ORIGINAL RESEARCH

Salmonella enterica serovars Typhimurium and Enteritidis causing mixed infections in febrile children in Mozambique

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Background and purpose: Invasive nontyphoidal salmonellosis, mostly caused by serovars Typhimurium and Enteritidis of *Salmonella enterica*, has emerged as a major public health problem in sub-Saharan Africa. The aim of this study was the clinical and microbiological characterization of nontyphoidal salmonellosis episodes affecting febrile children in Mozambique.
Patients and methods: The clinical records of the patients were evaluated, and *S. enterica* isolates were characterized with regard to serovar, phage type, antimicrobial resistance (phenotype/responsible genes), plasmid content, pulsed-field gel electrophoresis, and multilocus sequence typing.
Results: Fifteen *S.* Typhimurium and 21 *S.* Enteritidis isolates were recovered from blood samples of 25 children, the majority with underlying risk factors. With regard to phage typing, most isolates were either untypeable or reacted but did not conform, revealing that a number of previously unrecognized patterns are circulating in Mozambique. Most isolates were multidrug-resistant, with nearly all of the responsible genes located on derivatives of serovar-specific virulence plasmids. ST313 and ST11 were the predominant sequence types associated with *S.* Typhimurium and *S.* Enteritidis, respectively, and the uncommon ST1479 was also detected in *S.* Enteritidis. A distinct *Xba*I fragment of ~350 kb was associated with pulsed-field gel electrophoresis patterns of multidrug-resistant isolates of *S.* Enteritidis. Nearly half of the children were coinfected with both serovars, a fact expected to aggravate the disease and hamper the treatment. However, particularly poor outcomes were not observed for the coinfected patients.
Conclusion: Mixed *Salmonella* infections could frequently occur in febrile children in Mozambique. Additional studies are required to determine their actual impact and consequences, not only in this country, but also in other African countries.
Keywords: invasive nontyphoidal salmonellosis, bloodstream infection, multidrug resistance, virulence-resistance plasmid, ST313, ST1479

Introduction

Nontyphoidal serovars of *Salmonella enterica* are a prominent cause of bloodstream infections in sub-Saharan Africa, which mainly affect children with specific risk factors, including malnutrition, severe anemia, malaria, and HIV infection, as well as HIV-infected or immunocompromised adults.[1–3] *S.* Typhimurium and *S.* Enteritidis are the most common serovars involved in invasive nontyphoidal salmonellosis (iNTS).[4–6] Phylogenetic analysis based on single-nucleotide polymorphisms revealed that the majority of the invasive *S.* Typhimurium from sub-Saharan countries belong to sequence type (ST) 313 and are distributed into two closely related lineages designated I and II.[7,8] Both lineages comprise multidrug-resistant (MDR) isolates carrying distinct

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derivatives of pSLT, the ~94 kb virulence plasmid specific of *S*. Typhimurium, where multiple resistance genes are located.[7,8] Examples of such plasmids are pSLT-A130 (conferring resistance to ampicillin, kanamycin, sulfonamides, and trimethoprim), carried by the A130 isolate representative of lineage I, and pSLT-BT (responsible for resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, sulfonamides, and trimethoprim), found in D23580 of lineage II. With regard to *S*. Enteritidis, it is of note that MDR isolates of this serovar were recovered in the UK from blood and feces of patients who had an African name or had recently traveled to Africa.[9] The UK isolates were phage type (PT) 42 and belonged to ST11, the most common ST in *S*. Enteritidis, or to the infrequent ST1479. MDR in these isolates was encoded by derivatives of pSEV, the ~60 kb virulence plasmid of *S*. Enteritidis, related to pUO-SeVR1, a plasmid first detected in a PT14b, ST11 isolate collected in Spain from feces of a young child with gastroenteritis.[10] Recently, whole genome sequence analysis of *S*. Enteritidis from sub-Saharan Africa identified two related but phylogenetically and geographically distinct clades, the Western African clade and the Central/ Eastern African clade. In both clades, MDR was encoded by pSEV derivatives highly similar to pUO-SeVR1.[11]

In rural Mozambique, previous studies revealed that non-typhoidal *Salmonella* is the most frequent cause of bacteremia in children younger than 15 years admitted to the Manhiça District Hospital (MDH).[5,12,13] Like in other African countries, *S*. Typhimurium and *S*. Enteritidis were the most frequent serovars, accounting for 66% and 25% of the cases, respectively.[13] To broad our knowledge on iNTS in Mozambique, the present study focused on a number of isolates causing bacteremia in children, which were characterized with regard to serovar, PT, resistance properties (including phenotypes, genes, integrons, and plasmids involved), and genomic types established by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The study disclosed a relatively high number of mixed infections with the simultaneous detection of *S*. Typhimurium and *S*. Enteritidis isolates in a single episode of bacteremia.

