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Multilocus sequence typing of *Salmonella* Typhimurium reveals the presence of the highly invasive ST313 in Brazil

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Dear Editor,

Salmonella Typhimurium persists as one of the leading causes of gastroenteritis worldwide and has been extensively studied (Majowicz et al., 2010; Scallan et al., 2011). Multilocus sequence typing (MLST) has contributed to a better understanding of the epidemiology, evolution and genotypic diversity of clinically important bacterial pathogens, allowing comparison among strains isolated in different parts of the globe (Achtman et al., 2012). Specifically, among the *S.* Typhimurium strains isolated broadly, ST19 has been reported as the most prevalent ST followed by ST34 and ST313 (Achtman et al., 2012). ST34 was found mainly in multidrug resistant strains isolated from Asia and Europe (Sun et al., 2014; Wong et al., 2013). Interestingly, ST313 has been reported almost exclusively in isolates from sub-Saharan Africa presenting antibiotic resistance associated with bloodstream infections and mortality rates of >25% (Feasey et al., 2014; Ley et al., 2014).

S. Typhimurium pathogenicity has been well described (Fàbrega and Vila, 2013; Haraga et al., 2008; Herrero-Fresno et al., 2014; Galán, 1996; Galán and Curtiss, 1989; Shea et al., 1996). Most of the virulence features have been reported for prototype strains such as the SL1344 (Hoiseth and Stocker, 1981), virulence in mouse (Francis et al., 1992) and calf models (Wray and Sojka, 1978). The highly virulent prototype strains for *S.* Typhimurium pathogenesis such as 14028s and SL1344, a derivative from LT2, (McClelland et al., 2001) are all representatives of the gastroenteritis-associated classic ST19 (Singletary et al., 2016).

The aim of this study was to evaluate the genotypic diversity of 88 *S.* Typhimurium strains isolated from humans (40) between 1983 and 2010, and food (48) between 1995 and 2013 from different States of Brazil using MLST (Table 1). The strains isolated from human feces were isolated from patients with gastroenteritis diseases. Additionally, strains isolated from blood and brain abscess were isolated from patients with invasive diseases. Furthermore, the pathogenicity of some representative strains of ST313 was evaluated. Those strains were provided by two *Salmonella* spp. reference laboratories in Brazil including the Adolf Lutz Institute of Ribeirão Preto (IAL-RP) and the Oswaldo Cruz Foundation (FIOCRUZ). DNA samples were extracted according to the protocol described by Campioni and Falcão (2014) and sequenced using NextSeq Illumina chemistry (Illumina, San Diego, CA). The Illumina reads were assembled with SPAdes (Nurk et al., 2013). The sequences of all 88 *S.* Typhimurium strains were submitted to GenBank and the accession numbers are presented in Table 1.

The allele identifications for the seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*) were performed using the online *S. enterica* MLST scheme (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) and the corresponding STs were determined using the Center for Genomic Epidemiology MLST site (https://cge.cbs.dtu.dk/services/MLST/).

Moreover, we have evaluated respectively the HeLa cells invasion phenotypes and macrophage survival of the nine ST313 (STm 29, STm 30, STm 34, STm 35, STm 39, STm 40, STm 44, STm 47) and three ST19 (STm32, STm43, and STm48) strains of this study as depicted on Fig. 1A and B, these strains were compared with the SL1344 prototype to measure their *in vitro* pathogenesis within tissue cultures. The gentamycin protection assays were performed to measure the ability of these strains to invade epithelial HeLa cells after 90 min of bacteria-cell interaction and intramacrophage survival during 3 h, after 30 min bacteria-cell interaction in J774 murine macrophages both employing a 100:1 MOI, as previously described (Fierer et al., 1993; Finlay et al., 1991; Pfeifer et al., 1999) and detailed in the Supplemental material.

Among all 88 strains here studied (Table 1), 76 (86.4%) strains were typed as ST19, whereas this ST being the most common type among the *S.* Typhimurium strains in the *S. enterica* database. Additionally, 9 (10.2%) strains in this study isolated from humans ($n = 7$) and food ($n = 2$) were typed as ST313. Two (2.3%) strains isolated from food were typed as ST1921 and 1 (1.1%) strain was typed as ST1649.

