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Molecular analysis of clonally related *Salmonella* Typhi recovered from epidemiologically unrelated cases of typhoid fever, Brazil

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ABSTRACT

Background: The primary method of molecular subtyping for the identification and investigation of outbreaks has been pulsed-field gel electrophoresis (PFGE). In some cases, this technique has not been able to show discrimination between the unrelated strains that can be achieved by whole genome sequencing (WGS).
Methods: The aim of this study was to determine the strengths and drawbacks of WGS using different analytic approaches compared to traditional typing method, PFGE, for retrospectively typing clusters cases of 28 *S.* Typhi.
Results: We evaluated three analytical approaches on the WGS data set (Nucleotide Difference (ND), (SNPs) and Whole genome multi locus sequence typing (wgMLST) that identically classified the clusters-related strains into two clusters, cluster A (with strains from 2017), and Cluster B (with strains from 2007).
Conclusions: In this study WGS based typing, was able to compete with PFGE for differentiation of the clusters of *S.* Typhi strains.

Introduction

Typhoid fever is a systemic disease caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), clinically characterized in its most severe phase due to fever, fatigue, abdominal pain, diarrhea, and constipation or diarrhea. Mortality rates for typhoid fever remain high in population in Asia, especially in the Indian subcontinent (Crump and Mintz, 2010). Typhoid fever remains endemic in regions of limited access to sanitary conditions, and the transmission occurs through consumption of water and food contaminated by feces or urine of infected people (Parry and Threlfall, 2008; Sharma et al., 2016; Wong et al., 2016). Since human beings are the only reservoir of *S.* Typhi, outbreaks are associated to an asymptomatic carrier with risk behavior that spreads the organism (Cruickshank and Humphrey, 1987; Loharikar et al., 2012). *S.* Typhi outbreaks can occur in both endemic and non-endemic regions in developing countries (Yap et al., 2014).

Traditional serology is the first step to identify *Salmonella* serotypes associated to foodborne diseases. This technique is essential for outbreak detection, allowing the rapid and effective implementation of adequate control measures (Hendriksen et al., 2009). Currently, *Salmonella* strains presenting the same serotype can be subtyped by Pulsed Field Gel Eletrophoresis (PFGE), which has been the method for surveillance and investigation of foodborne diseases that allows the determination of the clonal relationship among strains, and identifying sources that originated a strain or an outbreak. This technique is the gold standard method used by Adolfo Lutz Institute for the molecular typing of *Salmonella* spp, following standardized procedures by the PulseNet Latin America and the Caribbean guidelines.

Complementarily, whole genome sequencing (WGS) has been used to identify genetic differences by the identification of single nucleotide polymorphisms (SNPs) that vary among strains, and can be highly informative markers, which are capable of revealing

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evolutionary histories of homogenous groups or through the whole genome multi locus sequence typing wgMLST that provides a gene-by-gene comparison method and is able to provide high-resolution typing results (Octavia and Lan, 2010; Liu et al., 2016). In addition, WGS allows the identification of virulence factors and antimicrobial resistance, and investigation of outbreaks that seem to be disconnected, especially those with geographically distant cases, as well as differentiation of outbreak and sporadic cases occurring at the same time, since that can be available (Wong et al., 2016; Bekal et al., 2016).

The aim of this study was to determine the strengths and drawbacks of WGS using different analytic approaches compared to traditional typing method, PFGE, for retrospectively typing cluster cases of *S.* Typhi isolated in São Paulo city, Brazil.

Material and methods

Bacterial strains

A total of 28 strains of *S.* Typhi isolated in the city of São Paulo were selected to be part of this study. Eighteen strains from the year 2017, representing all the strains recovered in the period, were included. In addition, nine strains recovered from a cluster of *S.* Typhi infections occurred in 2007 were selected since the common source was not identified. We also included an un-related strain isolated in 2005 (strain 385_05) in São Paulo area as a temporal outgroup case.

