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BACTERIAL FUNGAL AND VIRUS MOLECULAR BIOLOGY - RESEARCH PAPER

Phylogenetic analysis revealed that *Salmonella* Typhimurium ST313 isolated from humans and food in Brazil presented a high genomic similarity

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Abstract

Salmonella Typhimurium sequence type 313 (*S.* Typhimurium ST313) has caused invasive disease mainly in sub-Saharan Africa. In Brazil, ST313 strains have been recently described, and there is a lack of studies that assessed by whole genome sequencing (WGS)—the relationship of these strains. The aims of this work were to study the phylogenetic relationship of 70 *S.* Typhimurium genomes comparing strains of ST313 ($n = 9$) isolated from humans and food in Brazil among themselves, with other STs isolated in this country ($n = 31$) and in other parts of the globe ($n = 30$) by 16S rRNA sequences, the Gegenees software, whole genome multilocus sequence typing (wgMLST), and average nucleotide identity (ANI) for the genomes of ST313. Additionally, pangenome analysis was performed to verify the heterogeneity of these genomes. The phylogenetic analyses showed that the ST313 genomes were very similar among themselves. However, the ST313 genomes were usually clustered more distantly to other STs of strains isolated in Brazil and in other parts of the world. By pangenome calculation, the core genome was 2,880 CDSs and 4,171 CDSs singletons for all the 70 *S.* Typhimurium genomes studied. Considering the 10 ST313 genomes analyzed the core genome was 4,112 CDSs and 76 CDSs singletons. In conclusion, the ST313 genomes from Brazil showed a high similarity among them which information might eventually help in the development of vaccines and antibiotics. The pangenome analysis showed that the *S.* Typhimurium genomes studied presented an open pangenome, but specifically tending to become close for the ST313 strains.

Keywords *Salmonella* Typhimurium · ST313 · Phylogeny · Pangenome

Introduction

Salmonella enterica subsp. *enterica* serovar Typhimurium can cause gastroenteritis and invasive disease in humans and other animals around the world [1–3]. It is estimated that more than one million cases of salmonellosis, 23,000 hospitalizations and 450 deaths occur in the USA each year [4]. According to the European Center for Disease Prevention and Control

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(ECDC) [5], *Salmonella* is one of the leading bacteria found in foodborne infections in the European Union and *S.* Enteritidis and *S.* Typhimurium have been the most prevalent serovars [2].

In Brazil, *Salmonella enterica* has been reported as the main isolated pathogen in foodborne outbreaks and *S.* Typhimurium has been pointed as the first or second most isolated serovar in the country, depending on the location [6–8].

S. Typhimurium invades host cells through the type III secretion system (T3SS) and genes which encode this system and the effectors proteins are located mainly in two regions on the chromosome denominated pathogenicity islands 1 and 2 (SPI-1 and SPI-2) that have the capacity to modulate a series of cellular functions related to the survival and replication of *S.* Typhimurium in host cells [9, 10].

Clinical and epidemiological data indicate that *S.* Typhimurium sequence type (ST) ST313 has been frequently linked to invasive systemic disease, bacteremia, septicemia, and meningitis in Mali and West Africa [11, 12]. It has been reported that *S.* Typhimurium ST313 can also cause systemic infections in children and adults with HIV [11–13]. On the other hand, cases of gastroenteritis have been mainly caused by *S.* Typhimurium ST19 worldwide [14–16]. According to Gilchrist and Maclennan [17], *S.* Typhimurium ST313 strains is genetically distinct when compared to non-invasive *S.* Typhimurium ST19 strains, but it is not clear what these strains have of difference, some authors suggest the presence of plasmids, prophage-like elements, and the presence of different genes, such as, *st313-td* [18–20].

By multilocus sequence typing (MLST), nine *S.* Typhimurium ST313 strains isolated from humans and food between 1989 and 2003 have been recently described, for the first time, in Latin America and Hela cells invasion and intramacrophage survival assays were performed for those strains [1]. Comparative analyses using the complete genomes of *S.* Typhimurium ST313 and ST19 strains are of great importance and can help to elucidate the diversity and phylogenetic relations among the strains and can also improve epidemiologic data of this important global pathogen [21]. Furthermore, there is a lack in studies that characterized possible phylogenetic differences of *S.* Typhimurium strains isolated from diverse sources and distinct genetic subtypes in Brazil [1, 21].

Frequently, the 16S rRNA sequences analysis has been successfully used for taxonomic classification and in phylogenetic studies of different bacterial genus such as *Salmonella*, *Listeria*, and *Escherichia coli*, but in some cases, this approach cannot distinguish strains of the same species and is necessary to use other methods like some based on whole genome sequences [22–24].

Whole genome sequencing (WGS) has been proved to be a tool with a high discriminatory power capable to improve epidemiological and phylogenetic studies. Moreover, WGS has become financially more accessible in the last years, allowing the understanding of the genomic variability of some important foodborne pathogens such as *Salmonella* spp. [25–27].

The aims of this work were to study the phylogenetic relationship of 70 *S.* Typhimurium genomes comparing ST313 strains isolated from humans and food in Brazil among each other, with other STs isolated in this country and in other parts of the globe by using different phylogenetic strategies such as 16S rRNA sequences, the alignment of fragmented genomes for inference of phylogenetic distances using the Gegenees software [28], whole genome multilocus sequence typing (wgMLST), and average nucleotide identity (ANI). Furthermore, it was aimed to verify the heterogeneity of these genomes by pangenome analysis to better understand their genotypic diversity.

