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Genetic diversity, virulence genes and antimicrobial resistance of *Salmonella* Enteritidis isolated from food and humans over a 24-year period in Brazil

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ABSTRACT

Salmonellosis is a major health problem worldwide. Serovar Enteritidis has been a primary cause of *Salmonella* outbreaks in many countries. In Brazil, few molecular typing studies have been performed. The aims of this study were to molecularly type *Salmonella* Enteritidis strains isolated in Brazil in order to determine the genetic relationship between strains of food and human origin, as well as, to assess their pathogenic potential and antimicrobial resistance. A total of 128 *S.* Enteritidis strains isolated from human feces (67) and food (61) between 1986 and 2010 were studied. The genotypic diversity was assessed by ERIC-PCR and PFGE using *Xba*I, the antimicrobial resistance by the disc-diffusion assay and the presence of the SPI-1, SPI-2 and pSTV virulence genes assessed by PCR. The ERIC-PCR results revealed that 112 strains exhibited a similarity of >85.4% and the PFGE that 96 strains exhibited a similarity of >80.0%. Almost all strains (97.6%) harbored all 13 virulence genes investigated. Thirty-six strains (28.12%) were resistant to nalidixic acid. In conclusion, the nalidixic acid resistance observed after 1996 is indicative of an increase in the use of this drug. It may be suggested that these 128 strains might have descended from a common ancestor that differed little over 24 years and has been both contaminating food and humans and causing disease for more than two decades in Brazil.

1. Introduction

Salmonellosis is a major health problem worldwide and is responsible for high rates of morbidity. Infection with *Salmonella enterica* occurs mainly through the consumption of contaminated food, and the estimated number of human infections per year is greater than 93.8 million cases, with 155,000 deaths per year worldwide (Boyle et al., 2007; Majowicz et al., 2010; Hendriksen et al., 2011).

The *Salmonella* serovar Enteritidis emerged during the 1980s in many countries as a major cause of *Salmonella* outbreaks related to raw or undercooked chicken and eggs. Currently, *S.* Enteritidis remains the most frequently isolated serovar in African, Asian, European and Latin American countries and the second most common serovar in North America and Oceania, accounting for 43.5% of all *Salmonella* isolates (Humphrey, 2004; Gantois et al., 2009; Hendriksen et al., 2011).

In Brazil, the first outbreak of *S.* Enteritidis was reported by Mota et al. (1983); however, a higher prevalence of this serovar was not observed until the 1990s in São Paulo State and in the South Region, with an increase from 1% to 64.9% (Baú et al., 2001; Fernandes et al., 2003; Santos et al., 2003). Recent data show that between 1999 and 2008, *S.* Enteritidis was the causal agent of 119 documented outbreaks in Brazil. Moreover, another 1275 outbreaks were related to unidentified serovars of *Salmonella* spp., indicating that the *S.* Enteritidis prevalence is most likely underestimated in this country (Brazilian Ministry of Health, 2011).

The pathogenicity of *S.* Enteritidis strains has been related to genes present in a high-molecular-mass virulence-associated plasmid (pSTV) and in the chromosomal *Salmonella* Pathogenicity Islands (SPI). Genes such as *invA*, *sipA*, *sipD*, *sopA*, *sopB*, *sopD* and *sopE*2, found in SPI-1, allow *S.* Enteritidis to invade phagocytic and non-phagocytic cells. The genes *ssaR* and *sifA*, found in SPI-2, allow the survival and replication of *Salmonella* in the host cells. Other chromosomal genes, such as *flgK*, *fljB* and *flgL*, encode flagella-associated proteins and have been shown to be important for the invasiveness of *S.* Enteritidis. Moreover, plasmidial genes such as *spvB* and *prot6E* contribute to the colonization of deeper tissues among other functions (Coburn et al., 2007; Hadjinicolaou et al., 2009; Andrews-Polymenis et al., 2010; Hur et al., 2011; Shah et al., 2011a,b).

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The understanding of *S.* Enteritidis epidemiology comes from outbreaks and sporadic case studies, which revealed that the majority of human infections are caused by the consumption of contaminated poultry products (Mishu et al., 1994; Altekruse et al., 1997; Olsen et al., 2001).

The classical typing methods include serotyping, phagetyping and antimicrobial susceptibility, among others (Hickman-Brenner et al., 1991; Stubbs et al., 1994). These methods are limited by their low ability to differentiate subtypes within the same species and by their relatively low reproducibility (Olive and Bean, 1999). Furthermore, some techniques fail to discriminate related from unrelated *Salmonella* strains (Threlfall and Frost, 1990; Borrego et al., 1992; Chmielewski et al., 2002).

Molecular typing methods have been used successfully for the typing of *Salmonella* Enteritidis strains worldwide, complementing the phenotypic methods. Techniques such as Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Pulsed-Field Gel Electrophoresis (PFGE) have been shown to efficiently discriminate *S.* Enteritidis strains, with PFGE being considered the "gold standard" methodology to type these strains (Ridley et al., 1998; Chmielewski et al., 2002; Suh and Song, 2006; Aktas et al., 2007; Rivoal et al., 2009).

In Brazil, most of the few published studies focused on *S.* Enteritidis strains were performed with phenotypic methodologies, such as serotyping and phagetyping, that presented low discriminatory power (Hofer and dos reis, 1994; Kaku et al., 1995; Taunay et al., 1996; Irino et al., 1996; Tavechio et al., 1996, 2002; Peresi et al., 1998; Baú et al., 2001; Castro et al., 2002; Santos et al., 2003; Fernandes et al., 2003, 2006). In contrast, the few molecular typing studies performed in Brazil were able to more effectively discriminate *S.* Enteritidis strains than the studies that used classical methods (Fernandes et al., 2003; Matsuoka et al., 2004; Vaz et al., 2010; Kottwitz et al., 2011).