Clusters of S. *Typhi cases*

Cluster A (18 strains)

In August 2017, four children (aged from 4 to 5 years) attending in a day care center in the city of São Paulo, Brazil, were hospitalized with symptoms of typhoid fever. *Salmonella* sp. were isolated from the blood cultures and sent to Adolfo Lutz Institute (IAL). Somatic O and phase 1 and phase 2 of H flagellar antigens by agglutination were tested with antisera, as specified in the Kauffmann-White-Le Minor scheme for *Salmonella* serotyping. *S.* Typhi serovar was identified. The strains presented an indistinguishable pulsed-field gel electrophoresis (PFGE) pattern. Typhoid fever is a notifiable disease in Brazil. Laboratory networks report the isolation of *S.* Typhi to the Center for Epidemiologic Surveillance, São Paulo State Department of Health. Among the other 14 cases that occurred at the same period, IAL identified one *S.* Typhi strain (strain 134_17) from the blood of a biologist that works for the laboratory that initially recovered the *S.* Typhi strains from blood culture of day-care infants and 13 other samples that were received as sporadic cases in our laboratory.

Cluster B (9 strains)

For comparative purposes, nine strains of the last cluster of typhoid fever cases in the year 2007 were enrolled in this study. The strains were identified like *S.* Typhi by serotyping and presented an indistinguishable pulsotype pattern. The common source was not identified.

Antimicrobial susceptibility testing

An antimicrobial susceptibility test was performed for all strains (n = 28) by using the disk diffusion method according to the guidelines and interpretation criteria of the CLSI 2017. The following antimicrobial disks were tested: nalidixic acid, amoxicillin/clavulanic acid, ampicillin, amikacin, aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefepime, ciprofloxacin, chloramphenicol, streptomycin, gentamicin, imipenem, trimethoprim/sulfamethoxazole, sulfonamide and tetracycline.

Molecular typing by PFGE

Pulsed-Field Gel Electrophoresis was performed for all 28 strains according to the PulseNet protocol. *Xba*I macrorestriction fragments were resolved on 1% (w/w) agarose gels in 0.5X Tris/borate/EDTA buffer in a CHEF-DR III electrophoresis system (Bio-Rad Laboratories) for 16.5 h at 14 °C using a pulse ramping rate changing from 2.2 to 63.8 s at 6 V cm^{-1}. *S.* Braenderup H9812 was used as molecular sizes marker. The results were analyzed with Bionumerics (Applied Maths) software and a dendrogram using Dice similarity coefficient and Unweighted-Pair Group clustering method (UPGMA) was constructed based on tolerance and optimization set at 1.5%.

WGS analysis of *S.* Typhi strains

Genomic DNA was extracted according to the manufacturer's instructions using the Wizard Genomic DNA Purification kit (Promega) for bacterial cultures. Whole-genome shotgun sequencing was performed using an Ion Torrent S5. DNA libraries of 400 bp were generated and sequenced using Ion Torrent 530 Chip. Reads were de novo assembled using SPAdes v.5.8 to generate consensus files. Draft genomes covering each of the isolated chromosomes were predicted and annotated by the NCBI Prokaryotic Genome Annotation Pipeline.

Data analysis

In silico analyses were performed to detect the Multi Locus Sequence Type (MLST) through the Center for Genomic Epidemiology (CGE) pipeline of the Technical University of Denmark (Larsen et al., 2012). ResFinder tool was employed to detect the antimicrobial resistance genes (Zankari et al., 2012). The *Salmonella* pathogenicity islands (SPI finder) and virulence genes were detected using CGE server. PlasmidFinder and pMLST were used to a rapid detection of known plasmid types using on-line analysis pipeline available at CGE (Carattoli et al., 2014).

In this study, we used three recently developed procedures for identifying variations in whole genome sequencing reads and conducting phylogenetic analysis, ND tree and CSI Phylogeny (Leekitcharoenphon et al., 2014a; Kaas et al., 2014) and wgMLST (Li et al., 2017). The procedures have been made available as web tools at the following addresses: Nucleotide Difference (ND) method: http://cge.cbs.dtu.dk/services/NDtree/, and Novel SNP procedure: http://cge.cbs.dtu.dk/services/CSIPhylogeny/. wgMLST was performed by using Bionumerics version 7.6 (Applied Maths).

