

## Characterization of *Salmonella* Enteritidis strains isolated from poultry and farm environments in Brazil

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Received 9 April 2013; Final revision 10 January 2014; Accepted 14 February 2014;  
first published online 14 March 2014

### SUMMARY

*Salmonella* Enteritidis is a major causative agent of foodborne outbreaks worldwide. Using enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and pulsed-field gel electrophoresis (PFGE), this study assessed the genetic relatedness, the pathogenic potential, and antimicrobial resistance in 60 strains isolated from chickens and the farm environment in Brazil between 2004 and 2010. The resulting concatenated dendrogram of the two methodologies distinguished the strains into two clusters. Some strains isolated from the two sources were indistinguishable. All the strains contained the 13 virulence markers investigated. Forty-four strains were resistant to nalidixic acid. Quinolone resistance presented by many strains suggests that quinolones may have been used to treat chickens. The high prevalence of virulence markers highlights the importance of poultry as vehicles of *S. Enteritidis* strains that have the potential to cause disease.

**Key words:** Chickens, ERIC-PCR, quinolone resistance, PFGE, *Salmonella* Enteritidis, virulence genes.

### INTRODUCTION

Among the *Salmonella enterica* serovars, Enteritidis (*S. Enteritidis*) has characteristics that permit an interaction with the reproductive organs of chickens, as well as with egg compounds [1]. This link with animals related to human food products has resulted in *S. Enteritidis* emerging as the most frequently isolated serovar of *Salmonella* in several countries during the pandemic which began in the mid 1980s [1, 2].

Currently, *S. Enteritidis* remains the most frequently isolated serovar in several countries, impacting public health worldwide [3].

In Brazil, Mota and colleagues [4] reported the first outbreak of *S. Enteritidis* in 1983. However, a higher prevalence of the isolation of this serovar was observed after 1990. Subsequently, between 1991 and 1995, the isolation of *S. Enteritidis* in Brazil increased from 1·2% to 64·9% in human sources and from 0% to 40·7% in non-human sources [5–7]. Some epidemiological studies suggest that the introduction of *S. Enteritidis* in the country may be due to the introduction of contaminated poultry from the USA and Europe at the end of the 1980s [7–11]. Recent data shows that between 1999 and 2008, *S. Enteritidis* was the causal agent of 119 documented

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Table 1. Year, region and source of isolation of the *Salmonella* Enteritidis strains studied

Year of isolation	Region	Chickens*	Farm environment†	Total
2004	NE, S	8	2	10
2005	NE, MW, S, SE	8	–	8
2006	SE, NE	7	3	10
2007	NE, S, SE	8	1	9
2008	NE, SE	7	3	10
2009	NE, SE	5	4	9
2010	SE	1	3	4
Total no. of strains		44	16	60

MW, Midwest; NE, Northeast, S, South; SE, Southeast; SW, Southwest

\* Strains isolated from chickens include cloacal swabs, organs, eggs and meconium.

† Strains isolated from farm environment include drag swab, box swab and farmworkers' shoe covers.

foodborne outbreaks in Brazil, related primarily to raw or undercooked chicken and eggs [12].

The Brazilian production of chickens was the third largest in the world in 2011, surpassed only by the USA and China, respectively. Of the 13 million tons of chicken produced by Brazil, 30% was exported to other countries [13]. In light of the importance of Brazil as a producer and exporter of poultry products, studies regarding the epidemiology of *S. Enteritidis* in poultry and on farms are important in improving the surveillance and elucidating the contamination routes of this pathogen. Additionally, the analysis of the pathogenic potential and the antimicrobial resistance of the poultry-related strains can reveal important characteristics of the strains that can be transmitted to humans through food products.

