

Molecular characterisation of *Salmonella* strains isolated from outbreaks and sporadic cases of diarrhoea occurred in Paraná State, South of Brazil

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SUMMARY

A total of 46 strains of *Salmonella* isolated from patients with sporadic diarrhoea or involved in foodborne outbreaks were analysed by PCR for genus identification and serotyping. Subtyping was performed using pulsed-field gel electrophoresis (PFGE) and multiple amplification of phage locus typing (MAPLT) for seven variable loci. Bacteria were identified as belonging to serotype Enteritidis (33 strains; 71·7%) or Typhimurium (13 strains; 28·3%). A high similarity coefficient (94·6%) was observed in the *Salmonella* Enteritidis group for which were found three related PFGE profiles and only one MAPLT; strains representing profile PA/P1/MI were prevalent (27; 81·8%). Two *Salmonella* Typhimurium isolates were untypeable by PFGE. The remaining 11 strains had eight PFGE and three MAPLT profiles. The discriminatory power of MAPLT was lower than that of PFGE. *Salmonella* Enteritidis of clonal nature is predominant in Paraná State, with the most prevalent profile PA/P1/MI associated with sporadic diarrhoea and with seven of nine reported outbreaks. In conclusion, PFGE shows higher discriminatory power among *Salmonella* strains.

Key words: MAPLT, molecular epidemiology, PFGE, *Salmonella*.

INTRODUCTION

Salmonella enterica subspecies *enterica* is an important cause of foodborne diseases and a major cause of outbreaks and sporadic cases of diarrhoea in humans, representing a public health problem worldwide [1, 2]. It is estimated that *Salmonella* causes 93·8 million cases of gastroenteritis worldwide each year, with 155 000 deaths [1]. The diagnosis of

Salmonella infection is critical for detecting and controlling the illness. Although most infections caused by *Salmonella* are self-limiting and resolve without treatment, some patients develop a severe illness requiring pathogen-specific diagnosis, which is also important for a disease surveillance programme. Furthermore, *Salmonella* diagnosis is essential in investigations of outbreaks and of the source of associated infection, to prevent the spread of illness [1, 3, 4].

Classically, *Salmonella* infection diagnosis is based on phenotypical methods such as culture, biochemical profiling and serotyping [5, 6], which requires several days. In the last years, PCR-based methods for identification of most clinically relevant *Salmonella* serotypes were developed [7–10]. These molecular

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methods may offer an effective alternative to the traditional serotyping enabling faster diagnosis; this new approach may have a significant impact on the control of outbreaks of foodborne illnesses [6, 11]. Pulsed-field gel electrophoresis (PFGE), based on whole-genome cleavage by restriction enzymes generating large DNA fragments that are separated in agarose gels using alternating electric fields at different angles, is considered the gold standard for *Salmonella* subtyping and continues to be the most widely used method in outbreak investigations [3, 6, 11]. Nonetheless, alternative methods have been developed for subtyping *Salmonella* isolates [6, 11]. Among them, the multiple amplification of phage locus typing (MAPLT), a PCR-based assay for amplification of genomic sequences from temperate phages in *Salmonella* genome [12]. The assay was tested with different sets of primers and showed good discriminatory power for *Salmonella* serotypes Typhimurium and Enteritidis as well as the potential use as a tool for epidemiological studies [12, 13]. The aim of this work was to identify and characterise *Salmonella* strains previously isolated from patients with diarrhoea and to compare the discriminatory power of MAPLT and PFGE for subtyping the isolates.

METHODS

Bacterial isolates

A total of 46 *Salmonella* strains isolated from faecal cultures from patients with diarrhoea and identified by biochemical profile [5] were analysed. Samples were provided by Central State Laboratory (LACEN-PR), and were from patients with sporadic cases of diarrhoea or involved in foodborne outbreaks that occurred during the period of June 2010 to May 2013 in several localities of the Paraná state, southern Brazil [13; our unpublished data]. Thirty of these strains were serotyped and identified as serotype Enteritidis [14].

Growth conditions

Bacteria were grown in MacConkey agar (Oxoid; Basingstoke, UK) incubated overnight at 35°C or Tryptic Soy Broth (TSB, Difco; Sparks MD, USA), at 37°C for 14–16 h for PFGE.

DNA extraction

Overnight cultures were used for DNA extraction using the boiling method.