Nucleotide difference (ND) procedure

ND tree was constructed from assembled genomes. In brief, each read was mapped to the reference genome. We used the well-studied *S.* Typhi str. Ty2 as a reference genome (National Center for Biotechnology Information, accession: AE014613, length of 4,791,961 bp). The number of nucleotide differences in positions called in all sequences was counted, and a matrix with these counts was given as input to an UPGMA algorithm in order to construct the tree.

Single nucleotide polymorphisms (SNP) analysis

SNP tree was constructed from raw reads. CSI Phylogeny calls SNPs, filters the SNPs, does site validation and infers a phylogeny based on the concatenated alignment of the high-quality SNPs. SNPs were filtered out if the depth at the SNP position was not at least 10x or at least 10% of the average depth for the particular

genome mapping. SNPs were filtered out if the mapping quality was below 25 or the SNP quality was below 30 and a minimum Z-score of 1.96 was selected (Leekitcharoenphon et al., 2014a; Kaas et al., 2014). The validation includes both the depth check and the Z-score check as for the SNP filtering. Any position that fails validation is ignored in all mappings. Subsequently, multiple alignments were employed by MUSCLE from MEGA5. SNP tree was constructed by MEGA5 using maximum parsimony method.

Whole-genome MLST (wgMLST) analysis

In addition to online tools, raw data was uploaded to BioNumerics server and de novo assembly and based-calling bases were performed. The wgMLST analysis was conducted in all strains, to create a wgMLST clustering and to generate phylogenetic maximum-likelihood trees.

Nucleotide sequence accession numbers

Whole genome sequences (assembled by SPAdes v.5.8) of the 28 strains have been deposited in GenBank under the accession nos. PHKB00000000 (385/05), PHKC00000000 (118/17), PHKE00000000 (11/17F), PHKF00000000 (89/17), PHKG00000000 (144/17), PHKD00000000 (134/17), QAUZ00000000 (119/17), QAVA00000000 (197/17), QAVB00000000 (100/17), QAVC00000000 (50/17), QAVD00000000 (231/17), QAVE00000000 (117/17), QAVF00000000 (191/17), QAVU00000000 (206/17), QAVG00000000 (1099/07), QAVH00000000 (216/17), QAVI00000000 (211/17), QAVJ00000000 (207/17), QAVK00000000 (01/18), QAVL00000000 (1106/07), QAVM00000000 (116/17), QAVN00000000 (1066/07), QAVO00000000 (1056/07), QAVP00000000 (1055/07), QAVQ00000000 (1107/07), QAVR00000000 (1134/07), QAVS00000000 (1313/07), QAVT00000000 (1071/07). Strains numbers are in parentheses, and the versions described in this manuscript are the first versions. All the references applied in this study are available as complete assemblies from GenBank (AE014613; AL513382).

Results

A total of twenty-eight *S*. Typhi strains were studied: Cluster A: four from infants attended at a day-care center, one from the laboratory-acquired infection (laboratory technologist) and thirteen from epidemiologically unrelated cases occurring during 2017, Cluster B: nine strains of the *S*. Typhi cluster isolated in 2007, and an unrelated outgroup strain from 2005.

Antimicrobial susceptibility tested in all strains revealed susceptibility to all antibiotics, except of sulfonamide (144_17 and 191_17 strains) and streptomycin (144_17) antibiotics, according to CLSI. Macrorestriction analysis using pulsed-field gel electrophoresis (PFGE) showed that all strains of the 2017 cluster of *S*. Typhi (except 50_17 and 100_17) presented the same restriction profile (100% of similarity). When the analysis of the strains from 2007 and 2017 were compared, we found they share 84.1% similarity.

WGS allowed identification of the predictive serotype as *S*. Typhi, once SeqSero confirmed the predicted antigenic profile: O9: d: -. *Salmonella* pathogenicity islands (SPI) were identified as SPI1, SPI2, SPI3, SPI4, SPI5, SPI6, SPI-8, SPI-9 in some strains. All the strains belonged to ST1 using the MLST on-line analyses pipeline. Predicted antimicrobial resistance genes were analyzed using WGS data, and all strains were negative for the resistance genes investigated using ResFinder, correlating with the antimicrobial phenotypes determined by disk diffusion.