Due to the importance of *S.* Enteritidis as a major causative agent of foodborne diseases worldwide and due to the lack of molecular typing studies in Brazil, epidemiological studies with strains of this serovar are of great importance and may lead to the implementation of control measures.

Thus, the aims of this study were to analyze the genotypic diversity of *Salmonella* Enteritidis strains isolated from human feces and food in Brazil from 1986 to 2010 using ERIC-PCR and PFGE in order to determine the genetic relationship between those strains. In addition, the presence of 13 virulence genes was investigated by PCR, and the levels of resistance to classes of antimicrobials recommended by the Clinical and Laboratory Standards Institute (CLSI) for *Salmonella* treatment were evaluated. These data were analyzed to gain a deeper understanding of the epidemiology of the *S.* Enteritidis strains isolated over a 24-year period in Brazil and to verify the pathogenic potentials and antimicrobial resistance profiles of these strains.

2. Material and methods

2.1. Bacterial strains

A total of 128 *Salmonella* Enteritidis strains were studied; these strains were isolated from human feces (67 strains) and food (61 strains) from various cities in São Paulo State, Brazil, between 1986 and 2010. These strains were selected from the collection of the Adolfo Lutz Institute of Ribeirão Preto in Brazil, and they were systematically chosen to represent isolates from outbreaks and sporadic cases that occurred during different years. The serovar-specific *sdfI* gene was amplified by PCR to confirm the serovar molecularly, as described by Agron et al. (2001). Table 1 lists the year and the isolation source of the 128 *S.* Enteritidis strains used in this study.

2.2. ERIC-PCR typing and analysis

Genomic DNA was extracted as specified above. The ERIC-PCR assay was performed on all 128 strains presented in Table 1, and the results were analyzed as described by Souza et al. (2010), with few modifications. All amplifications were carried out in a total volume of 50 μL with 100 ng of DNA template. The PCR reaction mixture also contained each deoxyribonucleotide (Invitrogen) at 1.25 mM, 5 mM $MgCl_2$ (Invitrogen), 1.0 U of Klentaq™ DNA polymerase (Ab peptides), 1× PCR buffer (Invitrogen) and 50 pmol of each primer synthesized by IDT-Integrated DNA technologies (United States). The primers used were those described by Versalovic et al. (1991): ERIC1R (5′- ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′). The program used for the 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; and a final incubation 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). The ERIC-PCR amplicons were resolved by 1.5% agarose gel electrophoresis into bands, which were stained with ethidium bromide (0.5 μg mL^{-1}) and visualized under UV light.

Data were analyzed with the software package *BioNumerics* 5.1 (Applied Maths, Keistraat, Belgium). 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 from Invitrogen – Life technologies) was included three times on each gel, to normalize the images and thus allow valid comparisons of fingerprints on different gels.

2.3. PFGE typing and analysis

Agarose blocks of the 128 strains listed in Table 1 were prepared using the protocol described in Souza et al. (2010), with some modifications.

The modifications were made to the initial stage, during which pure cultures of the bacterial strains were grown in BHI broth (HiMedia Lab, India) O/N at 37 °C; the colonies were isolated on Muller–Hinton agar (HiMedia Lab, India) plates after incubation for 12–18 h at 37 °C. Some 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_{600nm} of 0.6–0.9 was reached.

Next, the cell suspension was warmed to 50 °C and mixed with 500 μL of a 2% low-melting-point agarose (Bio-Rad laboratories) at 50 °C, and 70 μL of the suspension was cast in each DNA-plug mold. Plugs were digested with 15 U of *XbaI* (Invitrogen) overnight at 37 °C.

Macrorestriction fragments were resolved by counter-clamped homogeneous electric field electrophoresis in a CHEF-DR III apparatus (Bio-Rad Laboratories), with an electric field of 6 V cm−1 and angle of 120°. The migration of fragments was performed at 14 °C in 0.5 × 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 Laboratories). The pulse times were ramped from 2.2 to 63.8 s over 19 h, as described by Ribot et al. (2006).

A standard molecular weight ladder (Lambda Ladder PFG Marker – New England BioLabs) was included three times on each gel, to allow comparison of the fingerprints over several gels.

The gels were stained with ethidium bromide (0.5 μg mL^{-1}) for 30 min and distained in distilled water for 20 min. The restriction fragments were visualized under UV light.

Table 1
Year and source of isolation of the 128 strains of *S.* Enteritidis studied isolated from various cities of the Sao Paulo State, Brazil.