Material and methods
Setting, patients, and bacterial isolates

Twenty-five blood samples positive for *Salmonella* were recovered during the period 2002–2004 from different children admitted to the MDH (the referral health facility for a rural area located in Southern Mozambique), and the clinical histories of the patients were retrospectively revised. The identification of *Salmonella* was based on Gram staining

and biochemical methods.[14] Serotyping was performed at the Centro de Investigação em Saúde da Manhiça (CISM, Maputo, Moçambique) by traditional slide agglutination with commercially available antisera (polyvalent and monoclonal; Bio-Rad, Marnes-la-Coquette, France) and by a PCR-based method.[15] Serotyping results were confirmed at the Centro Nacional de Microbiología (CNM, Madrid, Spain), where PTs were also determined. Isolates showing a pattern that did not conform to previously recognized PTs were designated as "reacted but did not conform" (RDNC). Isolates that did not react with any of the typing phages were designated as "untypeable" (UT).

Antimicrobial susceptibility testing and detection of resistance genes

Susceptibility to ampicillin, chloramphenicol, ciprofloxacin, cefotaxime, cefoxitin, cephalothin, gentamicin, kanamycin, nalidixic acid, sulfonamides, nitrofurantoin, streptomycin, tetracycline, and trimethoprim was tested by the disc diffusion method on Mueller–Hinton agar, using commercially available discs (Oxoid, Madrid, Spain). Results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute.[16]

Genes responsible for resistance to ampicillin ($bla_{OXA-1-like}$, bla_{PSE-1}, and $bla_{TEM-1-like}$), chloramphenicol (*catA1*, *catA2*, *cmlA1*-like, and *floR*), gentamicin/kanamycin [*aac(3)-II*, *aac(3)-IV*, and *aadB*], streptomycin (*aadA1*-like, *aadA2*, *strA*, and *strB*), sulfonamides (*sul1*, *sul2*, and *sul3*), tetracycline [*tet*(A), *tet*(B), and *tet*(G)], and trimethoprim (*dfrA1*-like, *dfrA7-dfrA17*, and *dfrA12*) were screened by PCR amplification using previously described primers and conditions,[17–19] except for *catA2* (catA2-F/R: 5'-AGCTTACGGAGGAC-GGAATG-3'/5'-CACCAATCCTGCCGGATACG-3'). The genetic bases of nalidixic acid resistance were investigated by using PCR amplification and sequencing of the quinolone resistance-determining region of the *gyrA* gene.[20] The presence of class 1 and 2 integrons was investigated by using the 5'-CS/3'-CS and Hep74/Hep51 primers, and gene cassettes located therein were identified by nested PCR amplification with primers selected according to the detected resistance genes.[21,22] Gene cassettes were sequenced at Macrogen Europe (Amsterdam, The Netherlands), when required for allele identification.

Plasmid analysis, conjugation, and transformation experiments

Plasmid DNA was extracted by using an alkaline lysis method.[23] Electrophoresis was carried out on 0.6% agarose

gels for 3–4 hours at 100 V. Plasmids obtained from *Escherichia coli* V517 (54, 7.3, 5.6, 5.1, 3.9, 3, 2.7, and 2.1 kb), *E. coli* 39R861 (150, 65, 37, and 7 kb), *S*. Typhimurium ATCC 14028 (pSLT; 94 kb), *S*. Enteritidis ATCC 13076 (pSEV; 60 kb), and *S*. Enteritidis CNM 4839/03 (which contains two plasmids, pUO-SeVR1 of 110 kb and a cryptic plasmid of ~80 kb) were used as size standards.

All isolates were screened for the IncFIIA and IncFIB replicons and the *spvC* gene of serovar-specific virulence plasmids, by PCR amplification using previously described primers and conditions.[10,24] pSLT and pSEV were included as positive controls. Isolates harboring small plasmids were also tested for the ColE replicon.[25]

Triparental conjugation experiments were performed on Luria–Bertani liquid medium at 37°C for 20 hours using the invasive *S*. Typhimurium and *S*. Enteritidis isolates as donors, a derivative of *E. coli* K12 J53 resistant to rifampicin as the recipient, and *E. coli* MT1694, resistant to kanamycin and containing the conjugative pRK2013 plasmid, as a helper strain. Transconjugants were selected on Eosin Methylene Blue agar (Oxoid) supplemented with rifampicin (50 mg/L) plus either ampicillin (100 mg/L), chloramphenicol (30 mg/L), streptomycin (30 mg/L), sulfonamides (300 mg/L), tetracycline (30 mg/L), or trimethoprim (10 mg/L). For transformation experiments, plasmid DNA obtained from selected isolates was purified by the High Pure Plasmid Isolation Kit (Roche, Madrid, Spain) and transformed into a nalidixic acid-resistant derivative of *E. coli* DH5α. The preparation of competent cells and transformation were as in Sambrook and Russell.[26] Transformants were selected in Luria–Bertani agar medium supplemented with nalidixic acid and ampicillin (30 and 100 mg/L, respectively). Transfer of additional resistance genes was tested by the disc diffusion method and PCR amplification.

PFGE analysis and MLST
PFGE was performed with the *Xba*I endonuclease (Thermo Fisher Scientific, Waltham, MA, USA), following the PulseNet protocol for *S. enterica* (www.pulsenetinternational.org). *Xba*I-digested DNA of *S*. Braenderup H9812 was used as a size marker. The resulting profiles were visually analyzed by recording the presence or absence of fragments >33 kb, and those with differences in two or more bands were designated using serial numbers (eg, X1 and X2). MLST was carried out by PCR amplification and sequencing, using the specific primers for seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) described in the MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). The STs were assigned according to the database in the same website.

