Molecular characterization and resistance profile of *Salmonella* Enteritidis PT4 and PT9 strains isolated in Brazil

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A total of 41 *Salmonella* Enteritidis strains, including phago-types (PTs) PT4 and PT9, were characterized by antimicrobial resistance profiles and PFGE. Of these strains, 34 were isolated from patients and foods, and 7 were of poultry origin. All strains were susceptible to ampicillin, chloramphenicol, cefotaxime, ciprofloxacin and trimethoprim/sulfamethoxazole, and 41.5% (n=17) were resistant to nalidixic acid. PFGE analysis using *Xba*I and *Spe*I restriction enzymes resulted in X1S1 as the prevalent pattern, which was present in 48.8% (n=20) of epidemic strains and in one strain isolated from discarded hatching eggs. Distinct patterns were found for the other strains isolated from poultry (X3S1, X8S8, X11S12, X11S13, X16S1 and X13S15).

The *S. Enteritidis* strains associated with outbreaks of salmonellosis were highly similar (¢0.90), suggesting clonality. The PFGE genotypes were related to the PTs, and it was possible to differentiate strains isolated from patients with salmonellosis from other strains of non-epidemic origin. The PFGE results suggested that the *S. Enteritidis* strains of poultry origin were a possible source of human salmonellosis during the study period.

INTRODUCTION

*Salmonella* species are associated with food-borne diseases worldwide. Foods of animal origin, such as poultry meat, eggs and their products, are frequently implicated in outbreaks (Clavijo et al., 2006). In the period 1999–2008, *Salmonella* species were responsible for 1408 (23.2%) of the 6062 outbreaks investigated in Brazil, and *Salmonella* species were responsible for 267 (43.8%) of the 609 outbreaks occurring in Paraná State between 2000 and 2005 (Brazilian Ministry of Health, 2010).

According to epidemiological data from the Central Laboratory of Paraná State (LACEN), egg products were associated with 45.0% of the salmonellosis outbreaks occurring from 1999 to 2008, and *Salmonella enterica* subsp. *enterica* serovar Enteritidis was the predominant serovar found in patient samples (87.8%) and foods (80.6%) (Kottwitz et al., 2010).

Phenotypic methods, such as phago-typing, antimicrobial resistance profiling and molecular methods, including analysis of the macrorestriction pattern of chromosomal DNA after PFGE, have been used to characterize *S. Enteritidis* strains. PFGE has been widely utilized in the molecular characterization of *Salmonella* species due to the stability of patterns, reproducibility and excellent discriminatory power (Ribot et al., 2002; Peters et al., 2003; Woo, 2005; Zou et al., 2010).

*Salmonella* strains isolated from different sources in the same temporal period and from the same geographical location are likely to be epidemiologically related and to have an identical PFGE restriction pattern independent of the restriction enzyme employed (Tenover et al., 1995). PFGE is capable of discriminating strains of *Salmonella* of the same serovar and phago-type (PT) (Ahmed et al., 2000; Liebana et al., 2001).

The aim of the present study was to characterize *S. Enteritidis* PT4 and PT9 strains of poultry origin or isolated from patients and foods involved in salmonellosis outbreaks by antimicrobial resistance profiles and PFGE patterns. An epidemiological correlation among the analysed strains based on genotype, geographical localization, source of isolation and period of isolation is also discussed.
METHODS

Salmonella strains. Twenty-six S. Enteritidis strains associated with outbreaks (epidemic strains) and eight S. Enteritidis strains isolated from sporadic cases of salmonellosis were provided by the Central State Laboratory (LACEN; Parana, Brazil). Additionally, seven strains of poultry origin were obtained from the Laboratory of Poultry Health accredited by the Ministry of Agriculture, and these strains were isolated from cloacae swabs, broiler breeder cloacae swabs and discarded hatching eggs. Serotyping and phago-typing were carried out in the Laboratory of Enterobacteria at the Oswaldo Cruz Institute (Fiocruz) in Rio de Janeiro, Brazil. The phenotype, origin of strains, year, source and geographical region of isolation are presented in Table 1.