Altogether, the results obtained in this work contributed for a better characterization of the *S.* Typhimurium strains studied regarding its genotypic diversity.

Materials and methods

Bacterial strains and genome sequencing

A total of 40 *S.* Typhimurium strains isolated from human diarrhoeic feces and food in the São Paulo State in Brazil, between 1983 and 2013 were selected from the collections of the Adolfo Lutz Institute of Ribeirão Preto (IAL-RP) and Oswaldo Cruz Foundation (FIOCRUZ-RJ). The genomic DNA extraction of these 40 *S.* Typhimurium strains was performed according to Campioni and Falcão [29]. The concentration of the genomic DNA was detected in NanoDrop 1000 (Thermo Scientific). Libraries were prepared using 1 ng of genomic DNA with the Nextera XT DNA library preparation kit (Illumina, San Diego, CA) [30].

The WGS was performed using the NextSeq 500 desktop sequencer with the NextSeq 500/500 high-output version 2 kit (Illumina) for 2 × 151 cycles according to the manufacturer's instructions in the US Food and Drug Administration (FDA), College Park, MD, USA [30].

The genomes were assembled using the SPAdes software [31] and the quality of the assemblies was evaluated using QUAST software [32]. The contigs for each isolate (draft genomes) were annotated using NCBI's Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) [33]. These draft genome sequences are available in GenBank database and their accession numbers are detailed in Table S1.

Beside these 40 genomes, 30 other ones of *S.* Typhimurium isolated from different sources, geographical areas, and sequence types (STs) were retrieved from GenBank database (Table S2). The genomes of all these strains were used in the

phylogenetic and pangenome analyses, except in the average nucleotide identity (ANI) analysis which was performed only for the ST313 strains.

Phylogeny

The genomes of all the 70 *S.* Typhimurium strains described in item 2.1 (Table S1 and S2) were used in the phylogenetic analyses.

For the phylogenetic analyses of 16S rRNA sequence, all sequences were retrieved from genomic annotation and aligned using the multiple sequence alignment CLUSTALW that is integrated in the MEGA6 software [34]. The appropriate evolutionary model was defined, and the evolutionary history was inferred using the maximum likelihood (ML) criterion, based on the Jukes-Cantor model and the rates among sites has invariant (I) with 1000 bootstrap replicates. *Escherichia coli* K12 (MG1655) was used to root the final tree.

The alignment of fragmented genomes for inference of phylogenetic distance was performed using the Gegenees software [28]. This software calculates the percentage of similarity among the genomes of all strains. The alignment method BLASTn was used with sequence fragmentation length of 200 bp and a step size of 100 bp. The heatmap resulting from this analysis was exported in the ".nexus" format for phylogenomic analysis using SplitsTree4 software [35], with NeighborNet and equal angle methods.

The wgMLST analysis was performed using the module *Build_PGAdb* on the software PGAdb-builder [36] for creating a *PGAdb* allelic profile. The wgMLST tree was constructed using the *Build_wgMLSTtree* module from uploaded genome contigs by *PGAdb* database. We used as input files the genomes contigs in the ".fasta" format. The parameters used for PGAdb were alignment coverage and identity ≥ 90% [36].

Average nucleotide identity (ANI)

The ANI analysis was performed using the whole genome sequences of nine Brazilian strains and the reference ST313-lineage II from Africa designated D23580.

ANI is based on the mean values of identity or similarity between homologous regions that are shared by two genomes. ANI values of 95–96% are equivalent to a DNA-DNA hybridization index of 70% and can be used as a threshold for species delineation [37, 38].

Pangenome calculation

The genomes of all 70 *S.* Typhimurium strains were used in the pangenome analysis. Furthermore, the 10 genomes *S.* Typhimurium ST313 had the pangenome performed separately. Initially, the amino-acid sequences from all DNA coding sequences (CDSs) in all genomes were used in the OrthoMCL software [39] for an all-vs.-all BLASTp analysis with an *e* value of 1e−6. The CDSs observed in all strains were considered as the core genome, while the CDSs harbored by only one strain were considered as singletons and those presented in more than one genome, but not in all, were classified as shared genome.