Molecular identification of *Salmonella*

The PCR system developed by Alvarez *et al.* [7] modified for identification only of the genus *Salmonella* and serotypes Enteritidis and Typhimurium was used. The triplex PCR was performed in a final volume of 25 µl, containing buffer Maxima Hot Start Taq DNA Polymerase[®] 1×, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1U of Maxima Hot Start Taq DNA Polymerase (Fermentas Life Sciences; Vilnius, LT), 1 µl of DNA and 0.4 µM of each primer (Table 1). The cycling program was as follows: 1 cycle at 95°C (2 min), 30 cycles at 95°C (1 min), 57°C (1 min), 72°C (2 min), and a final extension at 72°C (5 min). PCR was performed in a Veriti[®] Thermal Cycler (Applied Biosystems[®]) and detection of products was by electrophoresis in 2% agarose gel stained with ethidium bromide and visualised under UV light. *Escherichia coli* aEPEC 258FA [14], and *Salmonella* serotypes Typhimurium, Livingstone, Saint Paul, Newport, Muenster, London, Enteritidis, Javiana, Panama, Derby and Weltevreden – previously serotyped at the Brazilian National Reference Laboratory Fundação Oswaldo Cruz – served as controls.

PFGE

This procedure was performed according to the PulseNet protocol [16, 17]. XbaI (Fermentas Life Sciences; Vilnius, LT) was used as primary restriction enzyme, while AvrII (Thermo Scientific; Waltham, USA) was used only for strains presenting the same XbaI PFGE profile. DNA fragments were resolved using the system CHEF-DR[®] III (Bio-Rad, Hercules CA, USA) under the following conditions: 6 V/cm for 18:30 h at 14°C, and pulse time ranging from 2.2 to 63.8 s. *Salmonella* serotype Braenderup (ATCC[®] BAA-664TM) was used as a DNA size marker. DNA was stained with ethidium bromide and visualised under UV light. Gel images were analysed in software BioNumerics 7.5 (Applied Maths, Keijkstraat, Belgium), the banding pattern was compared by cluster analysis using the Dice similarity coefficient and UPGMA (unweighted pair group method with arithmetic mean) to generate a dendrogram with 1.5% optimisation and band position tolerance of 1.5%. Only DNA fragments ranging from 20.5 to 1135 kb were included. PFGE profiles were considered distinct when any difference in a DNA band pattern was observed [3].

Table 1. *Primer sequences and amplicon sizes*

<i>Salmonella</i> genus and serotype identification			
Primer	Sequence (5'→3')	Amplicon (bp)	Reference
OMPC (genus)	F-ATCGCTGACTTATGCAATCG R-CGGGTTGCGTTATAGGTCTG	204	[7]
ENT (Enteritidis)	F-TGTGTTTTATCTGATGCAAGAGG R-TGAACTACGTTTCGTTCTTCTGG	304	[7]
TYPH (Typhimurium)	F-TTGTTCACTTTTTACCCCTGAA R-CCCTGACAGCCGTTAGATATT	401	[7]
MAPLT subtyping			
<i>cI</i> _{ST64B}	F-TGTGTCGTTTGAGTGACTGCG R-TTCTAGGCTGGCTGACTGC	548	[13]
SB21 _{ST64B}	F-CTGTATGGTTATATCGATTATCTGG R-GATTTCTTTGCCAGATGACG	478	[13, 15]
SB26 _{ST64B}	F-GACACCATCAATGTATGGATCGC R-AGGTTATCTATAATTCCGACCTGG	477	[12]
SB28 _{ST64B}	F-TGCAGTCAAGAGGACGTCC R-TGCCGATATGCTGATCTGGC	589	[12]
SB46 _{ST64B}	F-CATTGATGGTATCGAAGTTTCGCC R-ATCTTATCGGCAGCCATAACC	401	[13]
<i>gtrA</i> _{p22}	F-AGACCTTCCGAATCCGCTG R-TAATTGCCGAGAAAGTGATAAGGG	336	[13]
<i>gtrB</i> _{p22}	F-CTTTCTCGGCAATTAGCCTG R-TTAGCCAGCACCATATCCGC	423	[13]

Multiple amplification of phage loci typing

This assay is based on the presence or absence of particular phage loci. A set of seven MAPLT primer pairs (Table 1) targeting prophage loci within the *Salmonella* genome were selected for subtyping of the isolates. These primers generated different MAPLT profiles with *Salmonella* Enteritidis or *Salmonella* Typhimurium and were capable of distinguishing the strains [12, 13, 15, 18]. The reagents and equipment used for PCR were the same described above. Primer pairs SB46_{ST64B} and SB28_{ST64B} were tested in a duplex PCR using the following cycling program: 1 cycle at 95°C (2 min), 30 cycles at 95°C (30 s), 58°C (1 min), 72°C (2 min), and a final extension at 72°C (5 min). Primer pairs *gtrB*_{p22}, *cI*_{ST64B}, SB21_{ST64B}, and SB26_{ST64B} were used in separated reactions under the same cycling conditions; for *gtrA*_{p22} the annealing temperature was 61°C (1 min).