Antimicrobial resistance profiles. The susceptibility of the strains to antimicrobials including ampicillin (10 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), cefotaxime (30 μg), ciprofloxacin (5 μg) and trimethoprim/sulfamethoxazole (25 μg) was evaluated by the disc diffusion technique (Bauer et al., 1966) following the recommendations of the CLSI (2006).

PFGE. PFGE was performed according to the CDC (1999). Gel blocks made of 2% low-melting-point agarose containing chromosomal DNA were separately subjected to restriction with 30 U XbaI and SpeI (1 mg ml⁻¹), and incubated in a 37 °C water bath for 90 min. After restriction, the material was subjected to PFGE with an initial pulse of 5 s and a final pulse of 45 s at 200 V for 24 h with an angle of 120° at 12 °C. The gels were stained with 1 μg ethidium bromide ml⁻¹ for 30 min followed by destaining in distilled water for 1 h, and the bands were visualized under UV light. A PFGE lambda ladder was used as a molecular mass marker.

Statistical analysis. The results were analysed using the Gel-Pro Analyser 4.0 software. The analysis of similarity between patterns was carried out with the NTSYS 2.02 program utilizing the Dice coefficient (Dice, 1945) and UPGMA grouping method to create dendrograms.

Table 1. Characteristics of Salmonella Enteritidis isolates analysed based on phago-type, origin, year, source and geographical region of isolation

<table>
<thead>
<tr>
<th>No. of isolate</th>
<th>Outbreak</th>
<th>PT</th>
<th>Year</th>
<th>Source</th>
<th>Geographical region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – OA*</td>
<td>4</td>
<td>2002</td>
<td>Mayonnaise</td>
<td>Central North</td>
<td></td>
</tr>
<tr>
<td>2 – OB*</td>
<td>4</td>
<td>2003</td>
<td>Sausage</td>
<td>West</td>
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<tr>
<td>3 – OC*</td>
<td>4</td>
<td>2003</td>
<td>Salami</td>
<td>South-west</td>
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<tr>
<td>4 21 OD†</td>
<td>4</td>
<td>2003</td>
<td>Cake</td>
<td>Central West</td>
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<tr>
<td>5 22 OE†</td>
<td>9</td>
<td>2003</td>
<td>Corn</td>
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<td>6 23 OF†</td>
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<td>8 25 OH†</td>
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<td>2004</td>
<td>Cake</td>
<td>South-west</td>
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<tr>
<td>9 26 OI†</td>
<td>9</td>
<td>2004</td>
<td>Cake</td>
<td>West</td>
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<td>10 27 OJ†</td>
<td>9</td>
<td>2004</td>
<td>Salad with chicken</td>
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<td>17 – OQ*</td>
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<td>2002</td>
<td>Human faeces</td>
<td>Central North</td>
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<td>– 19 SC</td>
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<td>2003</td>
<td>Human faeces</td>
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<td>– 20 SC</td>
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<td>2003</td>
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<td>– 34 SC</td>
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<td>2006</td>
<td>Human faeces</td>
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<td>– 41 PS</td>
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<td>2002</td>
<td>Poultry origin‡</td>
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<td></td>
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<tr>
<td>– 35 PS</td>
<td>9</td>
<td>2003</td>
<td>Discarded hatching eggs§</td>
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<tr>
<td>– 36 PS</td>
<td>9</td>
<td>2004</td>
<td>Discarded hatching eggs§</td>
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<td>– 37 PS</td>
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<td>2006</td>
<td>Poultry origin‡</td>
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<td>Poultry origin‡</td>
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<td>2006</td>
<td>Poultry origin‡</td>
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<td>– 40 PS</td>
<td>9</td>
<td>2006</td>
<td>Poultry origin‡</td>
<td>NI</td>
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</table>

*S. Enteritidis PT4 and S. Enteritidis PT9 strains isolated only from foods associated with outbreaks.
†S. Enteritidis PT4 and S. Enteritidis PT9 strains isolated from foods and patients involved with the same outbreak.
‡Strains isolated from broiler breeder cloacae swabs in routine exams.
§Discarded hatching eggs produced by broiler breeders in nests with wood shavings and destined for the production of 1-day-old chicks.
The discriminatory power ($D$) was calculated based on Simpson's diversity index (Hunter, 1990).