Hybridization experiments
PFGE and plasmid profiles were transferred into nylon membranes (Hybridon™ N+; GE Healthcare Life Sciences, Barcelona, Spain) and hybridized with probes for IncFIIA, IncFIB, and *spvC* selected as markers of serovar-specific virulence plasmids and for the identified resistance genes and integrons [$bla_{OXA-1-like}$, $bla_{TEM-1-like}$, *catA1*, *catA2*, *aac(3)-IV*, *aadB*, *aadA1*-like, *strA*, *strB*, *sul1*, *sul2*, *tet*(A), *dfrA7*, *dfrA12*, and *intI1*] to establish their location.[26] The probes were labeled using the PCR DIG labeling mix (Roche Applied Science, Barcelona, Spain), followed by gel extraction with the GFX™ DNA and Gel Band Purification Kit (GE Healthcare Life Sciences).

Ethical clearance
The strains characterized here were isolated from the ongoing invasive bacterial surveillance system that included several research protocols reviewed and approved by the Mozambican National Bioethics Committee for Health (IR00002657) and by Institutional Review Boards of Hospital Clinic of Barcelona, Spain; the US Centers for Disease Control and Prevention; and the School of Medicine, University of Maryland. Written informed consent was obtained from parents or caretakers of the eligible children.

Results
Serovars and PTs of the bloodstream isolates
From blood samples of 25 febrile children admitted to the MDH, 36 *Salmonella* isolates were recovered. Table 1 shows the relevant features of the patients, including sex and age, risk factors, symptoms and outcome. Fifteen isolates were assigned to *S*. Typhimurium and 21 isolates to *S*. Enteritidis. Both serovars were found in eleven samples, indicating a high frequency of mixed infections (Table 1). PT32 and U302 (shown by single isolates of *S*. Typhimurium), and PT1 (shown by two isolates of *S*. Enteritidis) were the only recognizable PTs associated with the detected isolates. In contrast, seven *S*. Typhimurium and 16 *S*. Enteritidis isolates displayed five and two RDNC patterns, respectively. The remaining isolates were UT (Tables 2 and 3).

Resistance properties of S. Typhimurium
As shown in Table 2, only three of the 15 invasive isolates of *S*. Typhimurium were susceptible to all antimicrobials tested. The remaining twelve were MDR, ie, resistant to three, five, or six unrelated antimicrobials, including ampicillin

Table 1 Features of 25 patients with invasive nontyphoidal salmonellosis and involved serovars

Patient/sex/age	Risk factor(s)	Symptoms	Outcome	Sample	Isolate(s)	Date of isolation
1/M/18 months	A, M, O	Fv, D	Discharged	MDH 156266,5	MDH-T1, MDH-E1	04/01/2002
2/F/18 months	M, SM, S, O	Fv	Died	MDH 160712,1	MDH-T2	28/01/2002
3/M/40 months	A, M, O	Fv, V	Discharged	MDH 160836,2	MDH-E3	01/02/2002
4/M/13 months	A, SM, M,	Fv	Discharged	MDH 165814,5	MDH-T4	25/02/2002
5/M/9 days	–	Fv, D	Discharged	MDH 172089,6	MDH-T5, MDH-E5	18/03/2002
6/unk	unk	unk	unk	MDH 173965.8	MDH-E6	08/04/2002
7/M/43 months	A, M	Fv, D, V	Discharged	MDH 175838,3	MDH-E7	08/05/2002
8/M/21 months	A, O	Fv, D, V, Dh	Discharged	MDH 183027,3	MDH-T8	10/07/2002
9/M/23 months	A, SM, S	Fv	Discharged	MDH 183092,1	MDH-T9	10/07/2002
10/F/3 months	A, SM, S, O	Fv	Discharged	MDH 193299,2	MDH-T10, MDH-E10	17/09/2002
11/M/14 months	A, O	Fv	Discharged	MDH 201596,3	MDH-E11	22/10/2002
12/M/14 months	A, M, O	Fv	Discharged	MDH 202045,9	MDH-E12	15/11/2002
13/M/4 months	A, SM, S	Fv	Discharged	MDH 202839,8	MDH-E13	10/12/2002
14/F/13 months	A, SM	Fv	Transferred	MDH 202944,7	MDH-T14, MDH-E14	10/12/2002
15M/16 months	A, SM, S	Fv, D, Dh	Discharged	MDH 213641,6	MDH-T15, MDH-E15	20/01/2003
16/F/19 months	A, M	Fv, D, V, Dh	Died	MDH 217168	MDH-E16	10/02/2003
17/M/29 months	A, M, S, T/PF	Fv, V	Discharged	MDH 233513,2	MDH-T17, MDH-E17	21/04/2003
18/F/63 months	A, SM, S	Fv	Discharged	MDH 233595,8	MDH-E18	22/04/2003
19/M/30 months	A, SM, M, O	Fv, D, Dh	Discharged	MDH 233819,9	MDH-T19, MDH-E19	09/05/2003
20/M/15 months	A, M, O	Fv	Left	MDH 252510,2	MDH-E20	21/07/2003
21/F/32 months	A, O	Fv	Discharged	MDH 252696,1	MDH-T21, MDH-E21	07/08/2003
22/F/15 months	–	Fv, D	Discharged	MDH 265314,1	MDH-T22, MDH-E22	07/10/2003
23/F/30 months	A, M, O	Fv	Discharged	MDH 265830,6	MDH-E23	03/11/2003
24/F/14 months	A, PI, O	Fv	Left	MDH 290256,7	MDH-T24, MDH-E24	11/12/2003
25/M/8 months	A, O	Fv, D	Discharged	MDH 282144,3	MDH-T25, MDH-E25	05/01/2004