Discriminatory index

The discriminatory ability (*D*) of PFGE was calculated according to the method of Hunter and Gaston [19].

RESULTS

Molecular identification of *Salmonella*

Results of PCR for identification of *Salmonella* indicated that all strains, including the isolates from patients with diarrhoea and the positive controls belonged to this genus, as expected. No amplification was observed for *E. coli* (not shown). On the other hand, among the *Salmonella* strains used as controls, PCR for molecular serotyping yielded positive results only for serotypes Enteritidis and *Salmonella* Typhimurium confirming the specificity of the primers. Among the 46 strains isolated from patients, 33 (71.7%) were characterised at the molecular level as belonging to serotype Enteritidis, including those previously identified using antisera [14]. The remaining 13 (28.3%) strains were identified as serotype Typhimurium (not shown).

PFGE profiling

A total of 11 distinct PFGE profiles containing from 10 to 19 DNA fragments were observed and named PA to PK (Fig. 1). Strains of serotype Enteritidis had three related profiles (PA, PB and PC). PA was predominant, shared by 27 (81.8%) strains of this

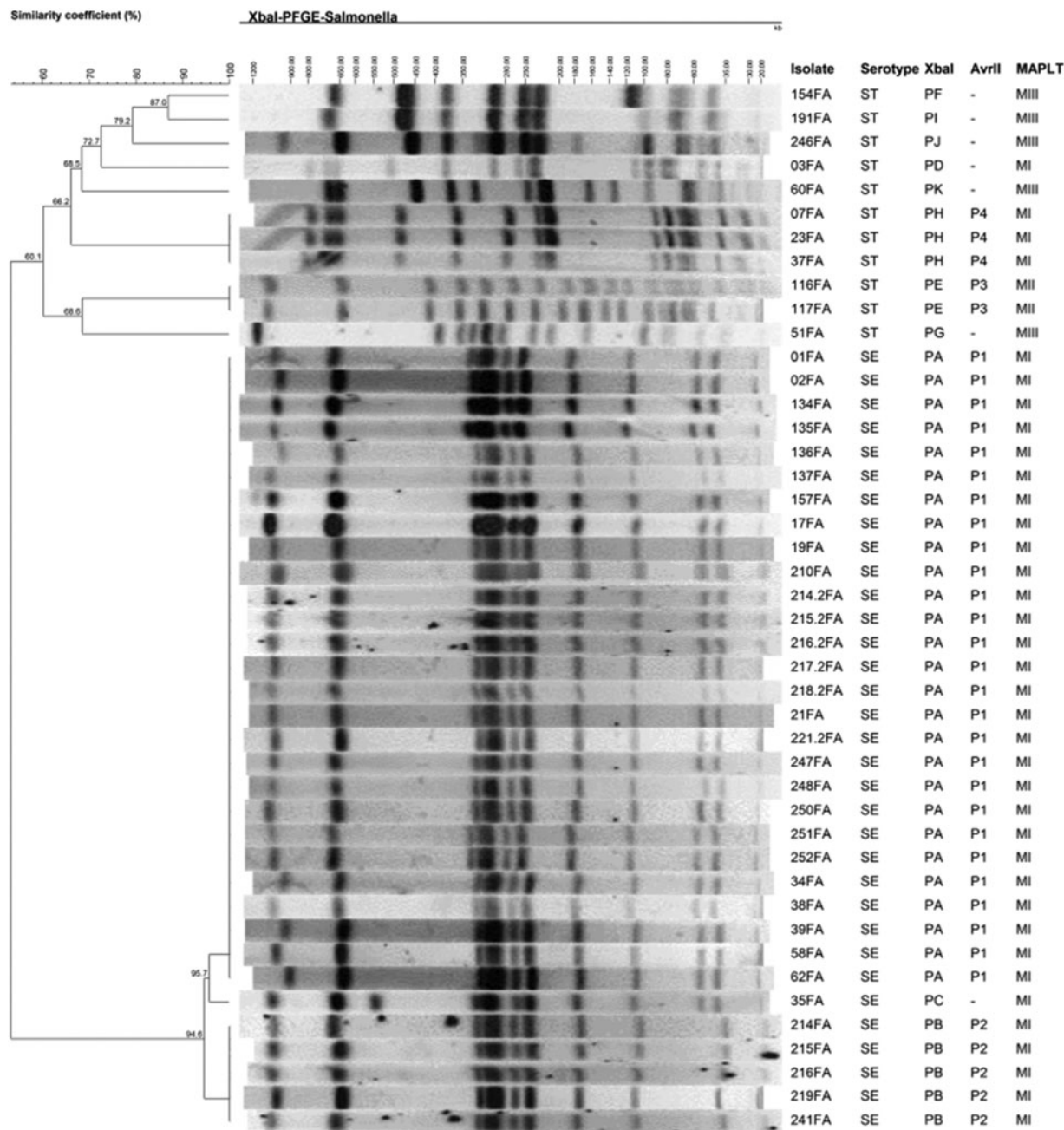


Fig. 1. Dendrogram depicting genetic similarity of *Salmonella* isolates based on PFGE profiles of chromosomal DNA digested with XbaI. Results of PFGE of AvrII-digested DNA and MAPLT are also indicated. Strains belonging to MAPLT group MI were positive for all loci tested: SB46_{ST64B}⁺, SB28_{ST64B}⁺, *gtrA*_{p22}⁺, *gtrB*_{p22}⁺, *cI*_{ST64B}⁺, SB21_{ST64B}⁺, SB26_{ST64B}⁺; MII: SB46_{ST64B}⁺, *cI*_{ST64B}⁺, SB21_{ST64B}⁺; MIII: *gtrA*_{p22}⁺, *gtrB*_{p22}⁺. SE and ST denote respectively *Salmonella* Enteritidis and *Salmonella* Typhimurium.

