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Genetically distinct lineages of *Salmonella* Typhimurium ST313 and ST19 are present in Brazil

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ABSTRACT

In sub-Saharan Africa, two genetically distinct lineages of multi-drug resistant non-typhoidal Salmonella (NTS) serovar Typhimurium sequence type 313 (ST313) are known to cause invasive disease among people. *S.* Typhimurium ST313 has evolved to become more human-adapted and is commonly isolated from systemic sites (eg., blood) from febrile patients in sub-Saharan Africa. Epidemiological studies indicate that *S.* Typhimurium is frequently isolated from systemic sites from human patients in Brazil, however, it is currently unknown if this pathogen has also evolved to become more invasive and human-adapted in this country. Here we determined genotypic and phenotypic divergence among clinical *S.* Typhimurium strains isolated from systemic and non-systemic sites from human patients in Brazil. We report that a subset (8/38, 20%) of epidemiologically diverse human clinical strains of *S.* Typhimurium recovered from systemic sites in Brazil show significantly higher intra-macrophage survival, indicating that this subset is likely more invasive. Using the whole genome sequencing and phylogenetic approaches, we identified *S.* Typhimurium ST313-lineage in Brazil that is genetically and phenotypically distinct from the known African ST313-lineages. We also report the identification of *S.* Typhimurium ST19-lineage in Brazil that is evolving similar to ST313 lineages from Africa but is genetically and phenotypically distinct from ST19-lineage commonly associated with the gastrointestinal disease worldwide. The identification of new *S.* Typhimurium ST313 and ST19 lineages responsible for human illnesses in Brazil warrants further epidemiological investigations to determine the incidence and spread of a genetically divergent population of this important human pathogen.

1. Introduction

Salmonella enterica sub sp. *enterica* is a Gram-negative, facultative intracellular human and animal pathogen and one of the four key causes of diarrheal diseases worldwide (WHO, 2016). *Salmonella enterica* is classically divided into typhoidal or non-typhoidal *Salmonella* (NTS) serovars (Feasey et al., 2012). The typhoidal category includes the human-restricted serovars such as *Salmonella* Typhi and Paratyphi that cause systemic (a.k.a. typhoidal) disease. The NTS category contains more than 1400 serovars including the most clinically significant serovars such as *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) and Typhimurium (*S.* Typhimurium) that primarily cause food-borne gastrointestinal disease in human (Batz et al., 2012; Hoffmann et al., 2012). However, in sub-Saharan African countries with a high prevalence of HIV and malaria, *S.* Typhimurium strains belonging to multi-locus sequence type ST313 (ST313) cause invasive disease and are commonly isolated from systemic sites such as blood or cerebrospinal fluid (CSF) (Ao et al., 2015; MacLennan, 2014). These invasive *S.* Typhimurium isolates generally display multi-drug resistance (MDR) and belong to two closely related but genetically distinct lineages, referred as African lineage-I and -II (Ao et al., 2015; Langridge et al., 2009; MacLennan, 2014; Okoro et al., 2015, 2012; Reddy et al., 2010; Uche et al., 2017; Zhang et al., 2003). These two African ST313-lineages have emerged independently in temporal association with HIV pandemic (Okoro et al., 2015). The *S.* Typhimurium strains within African ST313-lineages show a distinct metabolic signature and pathogenicity *in vitro* (cell culture models) and *in vivo* (murine and cattle model of colitis) when compared with the *S.* Typhimurium strains

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outside of the ST313 lineages (Okoro et al., 2015; Ramachandran et al., 2015). African ST313-lineages have undergone extensive genomic degradation that shares significant similarities with the genome degradation observed in human-adapted typhoidal *Salmonella* serovars *S.* Typhi and *S.* Paratyphi (Okoro et al., 2015). It has been proposed that series of micro-evolutionary processes have resulted in evolutionary convergence between *S.* Typhimurium ST313 and *S.* Typhi which has enabled African *S.* Typhimurium ST313 to become more human-adapted (Okoro et al., 2015).