Year of isolation	Food strains/outbreak number	Human feces strains/outbreak number	Food strains of sporadic cases	Human feces strains of sporadic cases	Total
1986	–	–	–	1	1
1992	–	–	–	1	1
1993	–	–	–	–	0
1994	1 – outbreak 2	1 – outbreak 1	1	5	8
1995	2 – outbreak 3 1 – outbreak 4 4 – outbreak 5 3 – outbreak 6	1 – outbreak 3	3	3	12
1996	1 – outbreak 8 1 – outbreak 9 1 – outbreak 11	2 – outbreak 7 2 – outbreak 8 1 – outbreak 9 1 – outbreak 11	4	3	16
1997	1 – outbreak 12 1 – outbreak 13 1 – outbreak 14 1 – outbreak 15	1 – outbreak 12	–	–	5
1998	1 – outbreak 17 1 – outbreak 18 1 – outbreak 19	1 – outbreak 16 2 – outbreak 17	–	1	25
1999	1 – outbreak 20 2 – outbreak 21 1 – outbreak 22 1 – outbreak 23 1 – outbreak 24 2 – outbreak 25 1 – outbreak 26 1 – outbreak 27	2 – outbreak 20	1	2	13
2000	1 – outbreak 28 1 – outbreak 30	2 – outbreak 28	1	2	7
2001	1 – outbreak 33 2 – outbreak 32 1 – outbreak 34	1 – outbreak 31 2 – outbreak 32	–	2	8
2002	1 – outbreak 38	1 – outbreak 35 1 – outbreak 37 1 – outbreak 38	1	4	9
2003	1 – outbreak 40 2 – outbreak 41 1 – outbreak 42	1 – outbreak 39	–	1	5
2004	1 – outbreak 45 1 – outbreak 46	1 – outbreak 43 1 – outbreak 44 1 – outbreak 45 2 – outbreak 47	1	1	9
2005	2 – outbreak 48	1 – outbreak 48	–	–	3
2006	–	1 – outbreak 49 1 – outbreak 50 1 – outbreak 51	–	2	5
2007	1 – outbreak 52	–	–	–	1
2009	–	1 – outbreak 53 1 – outbreak 54 1 – outbreak 55 1 – outbreak 56	–	1	5
2010	1 – outbreak 57	2 – outbreak 57	–	–	3
Total of strains	49 strains/37 outbreaks	38 strains/30 outbreaks	12 strains	29 strains	128

The relatedness among the PFGE profiles was analyzed with the software package BioNumerics 5.1 (Applied Maths). Only bands representing fragments between 48.5 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 and a position tolerance of 1.2.

2.4. Discrimination index (DI)

The discriminatory power of ERIC-PCR and PFGE was assessed by Simpson's diversity index, as presented by Hunter and Gaston (1988).

2.5. Antimicrobial resistance profiles

The susceptibility of the strains to antimicrobials was evaluated by the disc diffusion technique following the protocol of the Clinical and Laboratory Standards Institute – CLSI (2011). The tested antimicrobials were amikacin (30 μg), tetracycline (30 μg), ampicillin (10 μg), cephalothin (30 μg), trimethoprim-sulfamethoxazole (25 μg), nalidixic acid (30 μg), streptomycin (10 μg), ceftriaxone (30 μg) and chloramphenicol (30 μg), manufactured by CECON (Maranguape, Sao Paulo, Brazil).

2.6. Virulence genes

The genomic DNA of the 128 strains listed in Table 1 was extracted as described by Falcão et al. (2006) and the DNA concentration determined as described in Sambrook and Russel (2001). The general PCR procedure was performed according to the method described in Falcão et al. (2006) using 1.0 U of *Taq* DNA polymerase (Invitrogen). The primers, the size in base pairs of the respective amplification products (amplicons) and the references

used for the detection of 13 virulence gene markers are presented in Table 2. The virulence genes under study 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 analyzed by agarose gel electrophoresis and visualized by UV light after staining the gel with ethidium bromide (1.0 μg mL^{-1}).

3. Results

3.1. ERIC-PCR

The amplicons generated by ERIC-PCR ranged in size from 100 to 5000 bp. The 128 strains were differentiated into 55 ERIC types (Fig. 1).

The strains were grouped into three major clusters designated ERIC A, ERIC B and ERIC C. In the ERIC A cluster, 112 strains between SE 12 and SE 128 exhibited a similarity above 85.4%. These 112 strains were isolated between 1986 and 2006, from human feces (57) and food (55), either from outbreaks (74) or sporadic cases (38). The ERIC B contained eight strains between SE 135 and SE 133 in the dendrogram. These strains were isolated between 1999 and 2001 from human feces (3) and food (5), either from outbreaks (6) or sporadic cases (2), and exhibited a similarity above 88.6%. The ERIC C cluster contained eight strains, between SE 253 and SE 273 in the dendrogram that exhibited 100% similarity. These strains were isolated in 2009 and 2010 from human feces. All but one of these eight strains were isolated from different outbreaks (7) in different cities; the exception SE 253, was isolated from a sporadic case. The similarity among the strains of the three ERIC clusters was above 79.7% (Fig. 1).

Table 2
Primers and amplicons sizes of the genes *invA*, *sipA*, *sipD*, *sopB*, *sopD*, *sopE2*, *ssaR*, *sifA*, *spvB*, *prot6E*, *flgK*, *fljB*, *flgL* and *sdfI* searched in the 128 *S.* Enteritidis strains studied.