At the moment, the ST313 has been described and isolated exclusively from sub-Saharan African patients, consistently presenting a multi-drug resistance pattern, associated with bloodstream infections and mortality rates of >25% and often linked with HIV infection in adults, and/or linked with malaria, HIV, and malnutrition in children (Feasey et al., 2014; Ley et al., 2014). Herein, we found just one ST313 strain (STm29) that was resistant to ampicillin (Almeida et al., 2015). The other eight ST313 strains of this study (STm30, STm34, STm35, STm37, STm39, STm40, STm44 and STm47) were sensitive to all antimicrobials tested, as previously described in Almeida et al. (2015). Based on these observations on the Brazilian-isolated ST313 strains, they are not geographically exclusive to sub-Saharan Africa region, in addition the Brazilian ST313 strains have shown a different antibiotics resistance profile than typical isolates from Africa. However, more ST313 strains and further studies must be conducted to reinforce this specific observation. Besides bloodstream infections ST313 has been isolated from feces and food in Brazil similar to reports from Africa (Herrero-Fresno et al., 2014; Kariuki and Onsare, 2015).

The phenotypic comparisons between the *S.* Typhimurium SL1344 prototype strain and all nine ST313s, and additionally three selected ST19 strains were performed to evaluate their ability to invade HeLa cells and survive within J774 macrophages, as illustrated on Fig. 1A and B. All ST313 and ST19 strains here tested were able to invade epithelial cells at similar or slightly higher levels than SL1344 strain, as illustrated on Fig. 1A. All twelve strains employed have shown similar

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Table 1
Year, state and source of isolation of 88 *Salmonella* Typhimurium strains isolated from humans (43) and food (48) in Brazil.

CFSAN no.	Isolate name	GenBank accession no.	Source	Year of isolation	State	ST
CFSAN033849	STm 02	LVHB00000000	Human feces	1983	SP	19
CFSAN033850	STm 03	LVHA00000000	Human feces	1983	SP	19
CFSAN033851	STm 04	LVGZ00000000	Human feces	1983	SP	19
CFSAN033852	STm 05	LVGY00000000	Human feces	1983	SP	19
CFSAN033853	STm 06	LVGX00000000	Human feces	1983	SP	1649
CFSAN033854	STm 07	LVGW00000000	Human feces	1983	SP	19
CFSAN033855	STm 08	#N/D	Human feces	1983	SP	19
CFSAN033856	STm 09	LVGV00000000	Human feces	1984	SP	19
CFSAN033857	STm 10	LVGU00000000	Human feces	1984	SP	19
CFSAN033858	STm 11	LVGT00000000	Human feces	1984	SP	19
CFSAN033859	STm 12	LUJG00000000	Human feces	1984	SP	19
CFSAN033860	STm 13	LVGS00000000	Human feces	1984	SP	19
CFSAN033861	STm 14	LVGR00000000	Human feces	1984	SP	19
CFSAN033863	STm 16	LVGP00000000	Human feces	1985	SP	19
CFSAN033864	STm 17	LVGO00000000	Human feces	1985	SP	19
CFSAN033865	STm 18	LVGN00000000	Human feces	1985	SP	19
CFSAN033866	STm 19	LVGM00000000	Human feces	1986	SP	19
CFSAN033867	STm 20	LVGL00000000	Human feces	1986	SP	19
CFSAN033868	STm 21	LUJF00000000	Human feces	1986	SP	19
CFSAN033869	STm 22	LVGK00000000	Human feces	1986	SP	19
CFSAN033870	STm 23	LVGJ00000000	Human feces	1986	SP	19
CFSAN033871	STm 24	LVGI00000000	Human feces	1986	SP	19
CFSAN033872	STm 25	LVGH00000000	Human feces	1986	SP	19
CFSAN033873	STm 26	LVGG00000000	Human feces	1986	SP	19
CFSAN033874	STm 27	LVGF00000000	Human feces	1986	SP	19
CFSAN033876	STm 29	LVGE00000000	Human feces	1989	SP	313
CFSAN033877	STm 30	LVGD00000000	Human feces	1990	SP	313
CFSAN033878	STm 31	LUJD00000000	Human feces	1991	SP	19
CFSAN033879	STm 32	LVGC00000000	Human feces	1992	SP	19
CFSAN033880	STm 33	LVGB00000000	Human feces	1992	SP	19
CFSAN033881	STm 34	LVGA00000000	Human feces	1993	SP	313
CFSAN033882	STm 35	LVFZ00000000	Human feces	1995	SP	313
CFSAN033883	STm 36	LVFY00000000	Cold chicken	1995	SP	19
CFSAN033884	STm 37	LVFX00000000	Raw pork sausage	1996	SP	313
CFSAN033885	STm 38	LUJC00000000	Human feces	1997	SP	19
CFSAN033886	STm 39	LUJB00000000	Human feces	1998	SP	313
CFSAN033887	STm 40	LUJA00000000	Lettuce	1998	SP	313
CFSAN033888	STm 41	LVFW00000000	Raw kafta	1998	SP	19
CFSAN033889	STm 42	LUIZ00000000	Human feces	1999	SP	19
CFSAN033890	STm 43	LVFV00000000	Human feces	2000	SP	19
CFSAN033891	STm 44	LVFU00000000	Blood	2000	SP	313
CFSAN033892	STm 45	LUIY00000000	Raw pork sausage	2000	SP	19
CFSAN033893	STm 46	LVFT00000000	Raw Tuscan sausage	2002	SP	19
CFSAN033894	STm 47	LUIX00000000	Human feces	2003	SP	313
CFSAN033895	STm 48	LUIW00000000	Brain abscess	2005	SP	19
CFSAN033896	STm 49	LVFS00000000	Human feces	2010	SP	19
CFSAN033897	702/99	LVFR00000000	Final product	1999	SC	19
CFSAN033898	12288/06	LUIV00000000	Swine	2006	SC	19
CFSAN033899	12278/06	LUIU00000000	Swine	2006	SC	19
CFSAN033900	12290/06	LUIT00000000	Swine	2006	SC	19
CFSAN033901	12268/06	LVFQ00000000	Swine	2006	SC	19
CFSAN033902	12381/06	LUIS00000000	Swine	2006	SC	19
CFSAN033903	5936/06	LUIR00000000	Cold chicken	2006	SC	19
CFSAN033904	5937/06	LUIQ00000000	Cold chicken	2006	SC	19
CFSAN033905	5934/06	LUIP00000000	Swine	2006	SC	19
CFSAN033906	5961/06	LUIO00000000	Swine	2006	SC	19
CFSAN033907	5962/06	LVFP00000000	Swine	2006	SC	19
CFSAN033908	5929/06	LUIN00000000	Poultry	2006	SC	19
CFSAN033909	13609/06	LUIM00000000	Poultry	2006	SC	19
CFSAN033910	3848/08	LUIL00000000	Food	2008	SC	19
CFSAN033911	16238/09	LUIK00000000	Ready-to-eat dish	2009	MS	19
CFSAN033912	16239/09	LVFO00000000	Ready-to-eat dish	2009	MS	19
CFSAN033913	16240/09	LUIJ00000000	Ready-to-eat dish	2009	MS	19
CFSAN033914	16202/09	LUII00000000	Industrialized product	2009	RS	19
CFSAN033915	16251/09	LUIH00000000	Industrialized product	2009	GO	19
CFSAN033916	16273/09	LVFN00000000	Industrialized product	2009	GO	19
CFSAN033917	17307/09	LUIG00000000	Industrialized product	2009	–	19
CFSAN033918	9461/10	LUIF00000000	In natural meat	2010	SC	19
CFSAN033919	9479/10	LUIE00000000	In natural meat	2010	SC	19
CFSAN033920	7032/10	LVFM00000000	Poultry	2010	PR	19
CFSAN033921	3057/10	LUID00000000	Frozen chicken carcass	2010	PR	19
CFSAN033922	6346/10	LUIC00000000	Chicken	2010	SP	19
CFSAN033923	5635/10	LVFL00000000	Unknown	2010	RS	19
CFSAN033924	9109/10	LVFK00000000	Swine	2010	PR	19
CFSAN033925	426/10	LUIB00000000	Chicken	2010	SC	19