Until recently, *S.* Typhimurium ST313 was sporadically reported outside of sub-Saharan Africa. A recent study published by CDC showed that, in 2010, invasive NTS (iNTS) infections were most common in Africa (226 cases/100,000 population), with an increase in the incidence of iNTS infections in Europe (102 cases/100,000) Americas (23 cases/100,000), South East Asia (21 cases/100,000) and Middle East/Asia/Oceania (0.8/100,000) (Ao et al., 2015). The iNTS from sub-Saharan Africa are extensively characterized both genetically and phenotypically, however, there is a significant gap in the understanding of the biology of iNTS and iNTS-associated disease reported from the other parts of the world. For instance, *S.* Typhimurium is frequently isolated from systemic sites such as blood and CSF from human patients in Brazil (Fernandes et al., 2006). From 1985–1999, 14.7% (167 out of 1138) NTS were isolated from blood samples collected from human patients in São Paulo, Brazil (de Castro et al., 2002). Of all the NTS isolated from blood, *S.* Enteritidis and *S.* Typhimurium accounted for 24.5% (n = 41) isolates (de Castro et al., 2002). Similarly, from 1996 to 2003, 13.7% (465 out of 3414) NTS were isolated from blood and CSF of human patients (Fernandes et al., 2006). Of these, *S.* Enteritidis and *S.* Typhimurium accounted for 45.8% (n = 213) of the isolates (Fernandes et al., 2006). These studies show that *S.* Typhimurium and *S.* Enteritidis are more frequently isolated from systemic sites from human patients in Brazil than other NTS serovars. However, the underlying reasons are currently unknown.

The aim of this study was to perform comparative genotypic and phenotypic characterization of spatiotemporally, genetically and phenotypically diverse *S.* Typhimurium strains isolated specifically from systemic (blood/cerebrospinal fluid), non-systemic (stool) and extra-intestinal sites (urine, wound, and abdominal abscess) from human patients in Brazil. Our underlying hypothesis was that a subset of these *S.* Typhimurium strains may be phenotypically or genetically related to the iNTS serovar *S.* Typhimurium ST313 reported from sub-Saharan Africa. Here we report that irrespective of the source of isolation, a subset of Brazilian *S.* Typhimurium strains show significantly higher intra-macrophage survival, indicative of potentially invasive nature of this subset of strains. Whole-genome sequencing and comparative genomics analysis of representative strains with high and low intra-macrophage survival showed the presence of *S.* Typhimurium ST313 in Brazil that is genetically and phenotypically distinct from the African ST313-lineages. In addition, we report that a new *S.* Typhimurium ST19-lineage is circulating in Brazil that is genetically and phenotypically closely related to ST313-lineages, but distinct from ST19-lineage commonly associated with the gastrointestinal disease worldwide.

2. Materials and methods

2.1. Bacterial strains

A total of 38 spatiotemporally diverse clinical strains of *S.* Typhimurium isolated between 2006 and 2015 from different geographic regions of Brazil were obtained from a collection of Enterobacteria (CENT), located at the national reference laboratory for cholera and other bacterial intestinal infections (LRNCEB) at Oswaldo Cruz Foundation (Fig. 1). The sources of these strains included systemic (blood/CSF, N = 12), non-systemic (feces, n = 19), and extra-intestinal sites (n = 5) such as urine, wound secretion and abdominal abscess. All strains were stored in 15% (v/v) glycerol at −80 °C. Before each experiment, the strains were cultured in brain heart infusion (Difco, USA) or LB broth Lennox (LB) medium for 16 h at 37 °C. A single colony was inoculated in 5 ml of LB and incubated overnight (16 h) at 37 °C with shaking and 200 rpm.