Genes	Primers	Primers sequence (5′–3′)	Amplicon (bp)	References
InvA	Forward	ACA GTG CTC GTT TAC GAC CTG AAT	244	Hur et al. (2011)
	Reverse	AGA CGA CTG GTA CTG ATC GAT AAT		
sipA	Forward	ATGGTTACAAGTGTAAGGACTCAG	2055	Shah et al. (2011a,b)
	Reverse	ACGCTGCATGTGCAAGCCATC		
sipD	Forward	ATGC TCCTTGCAGGAAGCTTTTG	1029	Shah et al. (2011a,b)
	Reverse	TTAATATTCAAAATTATTCCG		
sopB	Forward	CCT CAA GAC TCA AGA TG	1987	Hur et al. (2011)
	Reverse	TAC GCA GGA GTA AAT CGG TG		
sopD	Forward	ACG ACC ATT TGC GGC G	1291	Hur et al. (2011)
	Reverse	GAG ACA CGC TTC TTC G		
sopE2	Forward	TAC TAC CAT CAG GAG G	995	Hur et al. (2011)
	Reverse	GAA TGT TTT ATG TGA CGC AG		
ssaR	Forward	GTT CGG ATT CAT TGC TTC GG	1628	Hur et al. (2011)
	Reverse	TCT CCA GTG ACT AAC CCT AAC CAA		
sifA	Forward	ATG CCG ATT ACT ATA GGC AAT GG	1011	Hur et al. (2011)
	Reverse	TTA TAA AAA ACA ACA TAA ACA GCCG		
spvB	Forward	CGGTTATAGAAGAGCTCCTGT	349	Rychlík et al. (2008)
	Reverse	CCGGTATACGACTCTGTGATC		
Prot6E	Forward	GGCACCGCAGCAATGGTTGG	135	Hadjinicolaou et al. (2009)
	Reverse	GGTCGAGCTACAGAGAGTCACAC		
flgK	Forward	ATGTCCAGCTTGATTAATCAC	1659	Shah et al. (2011a,b)
	Reverse	GCGAATATTCAATAACGCATC		
fljB	Forward	ATGGCACAAGTCATTAATACAAAC	1515	Shah et al. (2011a,b)
	Reverse	ACGCAGTAAAGAGAGGAC		
flgL	Forward	ATGCGTATCAGTACCCAGATG	951	Shah et al. (2011a,b)
	Reverse	CCGGTTCAACTGGAAAAGC		
sdfI	Forward	TGTGTTTTATCTGATGCAAGAGG	333	Agron et al. (2001)
	Reverse	CGTTCTTCTGGTACTTACGATGAC		

3.2. PFGE

The amplicons generated by PFGE ranged in size from 48.5 kb to 679 kb. The 128 strains were differentiated into 68 PFGE types (Fig. 2).

The strains were grouped into three major clusters designated PFGE A, PFGE B and PFGE C. In the PFGE A cluster, 96 strains between SE 119 and SE 86 in the dendrogram exhibited a similarity above 80.0%. These strains were isolated between 1994 and 2007 from human feces (44) and food (52), either from outbreaks (68) or sporadic cases (28). The PFGE B cluster contained three strains, between SE 4 and SE 60 in the dendrogram that were isolated from feces (2) and food (1), either from outbreaks (1) or sporadic cases (2); these three strains exhibited a similarity above 78.3%. These three strains were isolated between 1986 and 1996. The PFGE C cluster contained 28 strains, between SE 27 and SE 233 in the dendrogram, with a similarity above 74.5%. These strains were isolated between 1994 and 2010 from human feces (20) and food (8), either from outbreaks (17) or sporadic cases (11). The similarity among the strains of the three PFGE clusters was above 73.1% (Fig. 2).

3.3. Discrimination index

The DI of ERIC-PCR was 0.97, and that of PFGE 0.98.

3.4. Antimicrobial resistance profiles

All 128 strains listed in Table 1 were susceptible to the antimicrobials tetracycline, cephalothin, ampicillin, amikacin, ceftriaxone, chloramphenicol, trimethoprim-sulfamethoxazole and streptomycin. The only exception was SE43 (0.78%) that was resistant to trimethoprim-sulfamethoxazole and streptomycin. A total of 36 strains (28.12%) isolated from human feces (20) and from food (16) were resistant to nalidixic acid.

3.5. Virulence genes

Almost all 128 strains (97.6%) listed in Table 1 harbored all 13 virulence genes that were investigated. Only one strain (SE 76) was negative for the presence of the SPI-1 gene *sipA*, and two strains (SE75 and SE 183) were negative for the presence of the plasmidial gene *prot6E*.

4. Discussion

Salmonella Enteritidis emerged as the most frequently isolated serovar worldwide during the 1980s. Currently, this serovar still causes many economic losses due to the health problems. This bacterium is acquired primarily through the consumption of contaminated food. Therefore, epidemiological studies are important for elucidating contamination routes, improving monitoring and implementing control programs (Olive and Bean, 1999; Boyle et al., 2007; Hendriksen et al., 2011). Specifically, in Brazil, few molecular typing studies have been conducted (Fernandes et al., 2003; Matsuoka et al., 2004; Vaz et al., 2010; Kottwitz et al., 2011).

The present study used ERIC-PCR and PFGE to type 128 *S.* Enteritidis strains isolated in São Paulo State, Brazil, over a period of 24 years. Moreover, the presence of 13 virulence genes was investigated to verify the pathogenic potentials and the antimicrobial resistance profiles of the selected strains.

The dendrograms generated with the data from ERIC-PCR and PFGE grouped the strains into three major clusters. These clusters