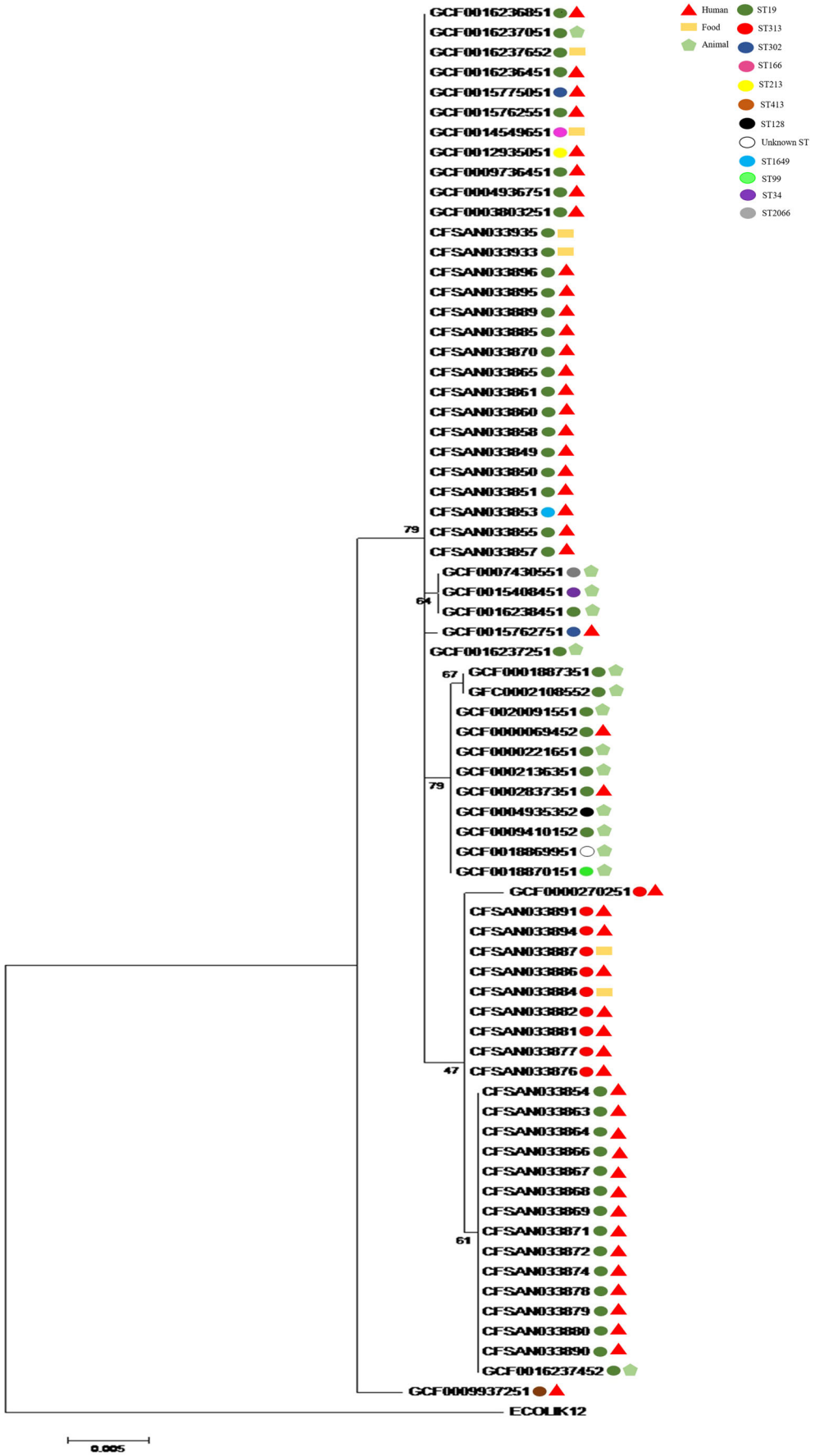
The pangenome development was calculated using the Heap's law and the extrapolations of the curves of the core genome and singletons were calculated using the least-squares fit of the exponential regression decay of the mean values, as described by Benevides and collaborators [40].

Results

Phylogeny

The phylogenetic analysis using the 16S rRNA sequences showed that 69 out of 70 *S.* Typhimurium strains analyzed were grouped in a single large cluster regardless of the source of isolation (Fig. 1). The dark green circle was designated for the ST19 *S.* Typhimurium strains that were widely distributed along different subclusters. In this analysis, we could observe the existence of many polytomies and some low bootstrap values. The 10 red circles were designated for the ST313 *S.* Typhimurium strains (CFSAN033876, CFSAN033877, CFSAN033881, CFSAN033882, CFSAN033884, CFSAN033886, CFSAN033887, CFSAN033891, CFSAN033894, and GCF0000270251). The nine ST313 sequences from Brazil were grouped closely among each other and with the reference ST313-lineage II from Africa D23580 (GCF0000270251) isolated in Malawi, Africa (Fig. 1). All the others STs (ST1649, ST34, ST99, ST128, ST213, ST302, ST2066, and ST166) were grouped in this large cluster, except the ST413 represented by a brown circle that was not grouped.

The Gegenees software generated a distance matrix based on the similarity among all genomes that was plotted as a heatmap (Fig. 2). In this matrix, the similarity varied between 100 and 79% among the 70 genomes. The nexus file exported from the Gegenees software was further used in the software SplitsTree4 to generate a phylogenetic tree. In this analysis, *S.* Typhimurium were grouped in two large clusters designated A and B (Fig. 3). The cluster A comprised six ST19 *S.* Typhimurium genomes (dark green circles) isolated from humans in Brazil. The cluster B comprised 62 *S.* Typhimurium genomes and this cluster was subdivided into B1 and B2 subclusters. The subcluster B1 grouped ST19 (dark green circles), ST1649 (light blue circle), and all the nine ST313 (red circles) *S.* Typhimurium genomes isolated from humans and food in Brazil. The subcluster B2 grouped ST19 (dark green circles), ST34 (purple circle), ST128


Human
Food
Animal
ST19
ST313
ST302
ST166
ST213
ST413
ST128
Unknown ST
ST1649
ST99
ST34
ST2066
GCF0016236851
GCF0016237051
GCF0016237652
GCF0016236451
GCF0015775051
GCF0015762551
GCF0014549651
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CFSAN033871
CFSAN033872
CFSAN033874
CFSAN033878
CFSAN033879
CFSAN033880
CFSAN033890
GCF0016237452
GCF0009937251
ECOLIK12
79
64
67
79
47
61
0.005

◀ **Fig. 1** Phylogenetic analysis based on 16S rRNA gene sequences of the 70 *Salmonella* Typhimurium strains studied. The bootstrap analysis was performed with 1000 replicates. Evolutionary analyses were conducted in MEGA6 [27]

(black circle), ST213 (yellow circle), ST302 (dark blue circles), ST99 (light green circle), unknown ST (white circle), ST2066 (gray circle), and ST313 (red circle) *S.* Typhimurium genomes isolated from humans, food, and animals in Brazil and in other parts of the world (Table S2). The ST166 (pink circle GCF001454965) and ST413 (brown circle GCF000993725) *S.* Typhimurium genomes were not grouped in any of the two clusters.