Table 1 (*continued*)

CFSAN no.	Isolate name	GenBank accession no.	Source	Year of isolation	State	ST
CFSAN033926	447/10	LUIA00000000	Chicken	2010	SC	19
CFSAN033927	2452/11	LUHZ00000000	Frozen chicken carcass	2011	SP	19
CFSAN033928	6709/11	LVFJ00000000	Cold chicken	2011	RS	19
CFSAN033929	948/12	LUHY00000000	Raw salad	2012	BA	19
CFSAN033930	1103/12	LUHX00000000	Swine (homemade salami)	2012	RS	19
CFSAN033931	1104/12	LVFI00000000	Swine (homemade salami)	2012	RS	19
CFSAN033932	3330/12	LUHW00000000	Roast beef	2012	SC	19
CFSAN033933	994/13	LUHV00000000	Final product sales (animal origin)	2013	SP	19
CFSAN033934	374/13	LUHU00000000	Final product sales (animal origin)	2013	SC	19
CFSAN033935	465/13	LUHT00000000	Final product sales (animal origin)	2013	SP	19
CFSAN033937	622/13	LUHS00000000	Final product sales (animal origin)	2013	SC	1921
CFSAN033938	583/13	LUHR00000000	Final product sales (animal origin)	2013	SC	19
CFSAN033939	623/13	LVFH00000000	Final product sales (animal origin)	2013	SC	1921

CFSAN, Center for Food Safety and Applied Nutrition; SP, Sao Paulo; SC, Santa Catarina; PR, Parana; MS, Mato Grosso do Sul; RS, Rio Grande do Sul; GO, Goias; BA, Bahia.

invasion levels, between 1×10^4–1×10^6 CFU/cm^2. The STm44 and STm47 strains peaked respectively with 5.9×10^5 and 5.8×10^5 CFU/cm^2 during HeLa invasion, which may be consider as high as the 5.7×10^5 CFU/cm^2 of the SL1344 prototype, although statistically they are not consider different. On the other hand, both strains may be contemplated to be as virulent as the *S.* Typhimurium prototype during HeLa cells invasion, when compared to DH5α strain control.