Therefore, the aim of the present study was to analyse the diversity of *S. Enteritidis* strains isolated from four regions in Brazil (Northeast, Midwest, South, Southeast) from chickens and from the farm environment. The analysis was performed using enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and pulsed-field gel electrophoresis (PFGE) to determine the genetic relationships in various strains isolated between 2004 and 2010. Additionally, the antimicrobial resistance and presence of some virulence markers were evaluated. These data were analysed to characterize strains isolated from the farm environment and chickens, and to reveal the pathogenic potential and antimicrobial resistance of strains isolated from poultry in Brazil.

## MATERIAL AND METHODS

### Bacterial strains

A total of 60 *S. Enteritidis* strains were studied. These strains were isolated from different chickens (44 strains) and the farm environment (16 strains) from five states in four regions in Brazil (Northeast, Midwest, South, Southeast) between 2004 and 2010. These strains were received from AVIPA (Avicultura Integral e Patologia S/A) in Brazil. The serovar-specific *sdfI* gene was amplified using PCR to confirm the serovar at the molecular level, as described previously [14]. Table 1 lists the year, isolation source and the region of the 60 *S. Enteritidis* strains studied.

AVIPA is a private laboratory that is accredited by the Brazilian Ministry of Agriculture, Livestock and Supply. AVIPA, among other accredited laboratories, receives samples from eggs, chickens and the farm environment for monitoring and advising poultry producers that send their samples to this institution. The activities of AVIPA are part of the National Poultry Health Programme which the producers must adhere to in order to maintain the quality of production of eggs and/or poultry meat. AVIPA is not the only laboratory accredited by the Brazilian Ministry of Agriculture, Livestock and Supply that performs testing for the National Poultry Health Programme.

### ERIC-PCR typing and analysis

The ERIC-PCR assay was performed on all 60 strains (see Table 1), and the results were analysed as

described previously [15] with a few modifications. Genomic DNA was extracted, and the DNA concentration was determined according to previously described methods [16, 17]. All amplifications were performed in a total reaction volume of 50  $\mu$ l with 100 ng DNA template. The PCR reaction mixture also contained each deoxyribonucleotide (Life Technologies, USA) at 1.25 mM, 5 mM MgCl<sub>2</sub> (Life Technologies), 1.0 U KlenTaq™ DNA polymerase (Ab peptides), 1  $\times$  PCR buffer (Life Technologies) and 50 pmol of each primer (IDT, USA). The primers used were as described previously [18]: ERIC1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3'). The programme used for ERIC-PCR was as follows: initial incubation at 94 °C for 7 min; 30 cycles of 30 s at 94 °C, 1 min at 52 °C and 8 min at 65 °C, with a final incubation at 65 °C for 10 min. The ERIC-PCR reaction was repeated at least twice for each strain to verify the reproducibility of the experiment. Reaction mixtures without the DNA template were used as negative controls. The PCR was performed in a DNA Engine® Peltier Thermal Cycler (Bio-Rad, USA). The ERIC-PCR amplicons were resolved in 1.5% agarose gel electrophoresis into bands, which were stained with ethidium bromide (0.5  $\mu$ g/ml) and visualized under UV light.

The data were analysed with BioNumerics software v. 5.1 (Applied Maths, USA). Only bands representing amplicons between 150 and 5000 bp in size were included in the analysis. A similarity dendrogram was constructed by the UPGMA method, using the DICE similarity coefficient and a position tolerance of 1.2. A standard molecular weight ladder (1 kb Plus DNA Ladder; Life Technologies) was included three times on each gel to normalize the images and allow for valid comparisons of fingerprints on different gels.

### PFGE typing and analysis

Agarose blocks of the 60 strains listed in Table 1 were prepared using the protocol described by Souza *et al.* [15] with some modifications. The modifications were made in the initial stage, during which pure cultures of the bacterial strains were grown in BHI broth (HiMedia Laboratories, India) O/N at 37 °C. The colonies were isolated on Mueller–Hinton agar (HiMedia Laboratories) plates after incubation for 12–18 h at 37 °C. Colonies were selected and suspended in a cell-suspension buffer [10 mM Tris (pH 7.2), 20 mM NaCl,

50 mM EDTA) until an OD<sub>600nm</sub> of 0.6–0.9 was reached.