The wgMLST analysis using a gene-by-gene approach showed two large clusters designated A and B (Fig. 4). The cluster A comprised 53 *S.* Typhimurium genomes containing all the ST19 (dark green circles), ST34 (purple circle), ST213 (yellow circle), ST1649 (light blue circle), and ST2066 (gray circle) isolated from humans, food, and animals in Brazil and in other parts of the world. The cluster B grouped 12 *S.* Typhimurium genomes including all the ST313 (red circles) isolated from humans and food in Brazil and Africa. Also, two ST302 (dark blue circles) *S.* Typhimurium genomes isolated from humans in Mexico. The ST166 (pink circle GCF001454965), ST413 (brown circle GCF000993725), ST128 (black circle GCF000493535), ST99 (light green circle GCF001887015), and unknown ST (white circle GCF001886995) *S.* Typhimurium genomes were not grouped in any of the two clusters.

Average nucleotide identity (ANI)

Using an identity cutoff of 95%, this analysis revealed that the nine ST313 *S.* Typhimurium genomes isolated from humans and food were very similar among

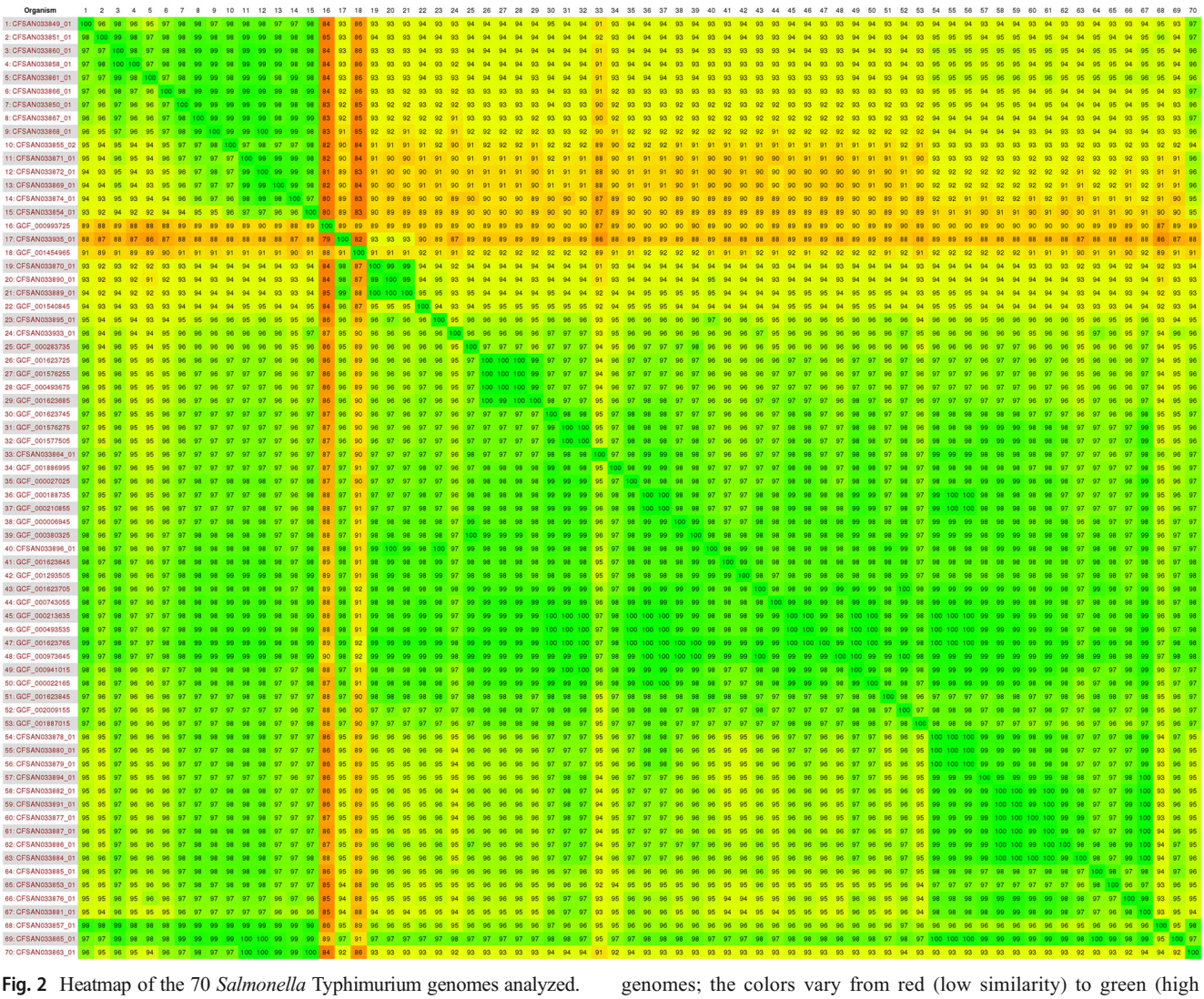
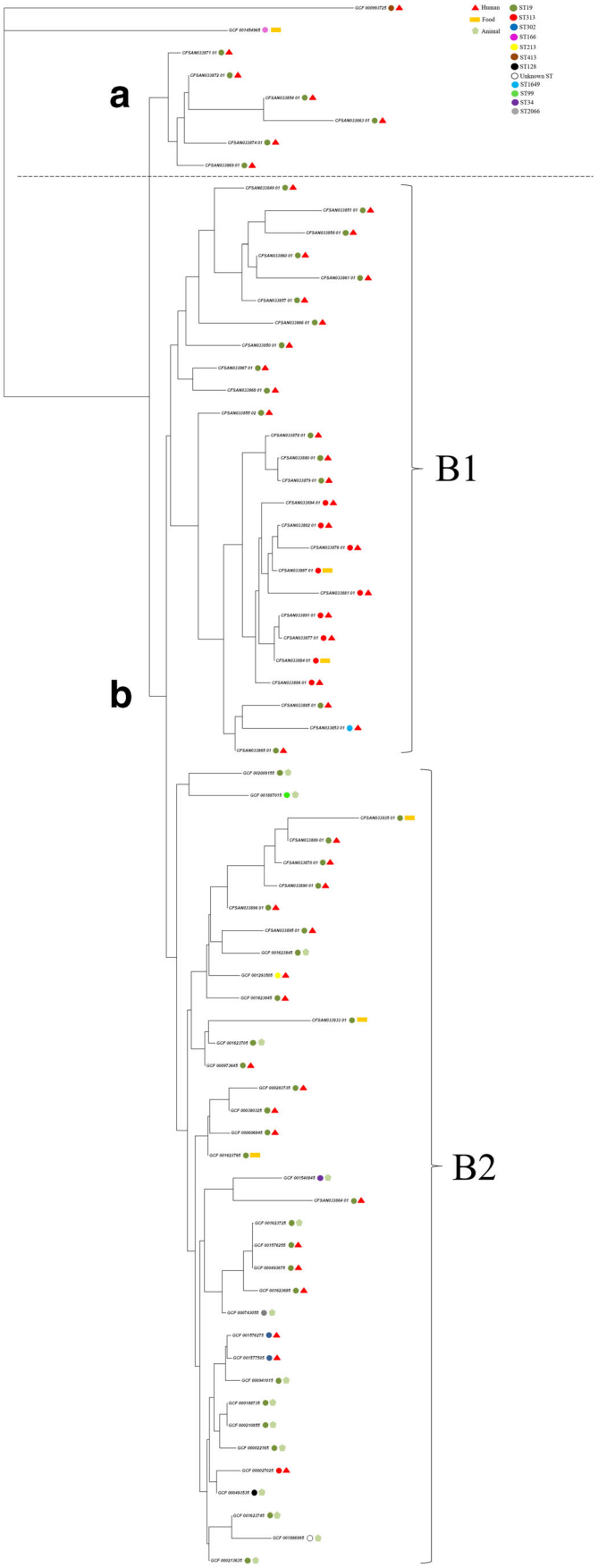