Notes: Patients are numbered from 1 to 25. Those coinfected with *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis are highlighted in bold. Clinical diagnosis of typhoid/paratyphoid fever could not be microbiologically confirmed.
Abbreviation: MDH, Manhiça District Hospital; F, female; M, male. A, anemia; SM, severe malnutrition; M, malaria; T/PF, typhoid/paratyphoid fever, S, septicemia; O, other (eye disorders; ear disease and mastoiditis; upper respiratory infections; influenza and pneumonia; tuberculosis; protozoal intestinal disease; and other infectious diseases). Fv, fever; D, diarrhea; V, vomiting, Dh, dehydration. T, *S. enterica* serovar Typhimurium; E, *S. enterica* serovar Enteritidis; unk, unknown.

($bla_{TEM-1} \pm bla_{OXA-1}$), chloramphenicol (*catA1*), gentamicin/kanamycin [*aac(3)-IV* and *aadB*], streptomycin (*aadA1* $\pm$ *strAB*), sulfonamides (*sul1* $\pm$ *sul2*), and trimethoprim (*dfrA1* or not identified). Resistance to cephalosporins, nalidixic acid, ciprofloxacin, nitrofurantoin, or tetracycline was not detected. All MDR isolates carried one or two class 1 integrons, with three different variable regions: 2000 bp/bla_{OXA-1}-*aadA1*, 700 bp/*aadB*, and 1600 bp/*dfrA1-aadA1*, but class 2 integrons were not found.

On the basis of plasmid content, the isolates could be distributed in three groups, characterized by the presence of pSLT (found in susceptible isolates), pSLT-A130-like plasmids or pSLT-BT-like plasmids (carried by seven and five MDR isolates, respectively; Figure 1A; Table 2). In agreement with this, all isolates were positive for the IncFIIA and IncFIB replicons and the *spvC* gene.

All except one of the pSLT-A130-like plasmids had a size of about 120 kb. These plasmids were found in *S.* Typhimurium isolates resistant to ampicillin, gentamicin/kanamycin, streptomycin, sulfonamides, and trimethoprim

and carried the bla_{OXA-1}, bla_{TEM-1}, *aadA1*, and *sul1* genes as well as the 2000 bp/bla_{OXA-1}-*aadA1* integron, as demonstrated by hybridization experiments of plasmid profiles (Table 2). In contrast, *aac(3)-IV* and the 700 bp/*aadB* integron, also present in these isolates, failed to hybridized with plasmid DNA. In a single isolate of the pSLT-A130-like group (resistant to ampicillin, streptomycin, and sulfonamides, but susceptible to gentamicin/kanamycin and trimethoprim), the plasmid was slightly smaller (~115 kb) but carried the same resistance genes and the 2000 bp/bla_{OXA-1}-*aadA1* integron of the 120 kb plasmids (Table 2). The pSLT derivatives were always accompanied by small ColE1 plasmids (~7 kb), which harbored a second copy of the bla_{TEM-1} gene and could be transferred into *E. coli* by transformation. Biparental and triparental conjugations, using four representative isolates as donors (Table 2), did not yield *E. coli* transconjugants under the applied conditions. This supports that the pSLT-A130-like plasmids are neither conjugative nor mobilizable.

pSLT-BT-like plasmids, of about 117 kb, were found in five isolates resistant to ampicillin, chloramphenicol,

Table 2 Characteristics of invasive *Salmonella enterica* serovar Typhimurium isolates from Mozambique

Patient	Isolate	PT	Resistance phenotype (n)	Resistance genotype	Class 1 integron	Plasmid(s)	XP	ST
5	MDH-T5	RDNC T3	Susceptible (3)	–	–	pSLT (IncFIIA+IncFIB; 94 kb)	X1	19
21	**MDH-T21**	**RDNC T4**	**–**	**–**	**–**	**–**	**X2**	**19**
24	**MDH-T24**	**RDNC T5**	**–**	**–**	**–**	**–**	**X2**	**19**
1	MDH-T1	RDNC T1	AG/KSSuTp (6)	[bla_{OXA-1}-bla_{TEM-1}]-aac(3)-IV- aadA1-aadB- sul1-ni	2000 bp/bla_{OXA-1}-aadA1; 700 bp/aadB	pSLT-A130-like (IncFIIA+IncFIB; 120 kb); (ColE1; 7 kb)	X3	313
4	MDH-T4	RDNC T2	–	–	–	–	X3	313
8	MDH-T8	32	–	–	–	–	X3	313
10	MDH-T10	UT	–	–	–	–	X3	313
14	MDH-T14	UT	–	–	–	–	X3	313
22	MDH-T22	UT	–	–	–	–	X4	313
2	MDH-T2	RDNC T2	ASSu	[bla_{OXA-1}-bla_{TEM-1}]-aadA1-sul1	2000 bp/bla_{OXA-1}-aadA1	pSLT-A130-like (IncFIIA+IncFIB; 115 kb); (ColE1; 7 kb)	X5	313
9	MDH-T9	U302	ACSSuTp (5)	bla_{TEM-1}-catA1-aadA1-strAB-[sul1-sul2]-dfrA1	1600 bp/dfrA1-aadA1	pSLT-BT-like (IncFIIA+IncFIB; 117 kb); (ni; 85 kb); (ColE1; 3 kb)	X6	313
15	MDH-T15	UT	–	–	–	pSLT-BT-like (IncFIIA+IncFIB; 117 kb);(ni; 50 kb); (ColE1; 3 kb)	X7	313
19	MDH-T19	UT	–	–	–	–	X7	313
17	MDH-T17	RDNC T2	–	–	–	–	X8	313
25	MDH-T25	UT	–	–	–	–	X8	313

