Identification of Invasive *Salmonella Enterica Serovar Typhimurium* ST313 in Ambulatory HIV-Infected Adults in Mozambique

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ABSTRACT

Introduction: Despite evidence describing the burden of invasive non-typhoidal salmonella (iNTS) disease in sub-Saharan Africa, iNTS is not recognized as a priority within global health policy institutions. Recently, *Salmonella enterica* serovar Typhimurium, sequence type (ST) 313, has been identified as the predominant cause of iNTS disease in multiple sub-Saharan African countries. **Materials and Methods:** We conducted multilocus sequence typing (MLST) to determine the prevalence of the ST313 genotype in a sample of blood isolates from ambulatory HIV-infected Mozambican adults with iNTS disease. **Results:** Of the 29 samples of NTS obtained and analyzed by MLST, all (29/29) were assigned the ST313 sequence type based on the set of allele types derived from each of the seven loci. For quality control, five randomly selected strains taken from the original cultures were confirmed as ST313, and the positive control strain SL3261 (taken from the original culture) was categorized as *S. Typhimurium* ST19. **Conclusion:** *S. Typhimurium* ST313 is an important example of a widely distributed pathogen that lacks a coordinated strategy for control. The highly vulnerable populations at risk for ST313 infection in Mozambique, and within the region, would benefit greatly from the development of new policy and on-the-ground capacity to support increased surveillance, prevention, and treatment initiatives.

Key Words: Invasive non-typhoidal salmonella, *Salmonella Typhimurium* ST313, Africa, Human immunodeficiency virus

INTRODUCTION

During the 20th century, nontyphoidal Salmonella (NTS) — previously associated primarily with localized gastrointestinal infections that often resolved without antibiotic treatment — evolved into an important cause of invasive and often fatal bacterial infections within specific subregions of sub-Saharan Africa. Invasive NTS (iNTS) disease most commonly affects immunosuppressed populations (young, human immunodeficiency virus (HIV)-infected, malnourished) and those with other specific comorbidities (especially malaria and anemia).[1-3] In parallel with NTS's evolution from a less-invasive to invasive pathogen, certain subtypes have become resistant to most first-line antibiotics in common use in Africa,[4,5] and the mode of transmission may be evolving from foodborne/zoonotic to person-to-person.[6]

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Recently, *Salmonella enterica* serovar Typhimurium, sequence type (ST) 313, has been identified as the predominant cause of iNTS disease in multiple sub-Saharan African countries, although other NTS strains have also been implicated. In an analysis of 129 specimens obtained from patients with invasive *Salmonella typhimurium (S. typhimurium)* in seven sub-Saharan African nations between 1988 and 2010, 120 (93.0%) were identified as ST313.[7]

This recent evolution in the characteristics and behavior of sub-Saharan African NTS strains has important implications for clinical and public health policy. Clinically, iNTS disease is difficult to distinguish from malaria and other bacterial bloodstream infections without recourse to blood cultures and other diagnostics that are often unavailable in the region. Because conventional first-line antibiotics are commonly ineffective and mortality rates are high in iNTS disease, countries lacking relevant diagnostic capacity must choose between overuse of expensive second- and third-line antimicrobials or undertreatment of iNTS infection. As there is yet no effective vaccine for NTS, uncertainty about the importance of person-to-person vs foodborne or zoonotic transmission also creates doubt about effective selection of prevention interventions.

We recently reported that multidrug resistant *S. typhimurium* was the predominant pathogen associated with bacteremia in febrile HIV-infected adults in Zambézia Province, Mozambique.[8] Multidrug resistant NTS has been shown to be an important and often lethal cause of bloodstream infection in Mozambican children of unknown HIV serostatus.[9,10] The ST313 genotype was identified in 11 of 12 NTS isolates acquired from Mozambican adults of unknown HIV serostatus between 2001 and 2004.[7] We hypothesized that ST313 might be represented among iNTS isolates acquired from our study subjects. Therefore, we performed further investigation to characterize the Zambézian isolates genetically.