The performance of typing methods was measured by percentage of concordance that was used to verify the consistence of the two clusters and their proximity to the background strain (outgroup strain). Both methods, ND tree and SNP tree, identically classified the clusters-related strains into two clusters, Cluster A (with strains from 2017), and Cluster B (with strains from 2007). They were both kept outside two strains, 50_17 and 100_17, that were also confirmed not to be part of 2017 cluster A cases after the epidemiological information (data not shown). ND and SNPs analysis showed 100% of concordance between typing methods. In addition, the identified SNPs were distributed thoroughly across core genes of the reference genome suggesting that mutations occurred randomly through the core genes.

The minimum and maximum number of SNP differences within the cluster strains were significantly less than those numbers between the two clusters-related strains and background strain. For ND difference the number of SNP difference between strains within 2007 or 2017 samples ranged from 0 to 19, and from 0 to 4, respectively. Comparing 2007 and 2017 strains, the number of SNP differences ranged from 115 to 123. Strains N50_17 and N_100_17, differ between them by 3 SNP difference, but they have a high number of SNP difference (ranging from 303 to 310) compared with both clusters of *S*. Typhi strains. The number of SNP differences between the background strain (385_05) and 2007 or 2017 strains ranged from 46 to 52, and from 139 to 141, respectively.

The number of SNP difference between strains within 2007 or 2017 ranged from 0 to 51, and from 0 to 7, respectively. Comparing 2007 and 2017 strains the number of SNP difference ranged from 122 to 151. Strains 50_17 and 100_17 differ by 8 SNP difference between them, but they have a high number of SNP difference (ranging from 343 to 338) in comparison with both clusters of *S*. Typhi strains. The number of SNP difference between the background strain (385_05) and 2007 or 2017 strains ranged from 54 to 62, and from 146 to 150, respectively.

The same clonal relationship observed by Nucleotide Difference (ND) procedure and SNP analysis was verified by wgMLST. The wgMLST analysis produced three sub-databases employed to generate the dendrograms: core, wgMLST and all loci. By the core allele analysis (Supplementary Figure S1), both the 2007 and the 2017 clusters of *S*.Typhi were grouped with 99.9% of similarity (differences equal or less than 0.1% or less), each other. The two clusters of *S*.Typhi were clearly separated. The background strain 385_05 grouped apart 0.4% of difference with clusters A and B and strains 50_17 and 100_17 presented 0.9% of difference in relation to the clusters A and B.

By the wgMLST loci (Figure 1), a more stringent clusterization was observed, in which difference between the 2017 cluster A and the background strain 385_05 was higher (1.2%). The difference between the 2007 and the 2017 samples reached 2.7%. Again, the two clusters of *S*. Typhi strains (the 2007 and the 2017 ones) clearly grouped apart.

In order to compare our isolates with well-known *S*. Typhi strains (CT18 and Ty2) and the worldwide disseminated H58 haplotype, we included four references available from the NCBI (Accession numbers AL513382 (CT18), AE014613 (Ty2), LT882486 (H58) and LT905060 (H58)) in the analysis of wgMLST of all loci. The 2017 isolates presented more similarity with the Ty2 strain. The H58 and the CT18 strains clustered apart from the both the 2007 and 2017 isolates of our study. It seems reasonable that the isolates H58 did not cluster with our isolates, since multidrug resistance was not observed in our isolates.

By using the all loci, the same results visualized in the wgMLST loci analysis was observed: the clusters of *S*. Typhi were clustered apart with a difference of 2.7%, and the outgroup strain 385_05 presented a difference of 1.2% compared to the 2017 cluster.