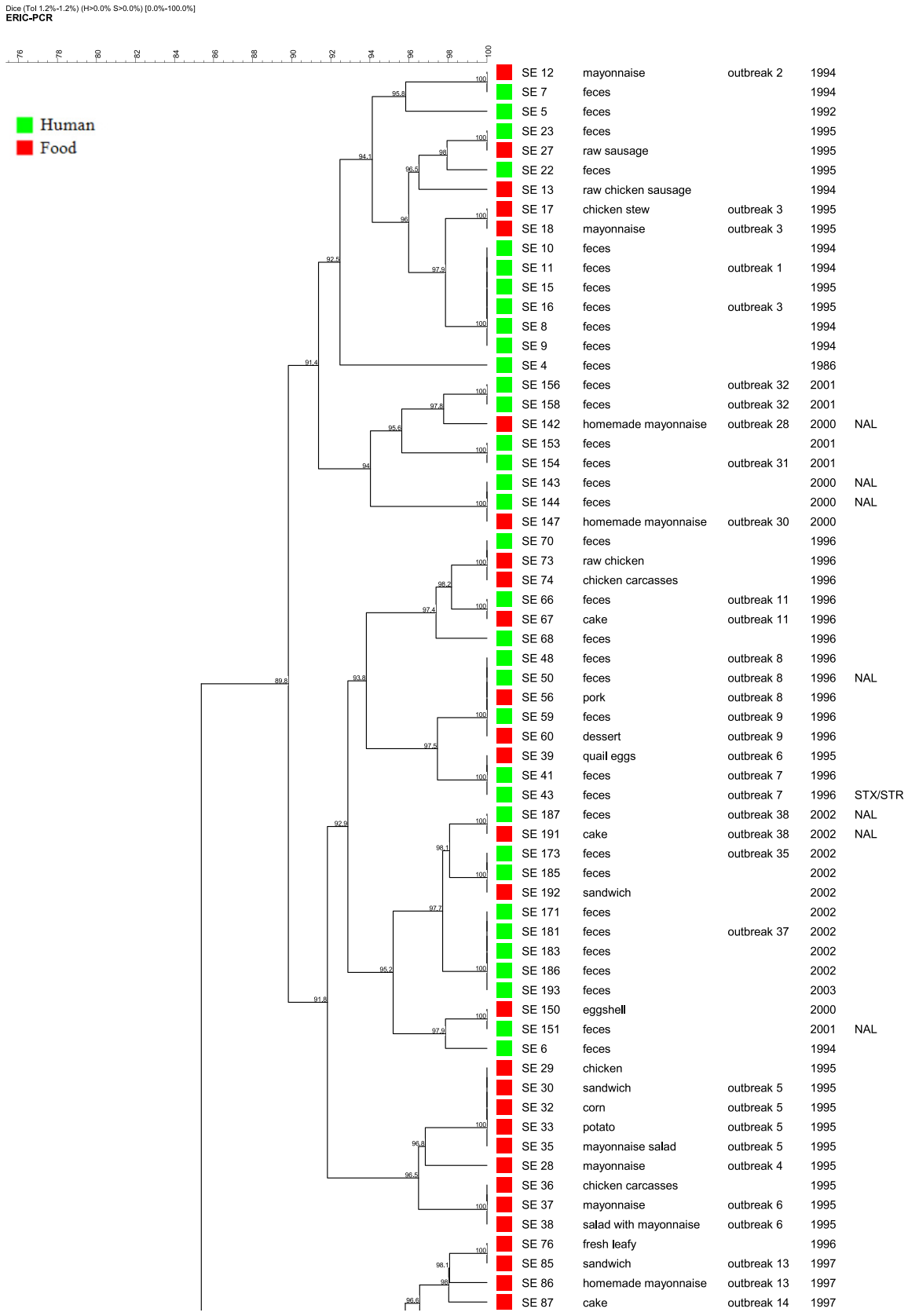

Fig. 1. Dendrogram representing genetic relationships among *Salmonella* Enteritidis strains based on ERIC-PCR fingerprints. Similarity (%) between patterns was calculated from the DICE index and is represented by the numbers beside the nodes. The data were sorted by the UPGMA method. NAL – nalidixic acid, STX – trimethoprim-sulfamethoxazole, STR – streptomycin.