**RESULTS AND DISCUSSION**

PFGE analysis of 41 $S$. Enteritidis strains using $XbaI$ and $SpeI$ identified 16 distinct patterns for each enzyme. The profiles were designated $Xn$ and $Sn$ where $n$ referred to the number of each pattern. In the restriction digest with $XbaI$, the X1 pattern was prevalent and present in 24 strains (58.5%) (Fig. 1a). In the restriction digest with $SpeI$, the S1 pattern was prevalent and present in 25 strains (61.0%) (Fig. 1b). The discriminatory powers ($D$) of $XbaI$ and $SpeI$ were 0.66 and 0.63, respectively. These results were similar to those reported by Weide-Botjes et al. (1998) for $S$. Enteritidis strains isolated in Germany and analysed with $XbaI$ ($D=0.71$) and $SpeI$ ($D=0.70$).

The $D$ value was increased to 0.77 when the $XbaI$ $S$. Enteritidis profiles were combined with the $SpeI$ $S$. Enteritidis profiles. X1S1 was the predominant pattern, and it was present in 20 (48.8%) of the analysed strains. The increase in discriminatory power among $S$. Enteritidis strains using the combination of $XbaI$ and $SpeI$ has also been reported by Lacochina et al. (2000) for 101 $S$. Enteritidis strains isolated from animals, foods, and humans. Liebisch & Schwarz (1996) reported that the combination of these enzymes in the PFGE analysis of 31 $S$. Enteritidis strains of poultry origin had a higher $D$ value than that when separate enzymes were used.

In the present study, PFGE patterns discriminated $S$. Enteritidis PT4 from PT9. Only strain 4 ($S$. Enteritidis PT4) had the same PFGE pattern as the $S$. Enteritidis PT9 strain when using $SpeI$. Weide-Botjes et al. (1998) also reported a correlation between phenotype and PFGE patterns in 76 $S$. Enteritidis isolates. Three $S$. Enteritidis PT4 strains isolated from foods and patients involved in outbreaks with X9S4, X4S1 and X5S5 profiles showed approximately 0.90 of similarity with the X1S1 pattern, which was characteristic of $S$. Enteritidis PT9 strains isolated from outbreaks. Similar results have been reported by Nauerby et al. (2000), who found identical PFGE patterns between $S$. Enteritidis PT11 and PT9 strains isolated in Denmark. Lapuz et al. (2007) also observed PFGE patterns with high similarity ($\geq 0.80$) for $S$. Enteritidis PT1b and PT6 strains isolated in Japan. According to Tenover et al. (1995), the random occurrence of genetic events, such as point mutations, insertions and deletions in DNA, may change PFGE patterns during the course of an outbreak. In some cases, distinct serovars or PTs show similar genotypes due to mutations associated with antimicrobial resistance that can occur by selection pressure after inadequate doses of treatment (Giraud et al., 2006; Shukun et al., 2010). In addition, $S$. Enteritidis strains can alter their phenotypes without changing their genotypes, and this can be done by the acquisition of antimicrobial resistance mechanisms (Nauerby et al., 2000).

$S$. Enteritidis PT9 strains isolated from human faeces and foods between 2003 and 2006 were highly similar ($\geq 0.90$), which indicated an epidemiological correlation. The same was observed between the $S$. Enteritidis PT4 strains isolated from patient 4 and food 21 in outbreak D (Fig. 1a). In relation to outbreak E, the $S$. Enteritidis PT9 strain isolated from patient 22 had a PFGE pattern distinct from the patterns found for the other 27 strains. However, the pattern of this strain differed only by three fragments from the pattern observed for the strain isolated from food 5 (data not shown), suggesting an epidemiological correlation. According to Tenover et al. (1995), isolates can be considered closely related when PFGE patterns differ by two or three fragments due to alterations compatible with genetic events. The high similarity found among the patterns of $S$. Enteritidis PT9 strains suggests a common origin and a possible clonality of the strains.