Notes: Patients coinfected with *S. enterica* serovar Enteritidis are highlighted in bold. Isolates used as donors in biparental and triparental conjugation experiments are underlined. Size of the variable region and gene cassette(s) located herein and identified by sequencing. Incompatibility groups; estimated plasmid sizes are shown in parentheses. Sequence type determined by multilocus sequence typing.
Abbreviations: MDH, Manhiça District Hospital; n, total number of isolates when more than one; ni, not identified; PT, phage type; RDNC, reacted but did not conform; UT, untypeable. A, ampicillin; C, chloramphenicol; G, gentamicin; K, kanamycin; S, streptomycin; Su, sulfonamides; Tp, trimethoprim; XP, *Xba*I profile; ST, sequence type.

streptomycin, sulfonamides, and trimethoprim (Table 2). All resistance genes (bla_{TEM-1}, *catA1*, *aadA1*, *strAB*, *sul1*, *sul2*, and *dfrA1*) as well as the 1600 bp/*dfrA1-aadA1* integron found in these isolates were mapped on the plasmids. Additional cryptic plasmids of variable sizes (~84 and 50 kb, of undetermined incompatibility group, and ColE1 of ~3 kb) were also detected. Although the pSLT-BT-like plasmids failed to conjugate through biparental mattings, they could be mobilized from representative isolates (Table 2) into *E. coli* by triparental mattings. The obtained transconjugants had the same resistance phenotype and genes as the donor isolates.

Resistance properties of S. Enteritidis

With regard to *S.* Enteritidis (Table 3), only two of 21 invasive isolates were susceptible to all antimicrobials tested, while the remaining 19 were resistant to five, six, or seven unrelated antimicrobials. Resistance to ampicillin (bla_{TEM-1}), chloramphenicol ($catA2 \pm catA1$), nalidixic acid (due to a single mutation in *gyrA*, changing TCC/Ser83 to TAC/Tyr, in three isolates tested), streptomycin ($strAB \pm aadA1$), sulfonamides

(*sul1* and *sul2*), and trimethoprim (*dfrA7*) was found in all MDR isolates, and all except two were also resistant to tetracycline [*tet*(A)]. In contrast, resistance to cephalosporins, ciprofloxacin, or nitrofurantoin was not detected. All isolates were negative for the integrase gene of class 1 and 2 integrons, but positive for the 700 bp/*dfrA7* variable region, consistent with the presence of a defective class 1 integron.

pSEV was present in the two susceptible isolates, together with a small ColE1 plasmid of ~5 kb (Figure 1B; Table 3). Larger plasmids, ranging in size from ~95 to 130 kb, were found in all MDR isolates, and all except three carried a small ColE1 plasmid of about 6 kb. Through hybridization experiments, the bla_{TEM-1}, *catA2*, *strAB*, *sul1*, *sul2*, *tet*(A), and *dfrA7*, and so the defective class 1 integron, were located on the larger plasmids. These plasmids also were hybridized with probes specific for the IncFIIA and IncFIB replicons, and for *spvC*, identifying them as derivatives of pSEV which, according to their resistance properties, appear to be related to pUO-SeVR1.[9,10,27] Also coinciding with pUO-SeVR1, the pSEV derivatives from Mozambique could not be transferred

Table 3 Characteristics of invasive *Salmonella enterica* serovar Enteritidis isolates from Mozambique

