, and incubated in a continuously monitored blood culture instrument (Bactec 2050, Becton Dickinson, Franklin Lakes, New Jersey; BacTAlert, bioMérieux, Durham, North Carolina). Broth from positive blood culture bottles was plated on MacConkey agar, Columbia agar enriched with 5% sheep blood, and chocolate agar (Oxoid, Hampshire, United Kingdom). *Salmonella enterica* was confirmed by API 20E biochemical testing (bioMérieux, Marcy L'Etoile, France) and the Oxoid *Salmonella* Latex Test. At the study laboratories, all *Salmonella* isolates were stored at −80°C before transportation on dry ice to the reference laboratory at the Bernhard Nocht Institute for Tropical Medicine in Hamburg, Germany (BNITM), where the isolates were again stored at −80°C until further processing.

Antimicrobial Susceptibility Testing

Identification of the *Salmonella* and antimicrobial susceptibility testing was repeated at BNITM. Antimicrobial susceptibility testing was performed by disk diffusion method according to the CLSI M100-S23 guidelines (www.clsi.org). All *Salmonella* isolates were tested against ampicillin, chloramphenicol, SXT, ciprofloxacin, and ceftriaxone. *Salmonella* Typhi that were resistant to ampicillin, chloramphenicol, and SXT were defined as MDR [4]. All *Salmonella* were further screened for ciprofloxacin resistance by Etest (Oxoid) to determine their MIC. According to CLSI M100-S23 guidelines, invasive *Salmonella* isolates were classified as having reduced susceptibility to ciprofloxacin if they had an MIC between 0.12 and 0.5 μg/mL [9].

Table 1. Demographic and Laboratory Investigation Results From Patients and Isolates, Typhoid Fever Surveillance in Africa Program, September 2011–December 2013

Country	Burkina Faso	Guinea Bissau	Senegal	Madagascar	Tanzania	Kenya
Demographics						
Patients recruited, No.	1674	1021	1058	2477	680	1251
Children aged <15 y, No. (%)	1227 (73)	914 (90)	287 (27)	641 (29)	362 (53)	950 (75)
Median age (25th, 75th percentile)	6 (2, 17)	3 (1, 7)	22 (14, 32)	24 (14, 37)	10 (1, 34)	7 (4, 14)
Female, No. (%)	871 (52)	487 (48)	468 (44)	1567 (63)	360 (53)	624 (50)
Inpatients, No. (%)	66 (4)	224 (22)	241 (23)	0 (0)[a]	376 (55)	0 (0)[a]
Blood culture results						
Total BCs performed, No.	1674	1021	1058	2477	680	1251
Total pathogen, No. (% of total BCs performed)	58 (3)	30 (3)	30 (3)	26 (1)	25 (4)	107 (9)
BCs positive for *S.* Typhi, No. (% of pathogens)	15 (26)	3 (10)	7 (23)	8 (31)	9 (36)	54 (50)
BCs positive for *S.* Paratyphi, No. (% of pathogens)	0 (0)	0 (0)	2 (7)	0 (0)	0 (0)	0 (0)
BCs positive for NTS, No. (% of pathogens)	11 (20)	7 (23)	1 (3)	1 (4)	2 (8)	6 (6)
Antibiotic resistance of *S.* Typhi						
Multidrug resistant[b] (% of available *S.* Typhi)	0 (0)	0 (0)	0 (0)	0 (0)	8 (89)	38 (79)
Ciprofloxacin nonsusceptible (% of available *S.* Typhi)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	11 (23)

Abbreviations: BC, blood culture; NTS, nontyphoidal *Salmonella*.

[a] Healthcare with outpatient department only.

[b] Resistant to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole.

Table 2. Alteration of Amino Acids in Quinolone Resistance-Determining Regions Among *Salmonella* Typhi With Reduced Susceptibility to Ciprofloxacin, Typhoid Fever Surveillance in Africa Program Sites, 2012–2013

Ciprofloxacin MIC, µg/mL	Isolate	Frequency	Position 1	Amino Acid (Codon) Change	Position 2	Amino Acid (Codon) Change
0.250	*S.* Typhi	3	*gyrA* 133	Glu to Gly (GAA to GGA)	gyrB 464	Ser to Phe (TCC to TTC)
0.500	*S.* Typhi	8	*gyrA* 133	Glu to Gly (GAA to GGA)	gyrA 83	Ser to Phe (TCC to TTC)
0.500	*S.* Paratyphi A	1	*parC* 57	Thr to Ser (ACC to AGC)	gyrA 83	Ser to Phe (TCC to TTC)

Abbreviations: Glu, glutamic acid; Gly, glycine; MIC, minimum inhibitory concentration; Phe, phenylalanine; Ser, serine; Thr, threonine.

DNA was extracted from all *Salmonella* isolates using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was stored at −20°C until polymerase chain reaction (PCR) amplification. *Salmonella* isolates were confirmed by a multiplex PCR assay using primers to detect *S. enterica* [11] and *S.* Typhi [12]. *Salmonella enterica* serovar Paratyphi A isolates were confirmed according to the White–Kauffmann–Le Minor serotyping scheme [13].