serotype; profile B, distinguished from PA by the absence of a fragment of approximately 54 kb, was detected in five (15.2%) strains, and only one strain (3.0%) had profile C which differed from PA by the presence of a band of approximately 550 kb (Fig. 1). The enzyme AvrII, used only for strains belonging to

the same XbaI PFGE profile, separated *Salmonella* Enteritidis into two groups (P1 and P2; Fig. 2), confirming the results obtained with XbaI. This finding resulted in PFGE groups: PA/P1, PB/P2 and PC/-. Amongst the 13 strains of *Salmonella* Typhimurium two were untypeable, and the remaining 11 strains

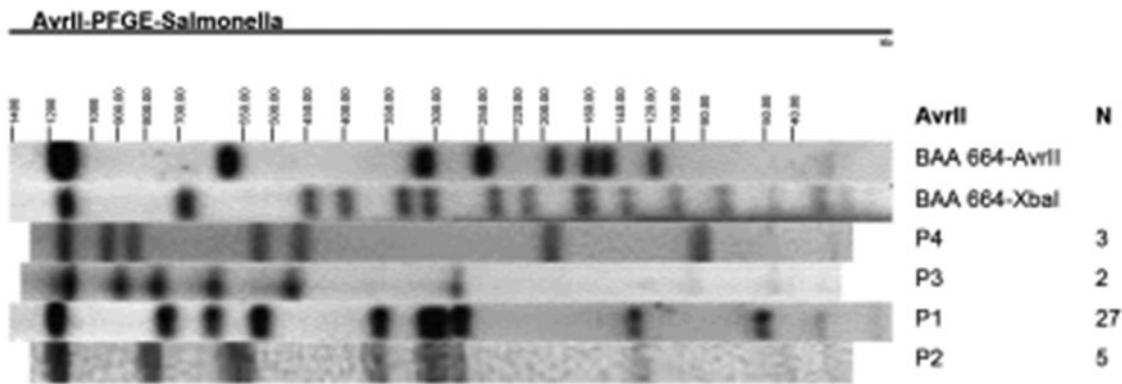


Fig. 2. PFGE profiles of AvrII-digested *Salmonella* isolates chromosomal DNA. PFGE profiles obtained with AvrII are indicated. N refers to number of strains exhibiting the profile.

produced eight PFGE profiles (PD to PK). Profiles PH and PE contained three and two strains, respectively. The other strains showed unique PFGE profiles (Fig. 1). AvrII profiling indicated that all strains of PE belonged to profile P3 (PE/P3), and strains PH to profile P4 (PH/P4). The coefficient of genetic similarity was 94.6% for *Salmonella* Enteritidis and 60.1% for *Salmonella* Typhimurium (Fig. 1). A low discriminatory index ($D=0.32$) of PFGE was found for *Salmonella* Enteritidis.