Next, the cell suspension was warmed to 50 °C and mixed with 500  $\mu$ l of a 2% low-melting-point agarose (Bio-Rad). Seventy microlitres of the suspension was cast in individual DNA-plug moulds. The plugs were digested with 30 U of *Xba*I (Life Technologies) overnight at 37 °C.

Macrorestriction fragments were resolved by counter-clamped homogeneous electric field electrophoresis in a CHEF-DR III apparatus (Bio-Rad) with an electric field of 6 V/cm and an angle of 120°. The migration of fragments was performed at 14 °C in 0.5  $\times$  TBE buffer [4.5 mM Tris, 45 mM boric acid, 1 mM EDTA (pH 8.0)] and 1.0% ultra-pure pulsed-field agarose (Bio-Rad). The pulse times were ramped from 2.2 s to 63.8 s over 19 h, as described previously [19].

A standard molecular weight ladder (Lambda Ladder PFG marker, New England BioLabs, USA) was included three times for each gel to allow for comparison of the fingerprints over several gels. The gels were stained with ethidium bromide (0.5  $\mu$ g/ml) for 30 min and de-stained in distilled water for 20 min. The restriction fragments were visualized under UV light.

The relatedness among the PFGE profiles was analysed with BioNumerics software v. 5.1 (Applied Maths). Only bands representing fragments between 48.5 kb and 630.0 kb in size were included in the analysis. A similarity dendrogram was constructed by the UPGMA method, using the DICE similarity coefficient with a position tolerance of 1.2.

### Discrimination index (DI)

The discriminatory power of ERIC-PCR and PFGE was assessed by Simpson's diversity index, as described by Hunter & Gaston [20].

### Antimicrobial resistance profiles

The susceptibility of the 60 strains listed in Table 1 to antimicrobials was evaluated using the disk diffusion technique following the protocol of the Clinical and Laboratory Standards Institute (CLSI) [21]. The tested antimicrobials were amikacin (30  $\mu$ g), tetracycline (30  $\mu$ g), ampicillin (10  $\mu$ g), cephalothin (30  $\mu$ g), trimethoprim-sulfamethoxazole (25  $\mu$ g), nalidixic acid (30  $\mu$ g), streptomycin (10  $\mu$ g), ceftriaxone (30  $\mu$ g) and chloramphenicol (30  $\mu$ g). The antimicrobials

were manufactured by CECON (Brazil). The strains used as standard controls were *Escherichia coli* ATCC 25922 *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 following CLSI guidelines [21].

### Virulence markers

The genomic DNA of the 60 strains listed in Table 1 was extracted as described above. The general PCR procedure was performed according to the method described in Falcão *et al.* [16] using 1.0 U *Taq* DNA polymerase (Life Technologies). The primers, the size of the respective amplification products (amplicons, assessed as base pairs) and the references used for the detection of 13 virulence gene markers are presented in Campioni *et al.* [22]. The virulence markers assayed were *invA*, *sipA*, *sipD*, *sopB*, *sopD*, *sopE2*, *ssaR*, *sifA*, *spvB*, *prot6E*, *flgK*, *fljB* and *flgL*. To evaluate the reproducibility of the experiments, the PCR reactions were repeated twice for some strains. The PCR products were analysed by agarose gel electrophoresis and visualized by UV light after staining the gel with ethidium bromide (1.0 µg/ml).

## RESULTS AND DISCUSSION

In the present study, we assessed the genotypic diversity of 60 *S. Enteritidis* strains isolated from chickens and the farm environment from diverse regions of Brazil from 2004 to 2010 using ERIC-PCR and PFGE. Moreover, we investigated 13 virulence markers to verify the pathogenic potential of those strains and examined antibiotic resistance profiles to nine antimicrobials.