All *S.* Typhi and *S.* Paratyphi isolates with ciprofloxacin MIC > 0.06 µg/mL were screened for mutations in the *gyrA*, *gyrB*, *parC*, and *parE* genes. DNA was amplified as described previously [14]. In brief, PCR amplification was performed using Expand Long Template enzyme (Roche Diagnostics) with 1.75 mM magnesium chloride, 350 mM deoxyribonucleotide triphosphate mix, and 1 µM of each primer. The PCR condition was 30 cycles of denaturation (92°C for 1 minute), annealing (62°C for 1 minute), and extension (68°C for 2 minutes), followed by a final extension of 10 minutes. The amplified PCR products were subjected to purification and bidirectional sequencing at Eurofins Genomics, Hamburg, Germany. The sequenced data were analyzed with SeqScape software version 2.1.1 (Applied Biosystems).

Detection of Plasmid-Mediated Quinolone Resistance Genes

Reduced ciprofloxacin-susceptible *S.* Typhi isolates were screened for the presence of PMQR determinants *qnrA*, *qnrB*, and *qnrS* [15] and the quinolone efflux pump determinant *qepA* [16]. A multiplex PCR assay was performed following the procedure described by Liu et al [17].

Statistical Analysis

All descriptive statistical analyses were performed with Stata software version 12 (StataCorp LP, College Station, Texas). Age was described using the median and interquartile range (IQR).

Research Ethics

The study was approved by the ethical review board of BNITM, the International Vaccine Institute (Seoul, Korea), all collaborating institutions, and the national ethical review bodies of each participating country.

RESULTS

A total of 8161 patients were enrolled in 9 study sites across 6 sub-Saharan African countries; 4376 (53.6%) were female. The median age of all patients was 12.5 years (IQR, 4–27 years), and 4381 (53.7%) were <15 years of age. Bacterial pathogens were identified in 276 (3.5%) blood cultures. Among them, 130 (47.1%) were confirmed to be *Salmonella enterica*, of which 100 (36.2%) were *S.* Typhi, 2 (0.7%) were *S.* Paratyphi A, and 28 (10.1%) were NTS. *Salmonella* Typhi were more commonly isolated in Kenya (n = 54), followed by Burkina Faso (n = 18), Tanzania (n = 9), Madagascar (n = 9), Senegal (n = 7), and Guinea-Bissau (n = 3) (Table 1). Both *S.* Paratyphi A were isolated from Senegal. Ten *S.* Typhi (6 from Kenya, 3 from Burkina Faso, and 1 from Madagascar) were not available for ciprofloxacin Etest.

Among the 90 available *S.* Typhi isolates, 38 of 48 (79.2%) from Kenya and 8 of 9 (88.9%) from Tanzania were MDR. In Kenya, the proportion of MDR *S.* Typhi was 78.0% (32 of 41) in 2012 and 85.7% (6 of 7) in 2013. Reduced ciprofloxacin susceptibility was detected in 11 *S.* Typhi isolates from Kenya only; their MICs were within the range of 0.25–0.5 µg/mL. The proportion of isolates with reduced susceptibility to ciprofloxacin was 19.5% (8/41) in 2012 and 42.9% (3/7) in 2013 ($P = .174$, proportion test). All *S.* Typhi isolates from other countries were identified as susceptible to ciprofloxacin. Both *S.* Paratyphi A isolates from Senegal were susceptible to ampicillin, chloramphenicol, SXT, and ceftriaxone. However, 1 of 2 *S.* Paratyphi A isolates was found to have reduced susceptibility to ciprofloxacin and had an MIC of 0.5 µg/mL.

Sequence analyses of *gyrA*, *gyrB*, *parC*, and *parE* genes from the *S.* Typhi and *S.* Paratyphi A isolates with reduced susceptibility to ciprofloxacin showed that all strains had at least 2 mutations associated with fluoroquinolone resistance. All *S.* Typhi isolates with reduced susceptibility to ciprofloxacin had a mutation in the *gyrA* gene at codon 133 (glutamic acid to glycine) along with a second mutation either in the *gyrA* gene at codon 83 (serine to phenylalanine) (n = 8) or within the *gyrB* gene at codon 464 (serine to phenylalanine) (n = 3). The *S.* Paratyphi A isolate with reduced susceptibility to ciprofloxacin had 1 mutation in *gyrA* at codon 83 (serine to phenylalanine) and an additional mutation in *parC* at codon 57 (threonine to serine) (Table 2). No mutations were identified in *parE*, and all strains were negative by PCR for the PMQR genes *qnrA*, *qnrB*, *qnrS*, and *qepA*.