2.2. Pulse field gel electrophoresis (PFGE)

In order to ensure that the selected strains were genetically diverse, PFGE was conducted following the standard CDC PulseNet PFGE protocol for NTS (Ribot et al., 2006). Briefly, overnight cultures (OD_{595} of 0.5) were incorporated into 1.0% (w/v) agarose plugs (Seakam Gold, Lonza, Rockland, ME, USA). The plugs were solidified at room temperature followed by digestion with Proteinase-K (20 mg/ml, Sigma–Aldrich) for 1 h in a 55 °C shaker water bath. Restriction endonuclease digestion was performed with 30 U of XbaI (TermoFisher®, USA) for 3 h at 37 °C. For optimization, 200 mM of thiourea (Sigma–Aldrich, USA) was added to the running buffer and the gel. The restriction fragments were separated by a CHEF-DR III System (Bio-Rad Laboratories, USA) in a 1% (w/v) agarose gel using pulse time at 2.2 s followed by 63.8 s at 6.0 V/cm for 24 h. For the cross-sectional study, gel images were analyzed by Bionumerics v.6.6 (Applied Maths Bionumerics®, Belgium) and cluster analysis was performed by UPGMA using dice similarity coefficient with optimization set at 0.5% and position tolerance at 1.5%. The strains were considered genetically identical or closely related when the PFGE band patterns were more than 95% similar. *S.* Braenderup strain H9812 served as a molecular standard.

2.3. Antimicrobial susceptibility testing

The antimicrobial susceptibility was determined by the disk diffusion method on Mueller Hinton agar (Difco, Becton Dickinson, MD, USA) following the clinical and laboratory standards institute guidelines (CLSI, 2010). Following antibiotic disks were used (BD, Becton Dickinson, MD, USA): Ampicillin – 10 µg (Am), Amoxicillin/Clavulanic Acid – 20/10 µg (Amc), Amikacin – 30 µg (An), Chloramphenicol – 30 µg (C), Ciprofloxacin – 5 µg (Cip), Sulfisoxazole – 0.25 µg (G), Gentamicin – 10 µg (Gm), Nalidixic Acid k 30 µg (Nal), Streptomycin – 10 µg (S), Sulfamethoxazole-Trimethoprim – 23.75/1.25 µg (Sxt), Tetracycline – 30 µg (Te) and Ceftiofur – 30 µg (XNL). *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* 10536 served as control strains.

2.4. Intra-macrophage survival assay

Intra-macrophage survival assay was performed as previously described with minor modifications (Ramachandran et al., 2015). The human macrophages (THP- 1 cells) were seeded into each well of a 48-well plate (2×10^5/well) in complete RPMI 1640 medium (HyClone Laboratories, USA) and activated with 10 mM phorbol 12-myristate 13-acetate (PMA) for 18 h at 37 °C with 5% CO_2. An aliquot from the overnight culture was transferred to LB and incubated at 37 °C for 3 h before cell infection. Activated THP-1 cells were infected at a MOI of 50:1 (bacteria:cell). The plates were centrifuged for 10 min at 400 x *g* at room temperature to bring the bacteria in contact with the cells. The cells were incubated for 30 min at 37 °C in a 5% CO_2 incubator following which cells were washed thrice with PBS. Next, RPMI 1640 containing 200 µg/ml gentamicin was added to each well and the cells were lysed using 200 µl of 0.5% Triton X-100 at 30 min (uptake/invasion), 2 h and 20 h (survival) of incubation. Serial 10-fold dilutions were plated on LB agar to determine viable counts. Each isolate was tested in triplicates in three independent experiments. The % intracellular counts were calculated as the CFU at t = 0 divided by the CFU at t = 30 min x 100 (% uptake/invasion); CFU at t = 30 min divided by the CFU at t = 2 h × 100 (% intracellular survival at 2 h) and CFU at t = 2 h divided by the CFU at t = 20 h × 100 (% intracellular survival at 20 h). One-way analysis of variance (ANOVA) with