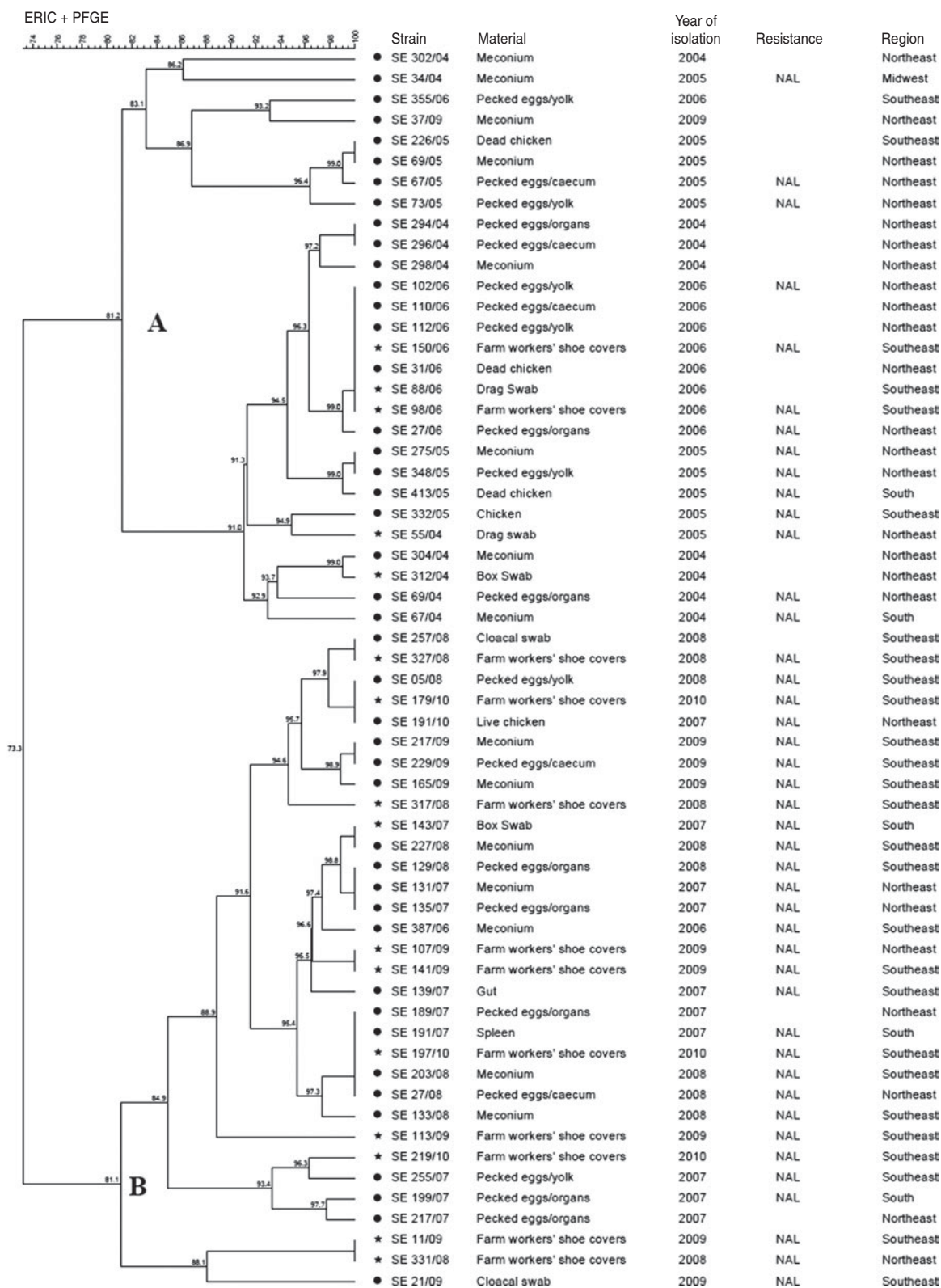
The amplicons generated by ERIC-PCR ranged in size from 100 bp to 5000 bp and the 60 strains were differentiated into 24 ERIC types. The amplicons generated by PFGE ranged in size from 48.5 kb to 679 kb and the 60 strains were differentiated into 17 PFGE types. Both methodologies presented a similar DI, 0.93 for ERIC-PCR and 0.90 for PFGE. This result is in agreement with a previous study conducted by our research group, which analysed 128 *S. Enteritidis* strains isolated from food and humans and showed a DI of 0.97 for ERIC-PCR and 0.98 for PFGE [22]. The high discriminatory power obtained in both studies confirms the potential of these methodologies to differentiate *S. Enteritidis* strains from various sources.