MATERIALS AND METHODS

We conducted a prospective observational study between May and September, 2012 (NCT 01681914, www.clinicaltrials.gov), at three health centers in Zambézia Province, Mozambique, to evaluate the performance of new Mozambican guidelines for non-physician clinician management of fever (or history of fever) in HIV-infected adults (previously described).[8,11] At enrollment, bacterial blood cultures were collected from all eligible subjects.

Briefly, 39 (15.1%) of 258 study subjects had blood-culture confirmed bacteremia. One subject had blood-culture confirmed bacteremia on two separate occasions. Thirty-two of 40 isolates (80%) were identified as NTS. We excluded the second specimen for the subject with two positive cultures. Based on serotyping (the only assay available in the field), 27 of the remaining 39 (69.2%) isolates were identified as *S. typhimurium*, nearly all resistant to multiple first-line antibiotics (ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole), one was identified as *S. enteritidis*, two as Salmonella Group O:4, and one as Salmonella spp.[8]

We conducted multilocus sequence typing (MLST) to determine the prevalence of the ST313 genotype in our 27 *S. typhimurium* isolates.[12] Additionally, we conducted MLST on one of the Salmonella Group O:4 isolates and the Salmonella spp. isolate. The second Salmonella Group O:4 was not tested due to contamination found at shipping. The *S. enteritidis* isolate was excluded from analysis.

Deoxyribonucleic acid (DNA) was extracted according to a protocol adapted from Stepan *et al.*,[13] Salmonella isolates were streaked onto Salmonella/Shigella (SS) agar and incubated at 37°C for 18 h. Colonies were resuspended in 40 mL of Proteinase K (Roche Diagnostics) and lysed at 80°C for 10 min, then 55°C for 10 min on a C1000 Touch™ (Bio-Rad) or a PTC-200 (MJ Research) thermocycler. Following incubation, 40 mL of sterile nuclease-free water were added, and the suspension was centrifuged at 10,000g for 1 min. The supernatant containing DNA was transferred to a sterile tube and stored at 4°C until further use.

Seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) were amplified using the recommended primer pairs listed on the University of Warwick's MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Senterica/documents/primersEnterica_html). Polymerase chain reaction (PCR) testing was performed in 40 mL reaction mixtures containing 1 U HotStarTaq® DNA polymerase (Qiagen), 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 0.4 mM each of forward and reverse primer (Sigma), and 2 mL of extracted DNA on a PTC-200. Cycling conditions consisted of an initial activation at 95°C for 15 min and 35 cycles of 30 s at 94°C, 45 s at 55°C, and 1 min at 72°C, followed by a final extension at 72°C for 10 min.

PCR products were diluted to 100 ng/mL and submitted to Vanderbilt Technologies for Advanced Genomics (VANTAGE) for sequencing. Samples were purified with ExoSAP-IT (Affymetrix) and sequenced in both directions with the appropriate forward and reverse

primers on an ABI 3730 × l DNA analyzer (Life Technologies). PeakTrace basecaller (Nucleics) was used to process trace data and designate bases. If forward and reverse sequences for a specimen were discordant, the specimen was resequenced. Sequence data were imported into MacVector 12.0 (MacVector, Inc.), trimmed, aligned, and compared to control sequences obtained from the MLST Warwick site (http://mlst.warwick.ac.uk/mlst/dbs/Senterica/Downloads_HTML). Sequence types for the Salmonella isolates were determined by using the Allele/ST Query tool of the *Salmonella enterica* MLST Database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica/).

For quality control, we also streaked five randomly selected strains and the positive control strain *S. typhimurium* SL3261 onto SS agar from original cultures, extracted, and amplified as described above.

Ethics

The Mozambican National Committee for Bioethics in Health and the Vanderbilt University Institutional Review Board had both approved the parent study and subsequently granted additional approval for the de-identified specimens to be sent to Vanderbilt University for MLST, a technique not then available in Mozambique.

RESULTS

Of the 29 samples of NTS obtained and analyzed by MLST, all (29/29) were assigned the ST313 sequence type based on the set of allele types derived from each of the seven loci [Table 1]. The five randomly selected strains taken from the original cultures were confirmed as ST313, and the positive control strain SL3261 (taken from the original culture) was categorized as *S. typhimurium* ST19.