Patient	Isolate	PT	Resistance phenotype (n)	Resistance genotype	Class I integron	Plasmid (s)	XP	ST
1	MDH-E1	I	Susceptible (2)	–	–	pSEV (IncFIIA+IncFIB; 60 kb) (ColE1; 5 kb)	X1	11
10	MDH-E10	I		–	–		X1	11
3	MDH-E3	RDNC E1	ACSSuTTp (12)	bla_{TEM-1}-catA2-strAB-[sul1-sul2]-tet(A)-dfrA7	700 bp/dfrA7	pUO-SeVR1-like (IncFIIA+IncFIB; 95 kb) (ColE1; 6 kb)	X2	11
5	MDH-E5	RDNC E1					X2	11
6	MDH-E6	RDNC E1					X2	11
14	MDH-E14	RDNC E1					X2	11
16	MDH-E16	UT					X2	11
17	MDH-E17	UT					X2	11
18	MDH-E18	RDNC E1					X2	11
19	MDH-E19	RDNC E1					X2	1479
21	MDH-E21	UT					X2	11
23	MDH-E23	RDNC E2					X2	11
7	MDH-E7	RDNC E1					X3	11
25	MDH-E25	RDNC E1					X4	11
12	MDH-E12	RDNC E2	ACSSuTTp	bla_{TEM-1}-catA2-[strAB-aadA1]-[sul1-sul2]-tet(A)-dfrA7	700 bp/dfrA7	pUO-SeVR1-like (IncFIIA+IncFIB; 95 kb)	X2	1479
15	MDH-E15	RDNC E1	ACSSuTTp	bla_{TEM-1}-[catA1-catA2]-[strAB-aadA1]-[sul1-sul2]-tet(A)-dfrA1-dfrA7	700 bp/dfrA7	pUO-SeVR1-like (IncFIIA+IncFIB; 95 kb) (ColE1; 6 kb)	X2	11
20	MDH-E20	RDNC E1	ACSSuTp (2)	bla_{TEM-1}-catA2-strAB-[sul1-sul2]-dfrA7	700 bp/dfrA7	pUO-SeVR1-like (IncFIIA+IncFIB; 130 kb) (ColE1; 6 kb)	X5	1479
22	MDH-E22	RDNC E1					X5	11
13	MDH-E13	RDNC E1	ACSSuTTpNal (3)	bla_{TEM-1}-catA2-strAB-[sul1-sul2]-tet(A)-dfrA7-Ser83Tyr	700 bp/dfrA7	pUO-SeVR1-like (IncFIIA+IncFIB; 110 kb) (ColE1; 6 kb)	X2	11
24	MDH-E24	RDNC E2				pUO-SeVR1-like (IncFIIA+IncFIB; 95 kb)	X2	11
11	MDH-E11	RDNC E2					X6	1479

Notes: Patients coinfected with *S. enterica* serovar Typhimurium are highlighted in bold. Size of the variable region and gene cassette(s) located herein and identified by sequencing. Incompatibility group (estimated plasmid size). Sequence type determined by multilocus sequence typing.
Abbreviations: MDH, Manhiça District Hospital; n, total number of isolates when more than one; PT, phage type; RDNC, reacted but did not conform; UT, untypeable. A, ampicillin; C, chloramphenicol; Nal, nalidixic acid; S, streptomycin; Su, sulfonamides; T, tetracycline; Tp, trimethoprim; XP, *Xba*I profile. ST, sequence type.

into *E. coli* by biparental conjugation. However, 17 of the 21 pUO-SeVR1-like plasmids could be mobilized through triparental mattings. The exceptions were the two plasmids lacking *tet*(A). The obtained transconjugants displayed the resistance phenotype of the corresponding donor strain and carried all resistance genes except *catA1*, *aadA1*, and *dfrA1*, which did not alter the resistance phenotype since they are redundant with the *catA2*, *strAB*, and *dfrA7* genes of plasmid origin.

PFGE and MLST analysis of the isolates

Eight *Xba*I-PFGE profiles were identified among invasive *S.* Typhimurium isolates (Figure 2A), differentially connected with susceptible (X1 and X2), pSLT-A130-like (X3, X4, and X5), and pSLT-BT-like (X6, X7, and X8) isolates. *S.* Enteritidis isolates showed six profiles (Figure 2B), with X1 associated with the susceptible isolates and X2–X6 with the MDR group. The latter profiles can readily be distinguished by a ~350 kb fragment, absent in susceptible isolates from Mozambique as well as in the control ATCC 13076. By MLST, the three susceptible *S.* Typhimurium isolates were assigned to ST19, while all MDR isolates belonged to ST313. In the case of *S.* Enteritidis, two susceptible and 15 MDR isolates were ST11. The remaining four MDR isolates were ST1479.