Fig. 2 Heatmap of the 70 *Salmonella* Typhimurium genomes analyzed. The numbers in the heatmap show the percentage of similarity among the genomes; the colors vary from red (low similarity) to green (high similarity)


Human
Food
Animal
ST19
ST313
ST302
ST166
ST213
ST413
ST128
Unknown ST
ST1649
ST99
ST34
ST2066
a
b
B1
B2

◀ **Fig. 3** Phylogenetic analysis based in the genomes of the 70 *Salmonella* Typhimurium strains. The network was constructed using SplitsTree software [28] with NeighborNet and equal angle methods, based on a distance matrix from Gegenees software [22]

themselves and with the reference ST313-lineage II from Africa D23580 (Table S3).

Pangenome calculation

To take a global view of the strains and to further explore the genome diversity of this genus, the size of the pangenome was calculated (i.e., the total number of non-redundant CDSs). The orthology analysis showed that the pangenome contained a total of 9,883 CDSs. The core genome showed that 2,880 CDSs were shared by all genomes and 4,171 CDSs singletons (i.e., unique to a single genome) were found in the studied genomes (Fig. 5a). Using the Heap's law and considering that $\alpha = 1\text{-}\gamma$, we inferred that the α value of the pangenome development was 0.722, indicating that the pangenome is open ($\alpha < 1$) but tending to become close ($\alpha \geq 1$). By examining the extrapolated curve of the core genome and singletons, we found that the size of the core genome tended to converge at ~ 960 genes and the singletons at ~ 782 (Fig. 6).

A separate analysis of the 10 genomes *S.* Typhimurium ST313 revealed that the core genome contained 4,112 CDSs and 76 CDSs singletons (Fig. 5b). Using the Heap's law and considering that $\alpha = 1\text{-}\gamma$, we inferred that the α value of the pangenome development was 0.970, indicating that the pangenome is open ($\alpha < 1$) but tending to become close ($\alpha \geq 1$) (Fig. 6).

Discussion

During the last decades, an epidemic of invasive infections of *S.* Typhimurium ST313 in Africa has been witnessed. Clinical observations and genomic studies suggested that such strains have been evolving concerning the known virulence patterns [16]. In addition, the presence of *S.* Typhimurium ST313 strains has been recently described in Brazil, being the first time that the highly invasive ST313 was reported in another continent than Africa [1, 21].

The 16S rRNA sequences analysis has been the method of choice of many researchers to study phylogenetic relationships and the investigation of microbial diversity, but it is important to consider not only these sequences. Therefore, the 16S rRNA sequences analysis can be used together with whole genome to complement studies of genomic diversity within the same genus or species [41, 42].