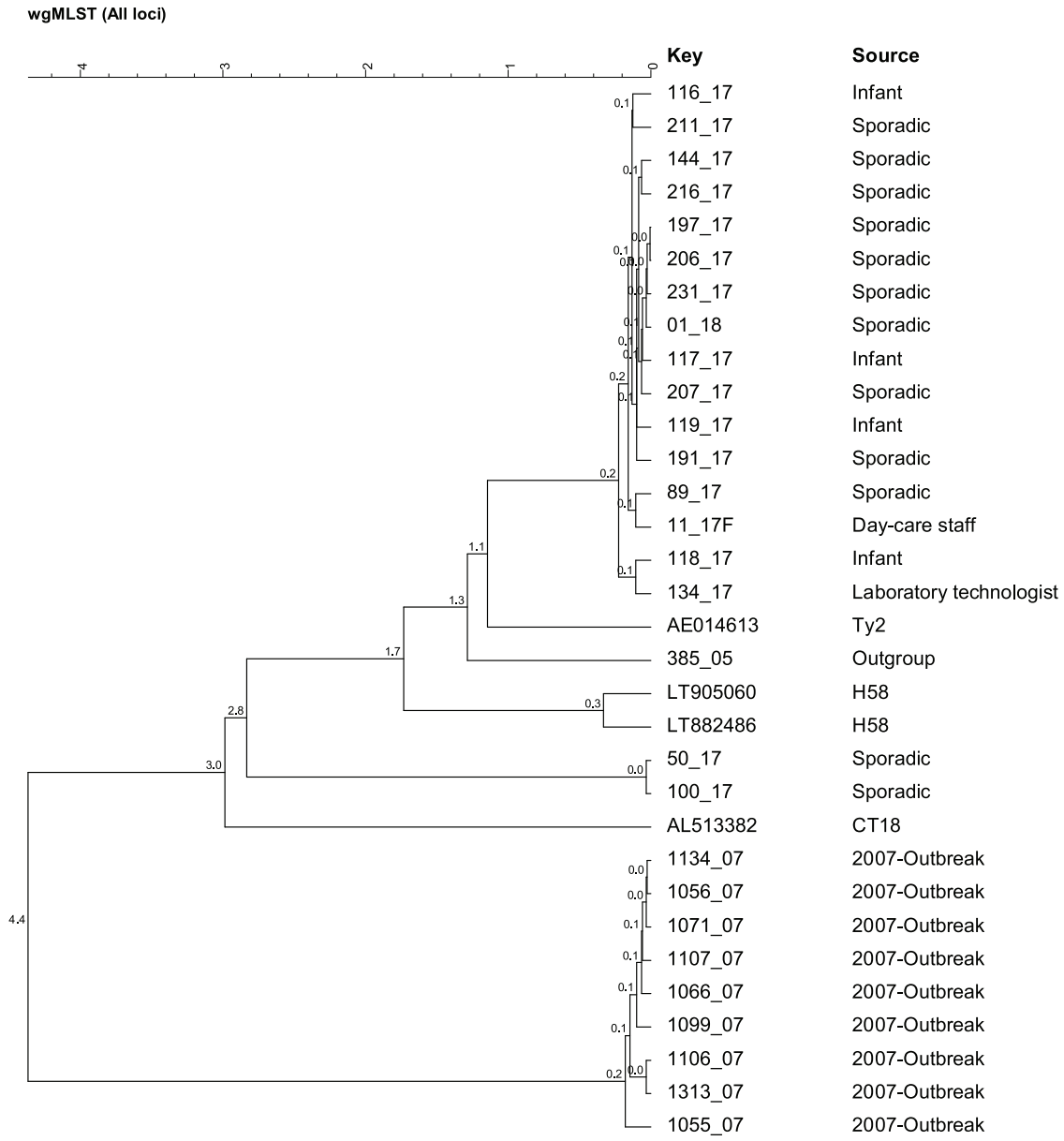

Figure 1. Whole genome MLST (wgMLST) dendrogram based on all loci, generated by BioNumerics software (similarity coefficient calculated by Categorical differences and clustering based on Complete Linkage. Numbers near to the branches mean show distances (1-similarity value, in%)).