DISCUSSION

The results of the study demonstrate a high prevalence of *S.* Typhi with reduced susceptibility to ciprofloxacin in Kenya;

only ciprofloxacin-susceptible isolates were detected at the other study sites. In Kenya, the proportion of *S.* Typhi isolates with reduced susceptibility to ciprofloxacin was 20% in 2012 and 43% in 2013, and MDR was 79% in 2012 and 86% in 2013. A previous Kenyan study conducted between 2004 and 2006 found that 13% of *S.* Typhi isolates had reduced susceptibility to ciprofloxacin and 70% were MDR [18]. These data suggest a trend of increasing prevalence of drug-resistant *S.* Typhi in recent years. This increase in the prevalence of *S.* Typhi with reduced susceptibility to ciprofloxacin has been additionally reported from other parts of the African continent, including the Democratic Republic of the Congo (15%) and South Africa (5%) [19, 20].

This study demonstrates also a high prevalence of MDR *S.* Typhi in Tanzania. In Burkina Faso, Guinea-Bissau, Madagascar, and Senegal, only low numbers of *S.* Typhi were isolated, and none were MDR. Data on antimicrobial resistance are limited from these countries. In a study conducted in Burkina Faso, 12 *S.* Typhi were isolated from 711 febrile patients; none were MDR or ciprofloxacin resistant [21]. A survey conducted in Guinea-Bissau in 2010 isolated neither MDR nor ciprofloxacin-resistant isolates among 3 *S.* Typhi strains [22]. Surveillance data from Senegal between 1999 and 2009 reported 127 *S.* Typhi; again, none were fluoroquinolone resistant or MDR [23], whereas another Senegalese study of data up to 2002 reported 1 of 232 isolates (0.4%) to be MDR [24]. These data supported our findings of a very low prevalence of antimicrobial resistance among *S.* Typhi in many parts of West Africa.

In this study, all the *S.* Typhi isolates with reduced susceptibility to ciprofloxacin had >1 mutation in genes associated with quinolone resistance. All isolates shared a common mutation in the *gyrA* gene at codon 133 combined with either a second mutation in the same gene at codon 83 or in the *gyrB* gene at codon 464. The double *gryA* mutant, with mutations at codon 83 (serine to phenylalanine/tyrosine) and codon 133 (glutamic acid to glycine), has been previously identified in *S.* Typhi with a ciprofloxacin MIC of >0.06 μg/mL in the Democratic Republic of the Congo [20]. It has been shown in *Salmonella enterica* serovar Typhimurium that triple mutations at codon 83 and 87 of *gyrA* and at codon 464 of *gyrB* confer complete ciprofloxacin resistance (MIC of 16–32 μg/mL) [25]. The double mutation in *gyrA* (codon 133) and *gyrB* (codon 464) has not been associated with complete ciprofloxacin resistance. Only single alterations to phenylalanine in *gyrB* at codon 464 have been reported previously from Bangladesh, India, and Vietnam in *S.* Typhi, with a ciprofloxacin MIC ranging from 0.12 to 0.5 μg/mL [26]. Taken together, these data suggest that double mutations at those positions cause only reduced susceptibility in *S.* Typhi.

The *S.* Paratyphi A isolate with reduced susceptibility to ciprofloxacin from Senegal, with a *gyrA* mutation at codon 83 and a *parC* mutation at codon 57, had little effect on MIC in this study. The second mutation, which alters threonine to serine, has been previously reported in Hong Kong [27] and South Africa [19], and also has a modest effect on the MIC to ciprofloxacin. To our knowledge, there is only 1 report from sub-Saharan Africa (Burkina Faso) on the identification of an *S.* Paratyphi A isolate with reduced susceptibility to ciprofloxacin with a mutation in the *gyrB* gene at position 464 [28]. However, ciprofloxacin-resistant *S.* Paratyphi A isolates have been reported since the mid-1990s from the Indian subcontinent [29–31].

In this study, we did not measure the effect of other resistance mechanisms that may have an effect of ciprofloxacin MIC, such as increased efflux pump activity. That underlying resistance mechanism might also play a role in decreasing the susceptibility of ciprofloxacin among the *S.* Typhi of this study.

CONCLUSIONS

This study identified a substantial number of *S.* Typhi isolates with reduced susceptibility to ciprofloxacin from Kenya and indicates a rapid increase in the proportion of *S.* Typhi strains displaying reduced ciprofloxacin susceptibility in recent years. A high prevalence of MDR *S.* Typhi was found in Kenya and Tanzania, whereas *S.* Typhi isolates from Burkina Faso, Guinea-Bissau, Madagascar, and Senegal were susceptible to all antimicrobial agents tested. In Kenya, physicians should be aware that empirical antimicrobial therapy with ampicillin, chloramphenicol, SXT, and ciprofloxacin for *S.* Typhi infections might not be effective. In Burkina Faso, Tanzania, Madagascar, Senegal, and Guinea-Bissau, continuous monitoring of antimicrobial susceptibility is required to detect the emergence of ciprofloxacin resistance to adjust treatment regimens in a timely manner.

Notes

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