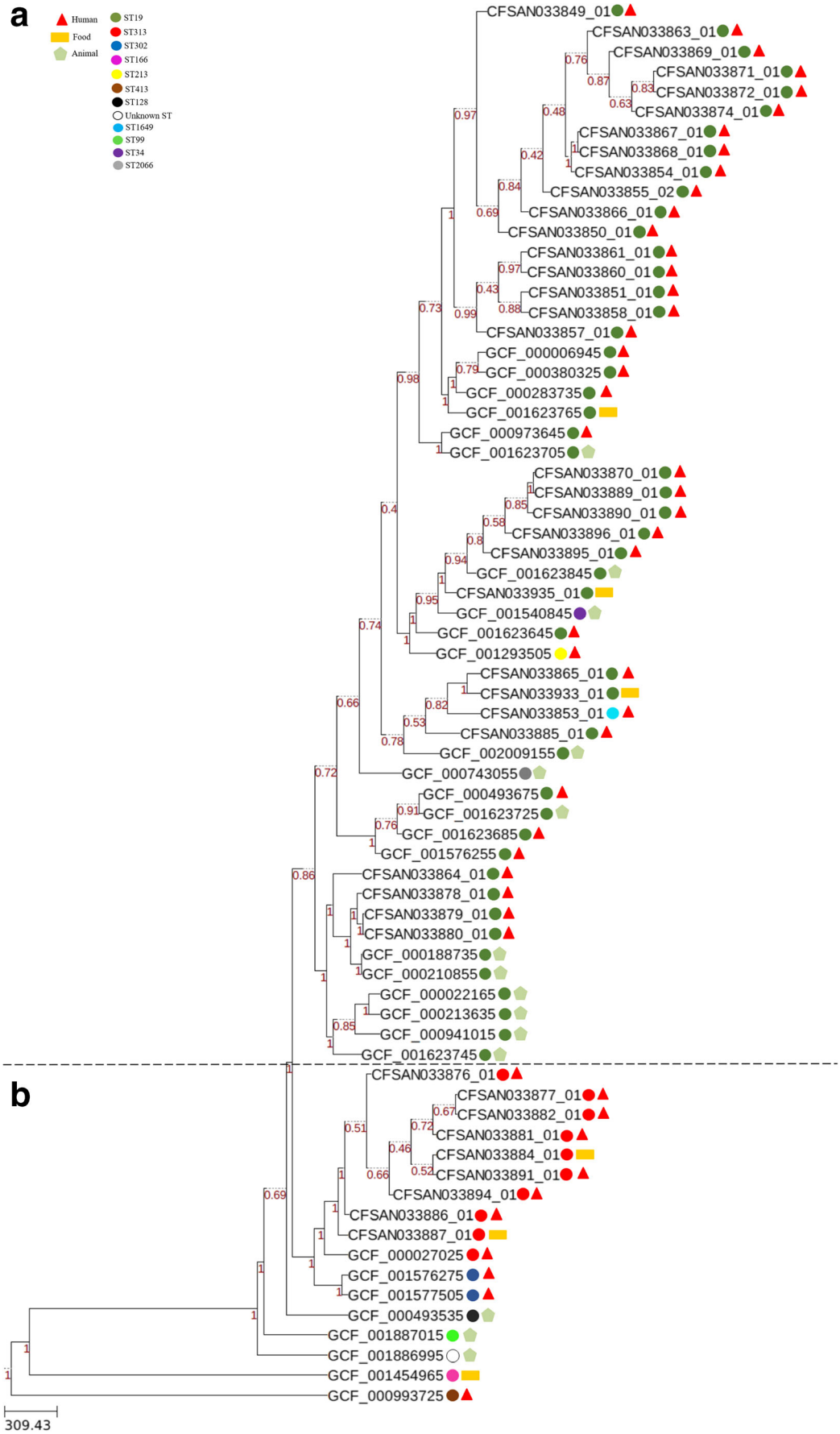
In the present work, the 16S rRNA gene sequencing was not able of accurately differentiating the *S.* Typhimurium strains analyzed, but it was important to confirm that all the strains studied are of the same serovarity. In addition, for the *Salmonella* genus, 16S rRNA gene sequencing has been widely used for its identification in diverse sources such as food, animals, and humans [43, 44].

The similarity matrix obtained with Gegenees software and used into the SplitsTree4 software for a phylogenomic analysis showed the evolutionary relationship among the strains, highlighting that all the nine ST313 strains from Brazil isolated from humans and food stayed grouped in subcluster B1. However, the ST313 strain from Africa was clustered in subcluster B2. This unexpected cluster pattern may be explained because the parameters used in Gegenees software does not use only the probably homologous genes, but the fragmented alignment of whole genomes, including repetitive regions, genomic islands, duplicated genes, and other elements that can create biases in this analysis. The analysis of this software can also be influenced by the different sizes in the genomes. To eliminate this bias, we used other methods.

The wgMLST tool was used to subtype the strains of this work. As opposed to conventional MLST analysis, which uses only a few housekeeping genes, the wgMLST approach takes advantage of a larger number of tracked loci, enabling higher resolution in intraspecies differentiation [45]. The constructed phylogenetic tree separated with accuracy the *S.* Typhimurium strains studied, showing that the 10 ST313 strains from Africa and Brazil were in a different cluster apart from all ST19 strains. The resolution of wgMLST resulting tree was better when compared to 16S rRNA sequences based phylogenetic tree and Gegenees, because more conserved genes were considered allowing a better differentiation among the strains. It is important to mention that the different sources of isolation of the strains studied did not influence their grouping in any of the phylogenetic trees constructed.