Discussion

The objective of this study was to determine the advantages and limitations of WGS using different analytic approaches compared to traditional typing method, PFGE, for *S.*Typhi infections retrospectively. A set of twenty-eight *S.* Typhi strains were used as test sets.

Enteric fever is a systemic disease which remains an important public health problem in developing countries. In this study, we monitored local clusters of *S.* Typhi strains using molecular typing methods based on whole genome sequencing to analysis of conserved genes, to understand the evolution and spread of typhoidal *Salmonella* and to contribute to detect virulence and antimicrobial resistance genes.

Molecular subtyping of bacterial strains is an effective methodology to investigate the relatedness of bacterial strains and to investigate the source of the infections. In this study, all strains were investigated with PFGE analysis that was indistinguishable, except two strains (50_17 and 100_17), since PFGE technique has limited discriminatory power. In order to maintain the ability of rapidly detect foodborne outbreaks, whole genome sequencing data analysis employ typing techniques in a more efficient way. Wide scale sequencing allows identification of genetic differences, including a phylogenetic analysis based upon a comparison of SNP profiles, which allowed determining the genetic similarity among the cases and the probable source, detection of virulence factors and antimicrobial resistance mechanisms. This technique allows the increase of the capacity to identify and investigate outbreaks that may seem disconnected, since they are geographically distant, as well as distinguish these cases from those of sporadic occurrence at the same time.

Our analysis of WGS methodology identified that 26 strains belonging to Multilocus Sequence typing (MLST) ST1, and two strains belonging to ST2 (50_17 and 100_17 strains) a sequence type mainly persisting in the Indian subcontinent (Sharma et al., 2016; Dahiya et al., 2013; Nüesch-Inderbinen et al., 2015). ST1 and

ST2 were both isolated from global sources, and ST1 are single locus variants of ST2 which is predicted to be a founder strain of *S.* Typhi and may be circulating internationally (Kidgell et al., 2002). In a study by C. Kidgell, ST1 and ST2 sequence types of *S.* Typhi were isolated from Eurasia, South America and Africa from 1918–1999 and 1981–2000 respectively.

Whole-genome sequencing of *S.* Typhi has demonstrated that the pathogen shows limited genetic variation with little evidence of antigenic variation or recombination between strains (Yap et al., 2014). Although not all genetic variations are essential for adaptation, some dispensable genes are believed to be responsible for conferring fitness advantages to the pathogen. WGS will be useful for detected fine variations among highly conserved S. Typhi strains.

Several recent studies have already used WGS for epidemiological typing of outbreaks (Leekitcharoenphon et al., 2014b; Gymoese et al., 2017; Ford et al., 2018). However, these studies have not so far used SNP analysis for typing *S.* Typhi. We evaluated three analytical approaches on the WGS data set and compared with PFGE typing – the current gold standard method for epidemiological studies.

By the PFGE, ND, SNPs analysis and wgMLST, we cannot accurately indicate the direction of the outbreak (i.e., we are not able to define the source or the index strain) of the 2017 samples. Epidemiological investigation also did not find a risk factor or attributable source for such outbreak (data not shown). Epidemiological unlinked cases were reported after the day-care center "index outbreak", puzzling the still ongoing investigation. On the other hand, those epidemiological unlinked cases were proven to be part of a laboratory-detected outbreak caused by the same *S.* Typhi strain, as determined by PFGE, ND, SNPs analysis and wgMLST.

Although any exposure factor could be associated with the cases in this study, a common unidentified source probably exists since different laboratorial tests and analysis of WGS identify a common pattern among the contemporary *S.* Typhi strains recovered from patients with typhoid fever. The same difficulty to identify the source of strains was already reported elsewhere (Purighalla et al., 2017; Song et al., 2017), highlighting the role of molecular tools to identify clustered strains in apparently "sporadic cases"

In our study, WGS based typing, using ND, SNP analysis and wgMLST, was able to compete with PFGE for outbreak clustering. The performance of the three selected WGS based typing methods was validated based on the clusters of *S.* Typhi strains.

Conflict of interest

The authors declare that they have no competing interests

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Ethical approval

Approval was not required.

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