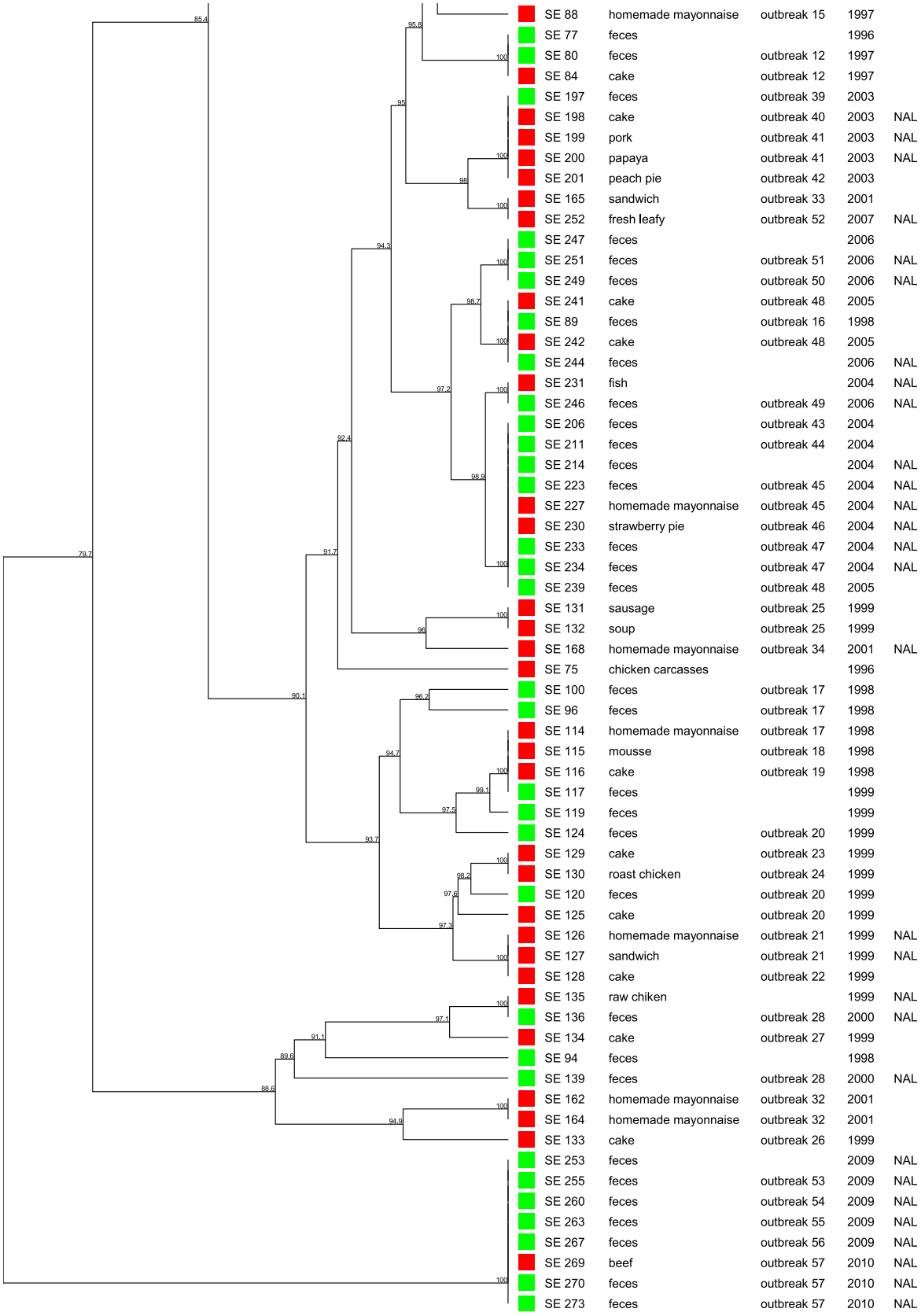

Fig. 1. (*continued*).

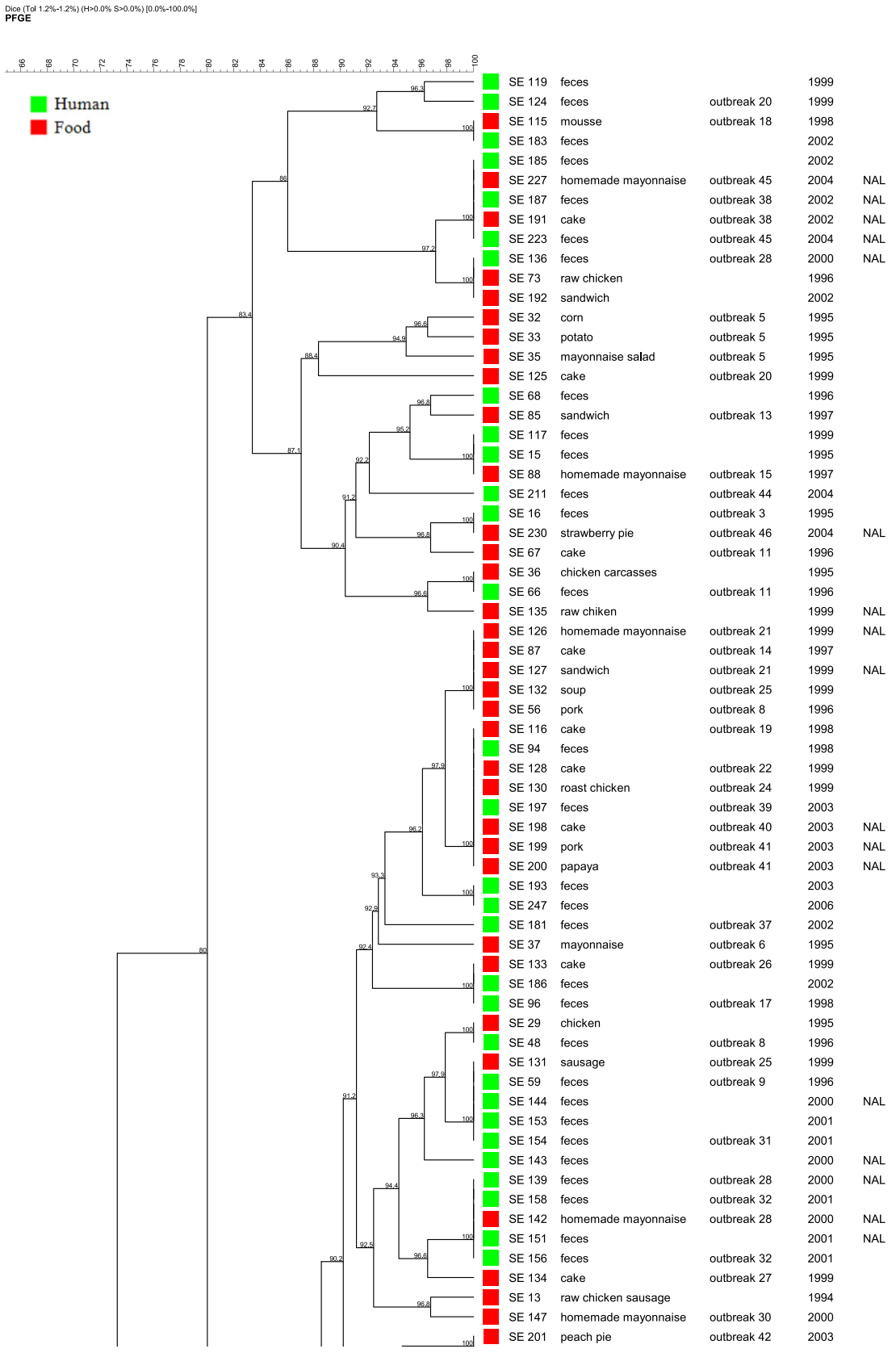

Fig. 2. Dendrogram representing genetic relationships among *Salmonella* Enteritidis strains based on PFGE fingerprints. Similarity (%) between patterns was calculated from the DICE index and is represented by the numbers beside the nodes. The data were sorted by the UPGMA method. NAL – nalidixic acid, STX – trimethoprim-sulfamethoxazole, STR – streptomycin.