In the present study, the ANI analysis showed a high similarity between the ST313 genomes isolated from humans and food in Brazil and Africa. ANI is based on the mean values of identity or similarity between homologous regions that are shared by two genomes. Furthermore, the ANI has been widely used to characterize and identify the genomic relationship of two or more strains, because it is a fast, easy, and reproducible method [46–48]. The ANI analysis has also been used for prokaryotic taxonomic classification studies and is considered the new gold standard for bacterial species determination [38, 49].

The present work provided additional information about *S.* Typhimurium ST 313, ST19, and ST1649 strains that were previously molecularly typed by pulsed-field gel electrophoresis (PFGE), enterobacterial repetitive


a
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ST128
Unknown ST
ST1649
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CFSAN033851_01
CFSAN033858_01
CFSAN033857_01
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GCF_000380325
GCF_000283735
GCF_001623765
GCF_000973645
GCF_001623705
CFSAN033870_01
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GCF_001623845
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GCF_000022165
GCF_000213635
GCF_000941015
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CFSAN033894_01
CFSAN033886_01
CFSAN033887_01
GCF_000027025
GCF_001576275
GCF_001577505
GCF_000493535
GCF_001887015
GCF_001886995
GCF_001454965
GCF_000993725
309.43

◀ **Fig. 4** Phylogenetic analysis with wgMLST profiles for 70 *Salmonella* Typhimurium genomes. The PGAdb profile from the genomes was used to construct a wgMLST tree using the *Build_wgMLSTtree* module [29]. Bootstrap values are shown next to the nodes. The dendrogram was constructed with the UPGMA clustering algorithm

intergenic consensus PCR (ERIC-PCR), multiple-locus variable-number tandem-repeat analysis (MLVA), and clustered regularly interspaced short palindromic repeats-multilocus virulence sequence typing (CRISPR-MVLST) [50–52]. Additionally, resistance genes were searched by the WGS in these strains and genes that confer resistance to aminoglycoside, tetracycline, sulphonamide, trimethoprim, beta-lactam, fluoroquinolone, and phenicol were found [53]. In sub-Saharan Africa, high levels of antibiotic resistance have been found in *S.* Typhimurium ST313 strains [12, 13, 19]. On the other hand, most of the *S.* Typhimurium ST313 strains isolated in Brazil showed sensitivity to different antimicrobials classes searched [50], and genes that confer resistance to aminoglycoside, sulphonamide, and beta-lactam were found in only one strain [53]. The other eight *S.* Typhimurium ST313 strains studied did not show any resistance genes [53].

Therefore, the ST313 genomes from Brazil presented a high similarity among themselves regardless of the source being from humans or food by 16S rRNA, wgMLST, and ANI analyses, which was also observed using single-nucleotide polymorphism (SNP) by [21].

The ST302 was genotipically similar to ST313 by wgMLST analysis in the present work (Fig. 4), being that in accordance to Vinuesa and colleagues [54] that reported ST302 strains to be closely related to ST313 human-invasive strains from Africa. The ST302 was first described in Mexico and isolated from humans. This ST was later described in two African strains being characterized as single locus variant (SLV) of ST19 that is the predominant ST among *S.* Typhimurium strains and is usually related to gastroenteritis worldwide [55, 56].

Furthermore, others STs of *S.* Typhimurium with different characteristics retrieved from GenBank database were studied in this work and compared with ST313 strains of this study. The ST128 was clonally related to ST313 and was described as a cause of systemic disease in pigeons [57]. In contrast, in this work, this close phylogenetic relationship was not observed between the ST128 and ST313. The ST213 and ST34 were related to resistance to multiple drugs, ST213 was also associated to invasive disease in humans and animals [58, 59]. Finally, the ST166 was described in poultry and the ST99 was reported in wild birds and pigs [60, 61]. All these STs abovementioned were not clonally related to the ST313, herein studied by the different tools used. According to the published literature, this is the first article that brings this epidemiological information comparing the genomes of ST313 strains isolated in Brazil with different STs isolated in other countries.

The pangenome analysis showed that *S.* Typhimurium genomes studied presented an open pangenome because the number of orthologous genes increased when other genomes were added in the analysis. According to Alikhan and collaborators [62], *Salmonella* is a recombinant bacterial genus characterized by an open pangenome. In addition, the two subsets showed α values close to 1, but is evident that when only the 10 genomes *S.* Typhimurium ST313 were analyzed in this study the α value was higher because these strains are very similar to each other, as it was observed in the other analyzes.

In conclusion, the ST313 genomes from Brazil showed a high similarity among them regardless of the source being from humans or food by all methods used which might eventually help in the development of vaccines and antibiotics. However, those ST313 genomes presented different similarities in comparison with other STs isolated in Brazil and from other parts of the world depending on the method performed. The pangenome analysis showed that the *S.* Typhimurium genomes studied presented an open pangenome in accordance