Molecular profiling by MAPLT

The MAPLT results classified the strains into three groups named MI to MIII distinguished by the number and type of loci present (Fig. 1). All *Salmonella* Enteritidis strains belonged to profile MI, resulting in groups PA/P1/MI, PB/P2/MI and PC/-/MI. All 13 *Salmonella* Typhimurium strains were typeable by MAPLT; four strains had profile MI, two profile MII and seven had profile MIII.

The molecular profiling revealed two clusters of *Salmonella* Enteritidis, the largest of them containing 27 strains with the profile PA/P1/MI (Fig. 1). Among these, six were isolated from patients with sporadic diarrhoea and 20 from patients involved in six outbreaks of foodborne diseases that occurred between July 2011 and February 2013 (respectively, strains 134–137FA; 214.2–218.2 and 221.2FA; 248, 250–252FA; 01–02FA; 38–39FA; 17 and 21FA). Of note, the other strain (34FA) with profile PA/P1/MI was isolated from a foodborne outbreak that occurred in January 2011. From patients of this outbreak, strain 35FA was also isolated which exhibited profile PC/-/MI (Fig. 1). The PFGE profiles PA/P1/MI and PC/-/MI are related, differing by only one DNA band,

while MAPLT was identical for these two strains. The second cluster of *Salmonella* Enteritidis contained strains 214–216FA, 219FA and 241FA (PB/P2/MI), isolated from patients from an outbreak that occurred in November 2010. Only one outbreak, in June 2010, was associated with *Salmonella* Typhimurium strains 116–117FA (PE/P3/MII; Fig. 1). All other *Salmonella* Typhimurium were isolated from sporadic cases of diarrhoea.

DISCUSSION

Salmonella is one of the most frequent bacterial causes of acute gastroenteritis, a major cause of morbidity and mortality worldwide, and an important public health concern [1, 2, 20, 21]. The development of rapid and sensitive methods for the detection and characterisation of *Salmonella* may have a significant impact on the disease burden caused by this pathogen [6].

Traditional serotyping is based on the identification of the somatic (O) and flagellar (H) antigens through bacterial agglutination with specific antisera [5, 6, 11]. Nonetheless, serotyping has several drawbacks, such as false-positive reactions resulting from weak, non-specific agglutination; untypeable isolates due to autoagglutination and loss of antigen expression in rough and immobile strains and the use of expensive antisera [6, 11]. Thus, alternative systems for serotyping *Salmonella* have been described. Alvarez *et al.* [7] developed a fast and reliable PCR system for detection of specific sequences of genus *Salmonella*, and from *Salmonella* serotypes 4,5,12:i:-, Enteritidis, Typhimurium, Typhimurium DT104 and U302, and *Salmonella* C2 serogroup. Considering that *S. enterica* are classified in more than 2500 serotypes, PCR serotyping methods are still limited in terms of the number of serotypes that can be identified. On the

other hand, these assays allow for identification of the most common serotypes and several multiplex assays are available [6–9]. Furthermore, PCR-based identification of genera and the most frequent serotypes in each geographic region may enable fast and cost-effective diagnosis of *Salmonella* besides being a valuable support for epidemiological surveillance. This notion is especially important in developing countries and remote regions where the serotyping is not performed on a routine basis. In this study, three of the primer pairs proposed by Alvarez *et al.* [7] were selected, which allowed for detection of *S. enterica* and the most common serotypes. For all the 46 isolates, two bands of amplified DNA were observed, enabling their classification as *Salmonella* serotype Enteritidis (33, 71.7%) or Typhimurium (13, 28.3%; Fig. 1). The serotyping PCR identified these serotypes with high specificity and may be useful for low-resource laboratories. *Salmonella* Enteritidis or Typhimurium are the most frequently isolated serotypes in Brazil [14, 22–24] and in several other regions worldwide [4, 20, 21, 25–29]. Moreover, eight of nine outbreaks analysed were associated to *Salmonella* Enteritidis.