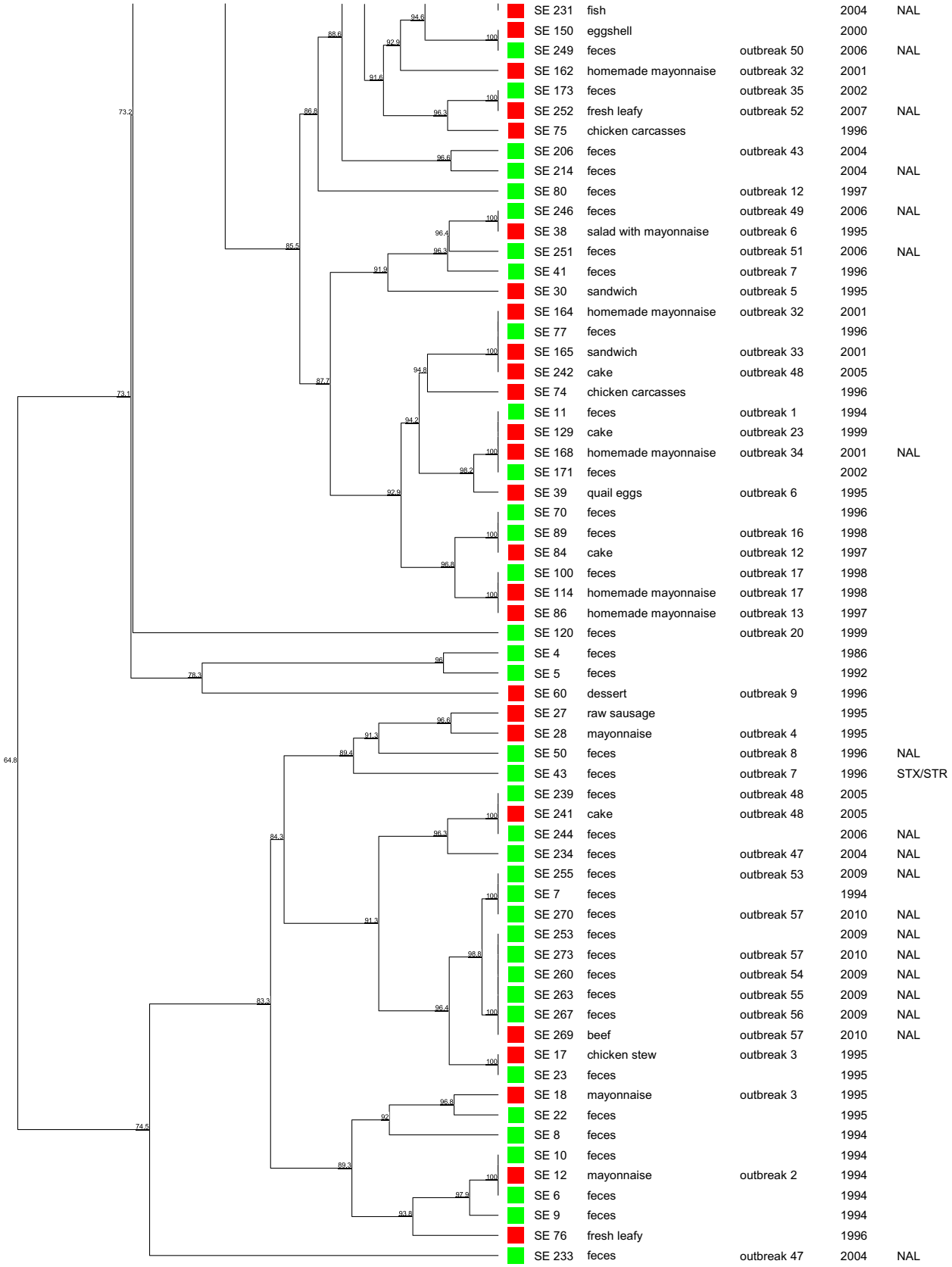

Fig. 2. (*continued*).

showed that the strains isolated from sporadic cases were undistinguishable from the outbreak strains. In addition, strains isolated during different outbreaks were grouped together. In general, both methodologies showed similar results grouping the strains into three major clusters; however, strains belonging to an ERIC cluster did not necessarily belong to the same PFGE cluster. Although the methodologies showed similar and high DIs (0.97 for ERIC-PCR and 0.98 for PFGE), the strains exhibited a high level of genetic similarity (above 79.7% in ERIC-PCR and above 73.1% in PFGE), which is notable given the long period of time over which the isolates were collected. The same high level of genetic similarity was observed in the study of Kottwitz et al. (2011), who found a high genetic similarity among *S.* Enteritidis strains isolated from humans and food, between 2002 and 2006 and typed by PFGE.

The published literature contains only few studies that used ERIC-PCR to type *S.* Enteritidis strains, and only one of these studies included strains isolated in Brazil (Milleman et al., 1996; Chmielewski et al., 2002; Suh and Song, 2006; Oliveira et al., 2007; Ammari et al., 2009).

In the study of Milleman et al. (1996), performed with 14 strains isolated in France between 1991 and 1993, and in the study of Suh and Song (2006), performed with 22 strains isolated in South Korea between 2001 and 2002, ERIC-PCR was found to have a low power of differentiation. In contrast, ERIC-PCR could efficiently discriminate *S.* Enteritidis strains in a study of 31 strains isolated in Poland that was performed by Chmielewski et al. (2002) and in another study by Ammari et al. (2009) with 15 strains isolated in Morocco between 2005 and 2006. In the study by Ammari et al. (2009), ERIC-PCR exhibited a higher discriminatory power than PFGE, which is considered the "gold standard" technique for the typing of *S.* Enteritidis strains. The present study corroborates studies performed by Chmielewski et al. (2002) and Ammari et al. (2009), which showed that ERIC-PCR is able to efficiently differentiate *S.* Enteritidis strains isolated over a short or long period of time.