Similarly, during intracellular replication among these twelve strains, both STm43 and STm44 strains have presented an order of magnitude higher during the macrophage survival, approximately 1×10^6 CFU/cm^2. Conversely the STm39 strain was the only strain that has presented lower intramacrophage replication compared to SL1344 (Fig. 1B).

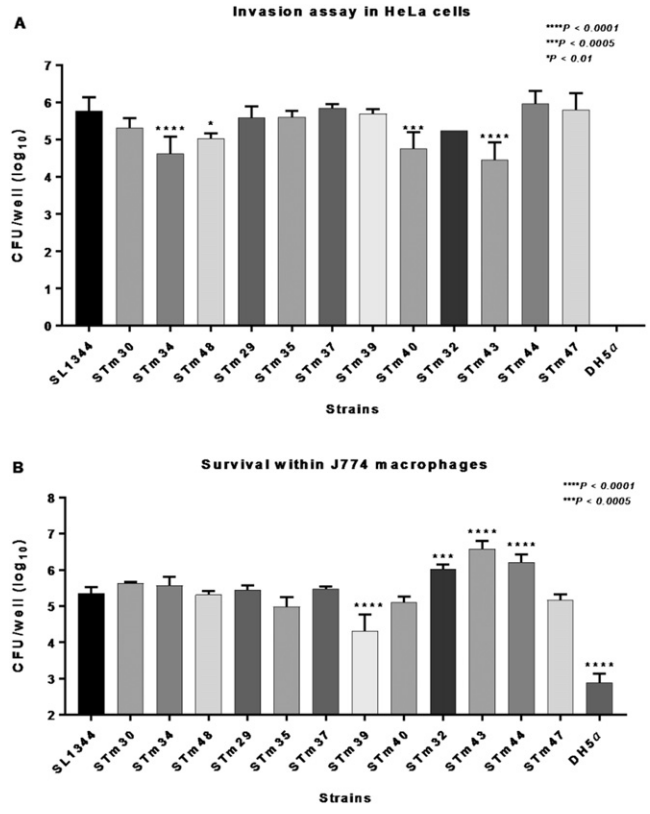

Fig. 1. Comparison of the selected *Salmonella* Typhimurium strains from both ST313 and ST19 isolated in Brazil with the prototype SL1344 strain. (A) Invasion of epithelial HeLa cells. (B) Intramacrophage survival and replication in J774 macrophage cells. ****$P < 0.0001$, *** $P < 0.0005$, * $P < 0.01$.

The blood-isolated STm44 strain has presented approximately an order of magnitude higher during both assays, thus it has presented the most prominent HeLa invasion and also a striking high replication levels within J774 macrophages replication when compared to SL1344 prototype, which is classically considerer as model pathogen. Both ST313 and ST19 typed strains have shown high levels of invasion of epithelial cells and intracellular replication in J774 macrophages comparable to SL1344 levels, respectively illustrated on Fig. 1A and B. Likewise, the 02-03/002 ST313 strain was as virulent as the 4/74 prototype strain in the macrophage assays (Herrero-Fresno et al., 2014). Herein, we present the first description and pathogenic characterization of these virulent ST313 strains isolated from clinical and food sources from Brazil pointing to the high virulence properties of these non-typhoidal strains. Recently, a study reported an African ST313 isolate to be more invasive than 14028s WT *S.* Typhimurium. The authors documented that ST19 *S.* Typhimurium strain SL1344 exhibits particularly high levels of SPI-1 expression (Singletary et al., 2016). Conversely, another study showed a less invasive phenotype for ST313 sub-Saharan Africa strains than ST19 *S.* Typhimurium isolates, with less expression of *sopE2*, from SPI-1, and *fliC*, which encodes for a *S.* Typhimurium flagellin (Carden et al., 2015). The complete role of T3SS-1 on the invasion progress, or the T3SS-2 intracellular replication of these ST313 infections is unclear. The data presented here on high survival rate within macrophages is an important step to understanding how these strains may be disseminated systemically in the patient's bloodstream. More studies are still necessary to fully clarify the process for the survival within the well-defined *Salmonella*-containing vacuole (SCV) (Haraga et al., 2008).

In summary, we have provided novel findings on the ST313 strains of *S.* Typhimurium reported for the first time in Brazil, including isolates from bloodstream infections, feces and foods.

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Conflict of interest

There is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2017.03.009.

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