The predominance of this serotype in food-borne illness outbreaks was also observed in Brazil by other authors [14, 23, 30] and was also described in other countries [20, 31]. *Salmonella* serotyping is useful in a surveillance programme as a starting point for identification and source tracking of outbreaks, and enables worldwide comparisons [4, 6, 11, 32]. Nonetheless, it does not provide the level of discrimination required for outbreak investigations because it does not have enough capacity to fingerprint strains [6, 11]. Typing methods must be able not only to identify outbreak-associated strains but also to distinguish those from closely related but genetically distinct strains of the same serotype, requiring a high-resolution typing system to accomplish this task [18]. Several DNA-based fingerprinting methods have been used for subtyping bacteria. Due to the high discriminating capacity, robustness and reproducibility, PFGE is considered the gold standard for subtyping of foodborne bacterial pathogens [6, 16]. Nevertheless, PFGE is time-consuming and labour-intensive and does not show the same sensitivity for all serotypes [6]. MAPLT is a PCR-based methodology which detects prophage loci within *Salmonella* genome [12]. It is fast and easy to perform and does not require specialised equipment; this feature is an advantage over PFGE. Additionally, the use of a PCR-based methodology may be useful in outbreak

investigations where the source must be readily identified [18].

When tested with phenotypically closely related but epidemiologically distinct *Salmonella* isolates, MAPLT has shown to provide good resolution with greater discriminative power in terms of the number of profiles in comparison with the profiles generated by PFGE [12, 13], or at least equal [18]. In the present study, we have selected seven phage loci amongst the most variable for *Salmonella* Enteritidis or *Salmonella* Typhimurium [12, 13, 15]. Although three PFGE profiles were observed for *Salmonella* Enteritidis, only one was obtained with MAPLT (Fig. 1) indicating that the assay was not able to distinguish these strains. For *Salmonella* Typhimurium, eight PFGE profiles were found compared with three by means of MAPLT (Fig. 1), also indicating that the latter assay has weaker discriminatory power than PFGE does. Often particular loci within an assay do not provide sufficient allelic diversity for maximum separation of isolates [18]; therefore, it is possible that a large number and/or a different set of MAPLT primers may enable better discrimination. By contrast, other PCR-based methods for *Salmonella* typing such as RAPD (randomly amplified polymorphic DNA) or VNTRs (variable number of tandem repeats) have a potential for use in low-resource settings and may be more discriminatory, thus deserving future research. Additionally, PFGE showed low discriminatory power ($D = 0.32$) for *Salmonella* Enteritidis with only three groups among the 33 strains suggesting high genetic homogeneity, with a similarity coefficient of 94.6% (Fig. 1). These results indicate that these strains are strongly related genetically. High similarity among *Salmonella* Enteritidis isolates was also observed in other studies [24, 33, 34], indicating the clonal nature of these bacteria. Strains with the PFGE profile PA (Fig. 1) were associated with seven of eight outbreaks caused by *Salmonella* Enteritidis. There is still few studies on molecular typing of *Salmonella* isolates in Brazil [35–38] but apparently the same PFGE profile was also the most common among *Salmonella* Enteritidis strains associated with foodborne outbreaks occurred in Rio Grande do Sul State, southern Brazil [24], suggesting the propagation of one clone of the bacteria in the south of the country. Interestingly, most *Salmonella* Enteritidis isolated from sporadic cases in Taiwan presented the PFGE profile apparently identical to PA found in this study [39]. The same pattern was also the most common pulsetype (SENTXB.0001) found

in an international multi-centre study including nine European countries [40], and was also observed among *Salmonella* Enteritidis presenting PFGE pattern 9 (strains 8, 14 and 16) isolated in Turkey [27]. In another study of human and animal isolates performed in Pennsylvania, USA, PA corresponded to pattern JEGX01-0002 one of the 20 most common human *Salmonella* PFGE patterns and found only amongst human isolates. Additionally, *Salmonella* Typhimurium pattern PD apparently corresponds to JPXX01-0001 of the same study which was found among humans and animal isolates [41].

These data suggest that *Salmonella* Enteritidis strains that have the PA PFGE profile are widely disseminated around the world. *Salmonella* serotypes differed regarding the PFGE profiles and the level of genetic similarity (94.6% for *Salmonella* Enteritidis and 60.1% for *Salmonella* Typhimurium). Although subtyping by MAPLT was less discriminatory than PFGE, the technique is simple with a potential for rapid subtyping of *Salmonella*, especially in countries where funds are in short supply. The strains with profile PA/P1/MI were predominant, representing 81.8% of *Salmonella* Enteritidis, thus supporting the common occurrence worldwide of this subtype in both sporadic and outbreak diarrhoea.

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DECLARATION OF INTEREST

None.

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