The PFGE patterns of the $S$. Enteritidis PT9 035 and 036 strains isolated from discarded hatching eggs resulting from $XbaI$ and $SpeI$ restriction digests were highly similar ($\geq 0.90$) to the patterns of the epidemic $S$. Enteritidis PT9 strains (Fig. 1a, b). Similar to the results in the present study, other investigators in Austria (Schmid et al., 2006), Chile (Fernandez et al., 2003), Korea (Woo, 2005) and Senegal (Cardinale et al., 2005) have found high similarity between PFGE patterns of $S$. Enteritidis strains isolated from poultry products and from patients with salmonellosis.

Commercial eggs are from small laying hens reared in cages. Hatching eggs are produced by broiler breeders in nests with wood shavings and destined for the production of 1-day-old chicks. They are not incubated when they have shell defects, cracks, dirt or thin shells. The condition of these eggs facilitates the passage of *Salmonella* from the surface of the eggs to internal structures of the eggs, increasing the risk of human contamination (Oliveira & Silva, 2000). The Brazilian legislation does not allow human consumption of discarded hatching eggs; however, these fertilized eggs have been donated or sold to food-producing establishments and farmers’ markets and their consumption could represent an important source of human salmonellosis.

PFGE with $XbaI$ and $SpeI$ discriminated eight genetic patterns of $S$. Enteritidis PT4 strains. However, the patterns observed for strains 18, 19 and 20 isolated from patients did not correlate with any of the outbreaks studied. Similar findings have been reported by Lukinmaa et al. (1999) for 43 $S$. Enteritidis PT4 strains isolated from patients in Finland with seven non-related PFGE patterns.

All of the 41 $S$. Enteritidis strains analysed were susceptible to all of the tested antimicrobial agents, except nalidixic acid; 38.5% of the strains isolated from foods and patients ($n=10$) and 100% of the strains of poultry origin ($n=7$) were resistant to nalidixic acid. Nalidixic acid-resistant strains were isolated in all geographical regions during the period of study. The characteristic PFGE profile was not observed for strains of poultry or epidemic origin that were either resistant or susceptible to nalidixic acid.
Fig. 1. Dendrogram of genetic similarity between Salmonella Enteritidis PT4 and PT9 strains isolated from foods (FOOD), human sources (HS), discarded hatching eggs (DE) and poultry sources (PS) that were either involved or not involved with outbreaks occurring in the State of Parana in the period 2002–2006. (a) X1–X16: different genetic patterns observed for the strains analysed after digestion with XbaI endonuclease. (b) S1–S16: different genetic patterns observed for the strains analysed after digestion with SpeI endonuclease. The matrix of genetic similarity based on PFGE patterns obtained with the XbaI and SpeI endonucleases was determined using the Dice coefficient, and the isolates were grouped by UPGMA. CW, Central West; M, Metropolitan; SW, South-west; W, West; CN, Central North.
The non-therapeutic use of quinolones in veterinary medicine, such as in prophylactic supplements or growth-promoting agents, can facilitate the selection of resistant bacteria or reduce susceptibility to these antimicrobials. Thus, the use of antimicrobial agents in poultry farming may be the main cause for the increase and spread of resistant Salmonella strains (Giraud et al., 2006; Vaz et al., 2010; Souza et al., 2010).

In this study, the antimicrobial resistance profiles had low discriminatory power ($D=0.50$). However, the antimicrobial resistance profiles in combination with PFGE had a higher discriminatory power ($D=0.88$). According to Zheng et al. (2007), methods with elevated discriminatory power and high reproducibility should be employed in the epidemiological characterization of S. Enteritidis due to the high genetic homogeneity of this serovar.

The results presented in this study reinforced the importance of the combination of phenotypic methods with genotypic methods for the characterization of S. Enteritidis strains. The results also indicated a high similarity of PFGE patterns among the strains of poultry origin and the strains isolated from patients or foods associated with outbreaks of salmonellosis during the period of this study.

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REFERENCES