The concatenated dendrogram of ERIC-PCR and PFGE (Fig. 1) grouped the strains into two major clusters, referred to as A and B (Fig. 1). The strains were grouped independently of whether they were isolated from chickens or the farm environment. In both groups, some strains from the farm environment were indistinguishable from the strains isolated from chickens. These strains were isolated in different Brazilian states, such as São Paulo and Pernambuco, and from different regions, such as Southeast and Northeast Brazil. This fact reinforces the previous results of our research group that suggested the prevalence of a subtype of *S. Enteritidis*, with high genetic homogeneity, which is contaminating food, humans, animals and the environment in Brazil [22, 23]. Additionally, because 15/44 strains isolated from chickens were isolated from meconium, it could be suggested that chicks received from the breeder were colonized with *Salmonella* and caused contamination of the farm environment and horizontal transmission in the flock.

Interestingly, cluster A, encompassing strains between SE302/04 and SE67/04 with >81.2% similarity, consisted of all but one strain isolated between 2004 and 2006, while cluster B, encompassing strains between SE257/08 and SE21/09 with >81.1% similarity, consisted of all but one strain isolated between 2007 and 2010. Furthermore, there was a high prevalence of strains from cluster A that were isolated from the Northeast and Midwest regions (20/28), while cluster B had a high prevalence of strains isolated from the South and Southeast regions of Brazil (24/32). The similarity between groups A and B were >73.3%. These data suggest that despite the high genetic homogeneity of the strains, ERIC-PCR and PFGE could cluster the strains in two groups, one composed of strains isolated between 2004 and 2006 and the other group composed of strains isolated between 2007 and 2010.

Of the studies published in Brazil focusing on the epidemiology of *S. Enteritidis*, few were related to strains isolated from poultry. The majority of these studies were performed with strains isolated from chicken carcasses and used phenotypic methodologies, which presented a low discriminatory power [24–30]. Only three studies used molecular methodologies to type *S. Enteritidis* strains isolated from poultry [23, 31, 32]. Additionally, the majority of these studies were performed with strains isolated only from the South and Southeast regions of Brazil.

Alcocer *et al.* [31] typed 18 strains of *Salmonella* serovars isolated in Southeast Brazil from chicken



**Fig. 1.** Dendrogram representing genetic relationships among *Salmonella* Enteritidis strains based on ERIC-PCR and PFGE fingerprints showing strains grouped into two major clusters, A and B. Similarity (%) between patterns was calculated from the DICE index and is represented by the numbers next to the nodes. The data were sorted by the UPGMA method. ●, Chicken, ★, farm environment.

carcasses in 1999 and 2000 by phage-typing, REP-PCR and ERIC-PCR. All of the 18 *S. Enteritidis* strains were phage type 4 and presented the same ERIC and REP-PCR profile, showing a high degree of genetic similarity. Similarly, in a study by Oliveira *et al.* [32], 111 (43 poultry-related) *S. Enteritidis* strains were isolated in southern Brazil between 1995 and 2001. Four different phage types were identified, and a high level of similarity of the strains typed by REP-PCR, ERIC-PCR and BOX-PCR was observed. In a previous study performed by our research group [23], some strains that were also examined in the present study were typed using multilocus variable-number tandem-repeat analysis (MLVA). In that previous study, some poultry strains were analysed in a different context, which did not permit a specific analysis of chicken and farm environment strains, in contrast to the present study. However, a high genetic similarity in the Brazilian strains was observed, as the majority of the 60 poultry-related strains were indistinguishable by MLVA [23]. By comparison, ERIC-PCR and PFGE discriminated the strains into more subtypes in the present study than in the previous study, which used only MLVA. The findings of the above studies reinforces the results of the present work and might suggest that a prevalent subtype of *S. Enteritidis* with high genetic homogeneity has been contaminating food, humans, animals and the environment in Brazil.