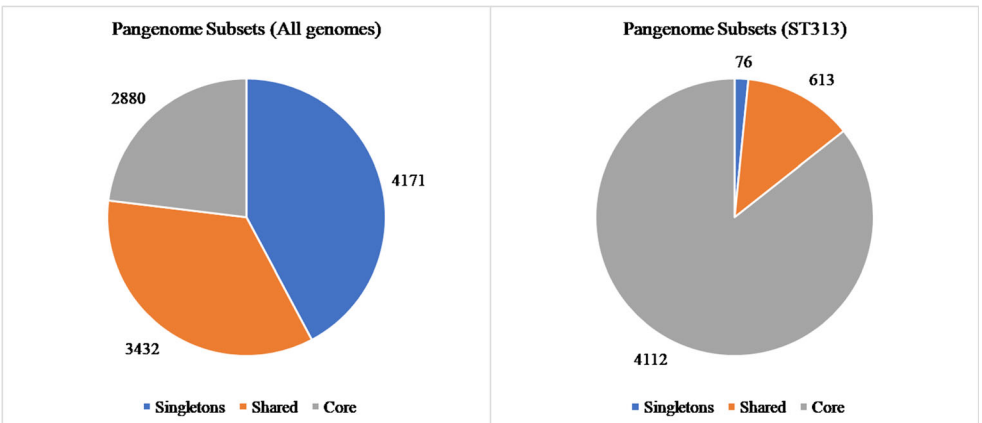

Fig. 5 Diagram depicting the subsets of the *Salmonella* Typhimurium pangenome. The numbers represent the coding sequences belonging to each subset. Left chart (**a**): pangenome subsets from an analysis based on all 70 *Salmonella* Typhimurium genomes. Right chart (**b**): subset based on analysis of 10 genomes *Salmonella* Typhimurium ST313

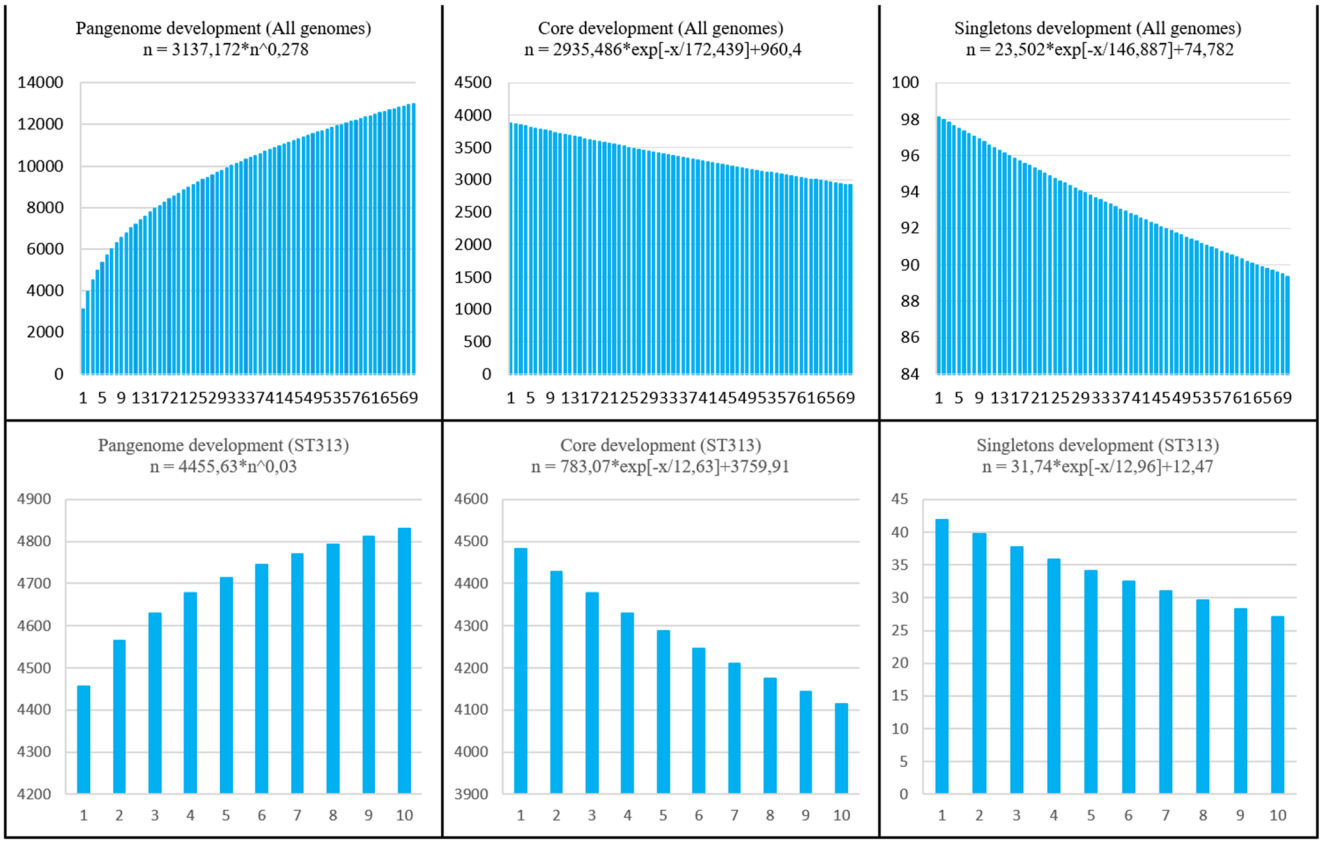

Fig. 6 Development of the pangenome, core genome and singletons. Upper chart (**a**): pangenome, core genome, and singleton development based on permutations of all 70 *Salmonella* Typhimurium genomes. Lower chart (**b**): development based on permutations of 10 genomes *Salmonella* Typhimurium ST313

to our results from the phylogenetic analyses. Altogether, the results obtained in this work contributed for a better characterization of the *S.* Typhimurium strains studied regarding its genotypic diversity. Detailed studies of the ST313 genomes should be performed in order to try to elucidate differences among them.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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