DISCUSSION

In our study, MLST analysis confirmed the presence of the highly invasive *S. typhimurium* ST313 in febrile HIV-infected adults in Mozambique, and showed genotypic homogeneity among all isolates of this multidrug resistant *S.* Typhimurium. ST313 was the only sequence type identified, and the predominant cause of bacteremia, in our subjects.

We believe that the finding of 100% ST313 is plausible because other studies conducted in sub-Saharan Africa have found ST313 prevalence of over 95% among invasive *S. typhimurium* isolates.[7,14] For example, in Malawi, which shares a border with Zambézia Province, ST313 represented 56/58 (96.6%) of isolates obtained from blood and cerebrospinal fluid.[7]

Table 1: Genetic characterization of *Salmonella enterica* serovar *S. typhimurium*

Sample ID	*aroC* (501 bp)	*dnaN* (501 bp)	*hemD* (432 bp)	*hisD* (501 bp)	*purE* (399 bp)	*sucA* (501 bp)	*thrA* (501 bp)	ST
Moz.1	10	7	12	9	112	9	2	ST313
Moz.2	10	7	12	9	112	9	2	ST313
Moz.3	10	7	12	9	112	9	2	ST313
Moz.4	10	7	12	9	112	9	2	ST313
Moz.5	10	7	12	9	112	9	2	ST313
Moz.6	10	7	12	9	112	9	2	ST313
Moz.7	10	7	12	9	112	9	2	ST313
Moz.8	10	7	12	9	112	9	2	ST313
Moz.9	10	7	12	9	112	9	2	ST313
Moz.10	10	7	12	9	112	9	2	ST313
Moz.11	10	7	12	9	112	9	2	ST313
Moz.12	10	7	12	9	112	9	2	ST313
Moz.13	10	7	12	9	112	9	2	ST313
Moz.14	10	7	12	9	112	9	2	ST313
Moz.15	10	7	12	9	112	9	2	ST313
Moz.16	10	7	12	9	112	9	2	ST313
Moz.17	10	7	12	9	112	9	2	ST313
Moz.18	10	7	12	9	112	9	2	ST313
Moz.19	10	7	12	9	112	9	2	ST313
Moz.20	10	7	12	9	112	9	2	ST313
Moz.21	10	7	12	9	112	9	2	ST313
Moz.22	10	7	12	9	112	9	2	ST313
Moz.23	10	7	12	9	112	9	2	ST313
Moz.24	10	7	12	9	112	9	2	ST313
Moz.25	10	7	12	9	112	9	2	ST313
Moz.26	10	7	12	9	112	9	2	ST313
Moz.27	10	7	12	9	112	9	2	ST313
Moz.28	10	7	12	9	112	9	2	ST313
Moz.29	10	7	12	9	112	9	2	ST313
SL3261	10	7	12	9	5	9	2	ST19

*Moz.1-29 represent patient specimens including 27 *S. Typhimurium*, 1 *Salmonella* Group O:4, and 1 *Salmonella* spp. SL3261 represents our positive control specimen

Because all of our specimens were derived from febrile, HIV-infected adults in three closely-approximated districts in a single province in Mozambique during one 5-month period, our results may not be generalizable either to other HIV-infected populations within Mozambique or to other immunocompromised Mozambican groups, such as severely malnourished children. However, our findings confirm that ST313 has persisted in Mozambique, where its presence was previously documented in samples acquired over a decade ago,[7] and that it now represents an important threat to the survival of HIV-infected Mozambican adults.

In December 2014, both the World Health Organization and the Review on Antimicrobial Resistance issued calls for

implementation of new worldwide initiatives for control of antimicrobial resistance, because of the associated and growing burdens of mortality, morbidity, and negative economic consequences.[15,16] *S. typhimurium* ST313 is an important example of a widely distributed pathogen that lacks a coordinated strategy for control. The highly vulnerable populations at risk for ST313 infection in Mozambique, and within the region, would benefit greatly from the development of new policy and on-the-ground capacity to support increased surveillance, prevention, and treatment initiatives, with particular attention to the needs of HIV-infected, malnourished, and other groups characterized by increased susceptibility to this often-fatal infection.

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Conflicts of interest

There are no conflicts of interest.

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