Discussion

Here we present the resistance properties and molecular types of *S.* Typhimurium and *S.* Enteritidis isolates recovered from
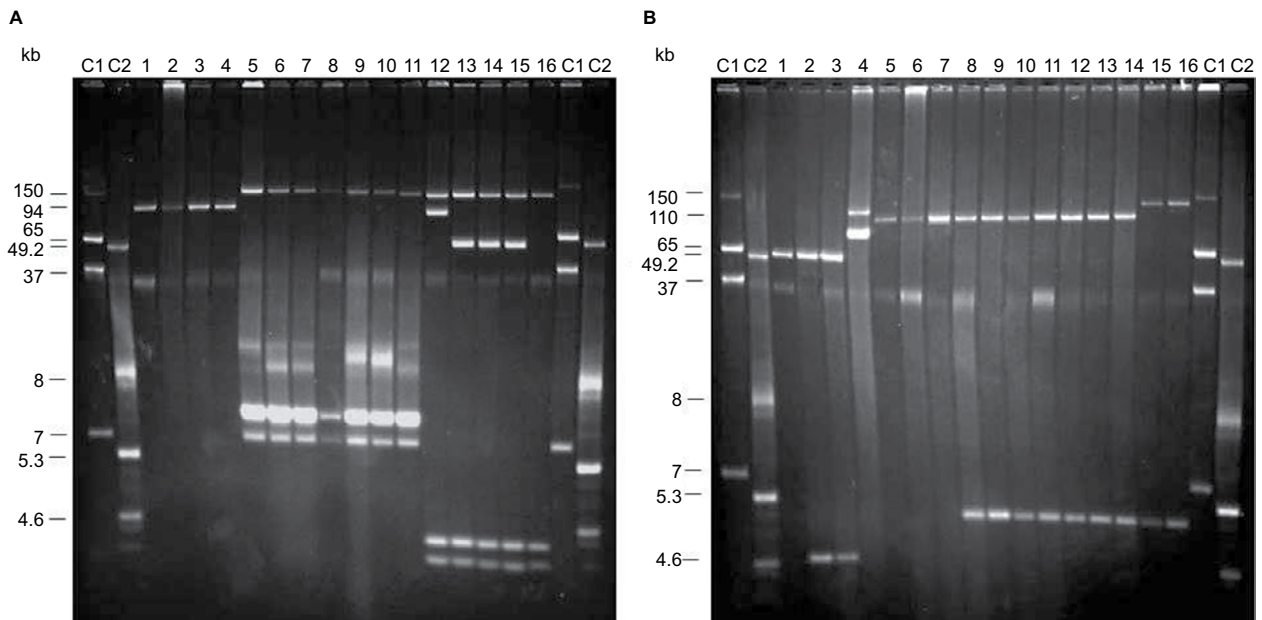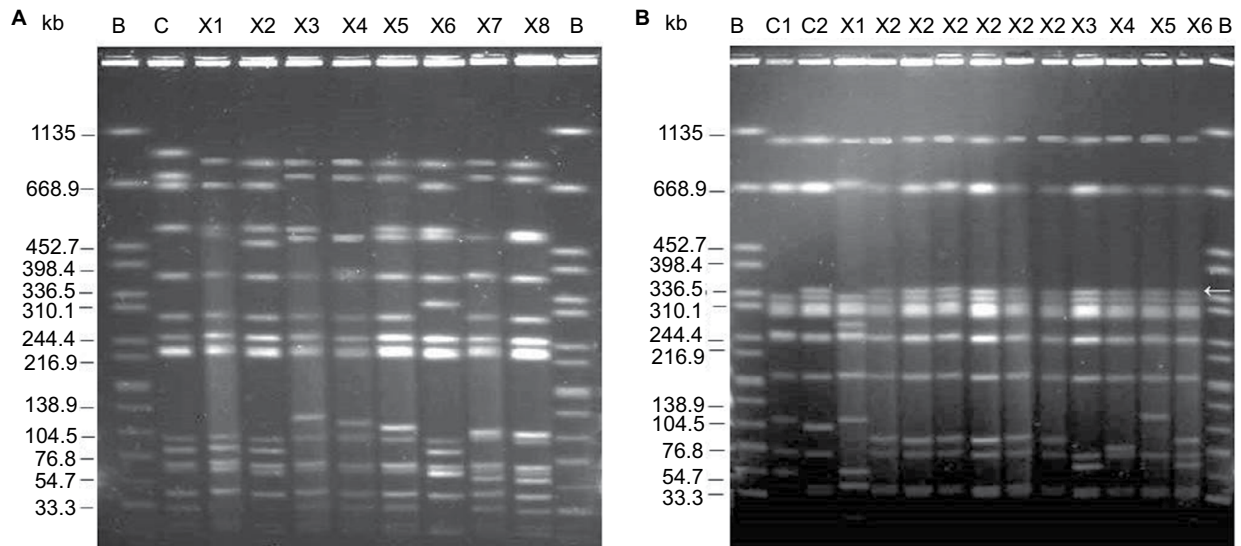
Figure 1 Plasmid profiles of invasive *Salmonella* Typhimurium and *S.* Enteritidis isolates from Mozambique. (**A**) *S.* Typhimurium. Lanes C1 and C2, plasmids obtained from *Escherichia coli* 39R861 and *E. coli* V517, used as size standards; lane 1, *S.* Typhimurium ATCC 14028 (pSLT); lane 2, MDH-T5; lane 3, MDH-T21; lane 4, MDH-T24; lane 5, MDH-T1; lane 6, MDH-T4; lane 7, MDH-T8; lane 8, MDH-T10; lane 9, MDH-T14; lane 10, MDH-T22; lane 11, MDH-T2; lane 12, MDH-T8; lane 13, MDH-T15; lane 14, MDH-T19; lane 15, MDH-T17; lane 16, MDH-T25. (**B**) *S.* Enteritidis. Lanes C1 and C2, size plasmids obtained from *E. coli* 39R861 and *E. coli* V517, used as size standards; lane 1, *S.* Enteritidis ATCC 13076 (pSEV); lane 2, MDH-E1; lane 3, MDH-E10; lane 4, CNM4839/03 (pUO-SeVR1); lane 5, MDH-E13; lane 6, MDH-E24; lane 7, MDH-E11; lane 8, MDH-E3; lane 9, MDH-E5; lane 10, MDH-E19; lane 11, MDH-E23; lane 12, MDH-E7; lane 13, MDH-E15; lane 14, MDH-E13; lane 15, MDH-E20; lane 16, MDH-E22.
Abbreviation: MDH, Manhiça District Hospital.