Considering studies of *S. Enteritidis* isolated in other countries, a study in Korea [33], showed a similarity of >80% in 66 *S. Enteritidis* strains isolated from humans and chickens between 1994 and 2002 using PFGE. The authors suggested that chickens have been a source of infection of humans in Korea. By contrast, other studies [34, 35] found a high diversity among the *S. Enteritidis* strains typed by ERIC-PCR and PFGE, respectively. In the study by Chmielewski *et al.* [34], results from ERIC-PCR grouped 31 *S. Enteritidis* chicken-associated strains isolated in Poland into three different genomic groups, with a maximum similarity of 60%. Similarly, in the study by Yang *et al.* [35], PFGE results indicated a high diversity in 109 strains isolated from chicken meat in several cities of China in 2007 and 2008 which presented a similarity of 55% and were distinguished into 52 PFGE types.

Gastroenteritis caused by *S. Enteritidis* is usually self-limiting, but sometimes the infection can lead to complications, and thus, antimicrobial treatment is necessary [36]. The antimicrobials used in these cases are typically fluoroquinolones. In veterinary medicine,

the overuse of quinolones to treat poultry has caused high levels of resistance in *S. Enteritidis* strains in some parts of the world, e.g. Korea, Iran, Brazil [28–30, 35–39]. Some studies have shown that strains resistant to quinolones are less responsive to fluoroquinolones, which have caused treatment failure in humans [35–39]. In Brazil in 2009, quinolones were prohibited for use in animal feed as growth promoters by the Ministry of Agriculture, Livestock and Supply. However, these drugs are still legally used as veterinary antimicrobials for treatment [40].

In the present study, the strains were resistant only to the antimicrobial nalidixic acid (44 strains, 73.3%), indicating high levels of quinolone resistance in the Brazilian strains. This result corroborates the results of other studies from Brazil [28–30]. In comparison with other countries, a study in the USA showed that food animals are a reservoir of *Salmonella* strains with plasmid-mediated quinolone-resistant mechanisms [41]. In China, a study with strains isolated from chickens in 2008 showed that 99.4% of the 178 *S. Enteritidis* strains studied were resistant to nalidixic acid. Additionally, the authors found that many of the strains were resistant to 16 other antimicrobials [42]. By contrast, in our study the strains were sensitive to all the other antimicrobials tested and only resistant to nalidixic acid.

Regarding virulence markers, all 60 strains examined in the present study contained the 13 virulence markers assessed, which highlights the pathogenic potential of strains isolated from Brazilian poultry. A high prevalence of certain virulence markers was also found in poultry strains isolated in the USA [43]. In a previous study by our research group, strains of *S. Enteritidis* isolated from food and humans also presented high levels of resistance to nalidixic acid and a high frequency of the virulence markers examined [22]. These results, together with the results of the present study, suggest that strains resistant to nalidixic acid isolated from poultry are potentially pathogenic.

In conclusion, the quinolone resistance presented by many strains suggests that quinolones may have been used to treat chickens. The high prevalence of virulence markers in the strains highlights the importance of poultry as a vehicle of *S. Enteritidis* strains that have the potential to cause disease.

## ACKNOWLEDGEMENTS

We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Proc. 2008/57478-1) for

financial support. During the course of this work, F. Campioni was supported by a scholarship from FAPESP (proc. 2009/09998-9).

## DECLARATION OF INTEREST

None.

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