Figure 2 *Xba*I pulsed-field electrophoresis profiles of invasive *Salmonella* Typhimurium and *S.* Enteritidis isolates from Mozambique. (**A**) *S.* Typhimurium. Lane B, *Xba*I-digested DNA of *S. enterica* serovar Braenderup H9812, used as size standard; lane C, *S.* Typhimurium ATCC 14028; lane X1, MDH-T5; lane X2, MDH-T21; lane X3, MDH-T4; lane X4, MDH-T22; lane X5, MDH-T2; lane X6, MDH-T9; lane X7, MDH-T15; lane X8, MDH-T17. (**B**) *S.* Enteritidis. Lane B, *Xba*I-digested DNA of *S. enterica* serovar Braenderup H9812, used as size standard; lane C1, *S.* Enteritidis ATCC 13076; lane C2, CNM4839/03; lane X1, MDH-E1; lanes X2, MDH-E3, MDH-E19; MDH-E12; MDH-E15; MDH-E13; MDH-E24; lane X3, MDH-E7; lane X4, MDH-E25; lane X5, MDH-E20; lane X6, MDH-E11. The arrow points the ~350 kb fragment distinctive of MDR isolates.
Abbreviations: MDH, Manhiça District Hospital; MDR, multidrug-resistant.

blood samples taken from febrile children in rural Mozambique. As previously reported for iNTS, diarrhea, vomiting, and dehydration were infrequent, and most children presented risk factors, particularly anemia, severe malnutrition, and/or malaria.[3] Like in other sub-Saharan countries, HIV infection is a well-recognized risk factor for iNTS in Mozambique, being highly prevalent in the population, with a high rate of mother-to-child transmission in the community of Manhiça.[28–30] Unfortunately, no HIV data were available for the study period (2002–2004), since HIV counseling and

testing in Manhiça District (southern Mozambique) were not established until 2005.

Blood infections with simultaneous detection of two *Salmonella* serovars have rarely been reported and mainly concerned *S*. Typhi and *S*. Paratyphi A coinfecting Asian patients or patients from developed countries who had traveled to Asia.[31–35] Thus, a surprising result of the present study was the high number of patients coinfected with both *S*. Typhimurium and *S*. Enteritidis. However, a systematic assessment of mixed infections was not attempted, so well-designed comprehensive studies are required to determine the actual frequency and impact of coinfections, not only in Mozambique, but also in other African countries. Coinfections could increase the severity of the disease and led to treatment failure. The latter is of special relevance in low-income countries, due to the limited number of cost-effective therapeutic alternatives. Nonetheless, the two deaths reported in our series were not related with this type of infections.

MDR isolates of *S*. Typhimurium were ST313 and contained two kinds of pSLT derivatives that probably correspond to the pSLT-A130-like and pSLT-BT-like plasmids previously reported in lineages I and II of this invasive clone.[7,8] According to Okoro et al[8], chloramphenicol resistance conferred by pSLT-BT-like plasmids together with the use of this antibiotic as a drug of choice for the treatment of iNTS in Africa could have led to the replacement of lineage I by lineage II, which took place around 2003–2005. As indicated above, the isolates from Mozambique were collected along the period 2002–2004, which overlaps the time proposed for the replacement; therefore, temporal coincidence of the two kinds of plasmids was to be expected. Interestingly, despite being invasive, the three susceptible *S*. Typhimurium isolates belonged to ST19 and carried pSLT.

In contrast to *S*. Typhimurium, information on the STs of invasive *S*. Enteritidis from African countries is still limited. ST11, the most prevalent ST in this serovar, was detected in both susceptible and MDR isolates from Mozambique. Interestingly, four MDR isolates were assigned to ST1479, an uncommon ST that has only been reported in invasive UK isolates from patients linked to Africa.[9] All MDR isolates carried pSEV derivatives that appear to be related to pUO-SeVR1, like those found in the UK isolates, as well as in those belonging to the Central/Eastern African clade, which characteristically carry the *catA2* and *tet*(A) genes for chloramphenicol and tetracycline resistance.[9–11,27] Interestingly, *Xba*I profiles of all *S*. Enteritidis isolates positive for pUO-SeVR1-like plasmids that were analyzed thus far included a distinct ~350 kb fragment, absent in susceptible isolates.[10,36]

Up till now little information exists on the PTs of *S*. Typhimurium and *S*. Enteritidis causing iNTS in Africa. In the present study, most isolates were UT or showed RDNC patterns, which did not coincide with any of those previously recognized in *S*. Typhimurium or *S*. Enteritidis. However, data available on PTs mainly were from European countries. In Kenya, UT and RDNC isolates have also been recovered from patients with gastroenteritis.[37] Therefore, distinctive PTs appear to be circulating in Africa. PT42, the PT shown by invasive UK *S*. Enteritidis related to Africa,[9] was not represented among the limited number of isolates tested in this study, although it has been reported previously in the study community.[12]

Conclusion

Results of the present study have revealed that 1) serovar-specific virulence plasmids were responsible for the MDR phenotype of *S*. Typhimurium and *S*. Enteritidis recovered from febrile children in Mozambique; 2) ST313 (*S*. Typhimurium), ST11, and the uncommon ST1479 (*S*. Enteritidis), as well as previously unrecognized PTs (UT and RDNC) were associated with these isolates; and 3) nearly half of the children were coinfected with both serovars, although there were no particularly poor outcomes in the affected patients.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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