To our knowledge, there is only one published study that used ERIC-PCR to type *S.* Enteritidis strains in Brazil. The study performed by Oliveira et al. (2007) used rep-PCR methodologies to study *S.* Enteritidis strains isolated in the states of the South Region of Brazil. The authors typed 102 strains isolated from outbreaks, between 1995 and 2001, using REP-PCR, ERIC-PCR and BOX-PCR, and these methodologies could not efficiently differentiate those *S.* Enteritidis strains. Those results may be explained in part by the fact that the strains studied were isolated only from outbreaks that occurred over a much shorter period of time than encompassed by the present study. Therefore, the present study contributes to the elucidation of the epidemiology of *S.* Enteritidis strains in Brazil over a period of more than two decades.

The PFGE methodology is widely used to discriminate outbreak strains and has been shown to be an important tool to type *S.* Enteritidis strains (Ridley et al., 1998; Aktas et al., 2007; Chu et al., 2009; Kang et al., 2009; Rivoal et al., 2009; Kober et al., 2011).

The use of PFGE to type 60 phagetype reference strains of *S.* Enteritidis in the study of Ridley et al. (1998) showed that this technique had a better discriminatory power than ribotyping or plasmid analysis. Furthermore, Aktas et al. (2007) typed 26 strains of *S.* Enteritidis isolated from pediatric clinical samples in Turkey and showed that PFGE exhibited greater discriminatory power than the plasmid analysis.

In Brazil, studies that used PFGE to type *S.* Enteritidis strains isolated in the South Region of the country found moderate DI values; a DI of 0.55 was obtained in the study of Kober et al. (2011) performed with 31 strains isolated from several sources, between 1995 and 2001, and a DI of 0.66 was obtained in the study of Kottwitz et al. (2011) that typed 41 strains from human feces and food isolated between 2002 and 2006. Thus, the present study was able to more effectively discriminate the strains by PFGE (DI 0.98) than the other two studies mentioned above.

Few molecular typing studies have been performed with *S.* Enteritidis strains isolated in the São Paulo State in Brazil. None of them used ERIC-PCR or PFGE, making it difficult to compare those studies with this present study (Fernandes et al., 2003; Matsuoka et al., 2004; Martins et al., 2006).

In the present study, ERIC-PCR differentiated the 128 strains into 55 ERIC types (Fig. 1), and PFGE differentiated the 128 strains into 68 PFGE types (Fig. 2). Thus, the techniques used in this study showed a higher discriminatory power than the ribotyping method used by Fernandes et al. (2003), which discriminated 105 strains isolated from 1975 to 1995 into 14 ribotypes. Fernandes et al. (2003) observed that the 25 studied strains isolated between 1975 and 1992 were mostly of phagetype 8 and that the 80 studied *S.* Enteritidis strains isolated from 1993 to 1995 were mostly phagetype 4 and ribotype 11.

In the present study, given the similar results provided by both techniques used and the high genotypic similarity among the strains isolated over the 24-year period, it may be suggested and hypothesized that the majority of the *S.* Enteritidis strains studied might have descended from a common ancestor that differed little over those 24 years and that has been infecting humans and contaminating food from outbreaks and sporadic cases in Brazil. This hypothesis is reinforced by the studies of Fernandes et al. (2003) and Kottwitz et al. (2011), which found a high clonality in strains isolated after 1993 in Brazil.

The use of antimicrobials is not usually recommended to treat salmonellosis in general; however, in some cases, the infection can evolve into a severe infection such as bacteremia or meningitis. In these cases, the use of antimicrobials is mandatory. The antimicrobial resistance of the strains analyzed in the present study was tested against some classes of drugs recommended by the CLSI to treat *Salmonella* infections. The most common antimicrobial used in human and veterinary treatments has been nalidixic acid, a quinolone that targets DNA gyrase (Stevenson et al., 2007; Tamang et al., 2011). In the present study, the antimicrobial resistance profile showed that 28.1% of the strains were resistant to nalidixic acid and that only 0.8% were resistant to trimethoprim-sulfamethoxazole and streptomycin. The resistant strains were isolated since 1996 in Brazil, and the evolution of resistant strains most likely reflects the use of antimicrobials to treat *S.* Enteritidis infections after pandemics.

In a study performed with 128 strains isolated in São Paulo State between 1985 and 1999, Castro et al. (2002) found resistance to nalidixic acid in 0.8% of the strains and to trimethoprim-sulfamethoxazole in 8.6%. Fernandes et al. (2003) studied 105 strains, isolated between 1975 and 1995 and also found resistance to nalidixic acid (20.9%), streptomycin (40.9%) and trimethoprim-sulfamethoxazole (3.8%).

Similar resistance profiles were found in strains isolated in the South Region of Brazil. In a study with 79 strains isolated between 2001 and 2002, Oliveira et al. (2006) found resistance to nalidixic acid in 21.5% of the strains and resistance to trimethoprim-sulfamethoxazole in 13.9%. Kottwitz et al. (2011) studied 41 strains isolated between 2002 and 2006 and found resistance to nalidixic acid in 41.5% of the strains. The nalidixic acid resistance found in the present study (28.1) and in the studies mentioned above show that the percentage of *S.* Enteritidis strains resistant to this antimicrobial has increased over time in Brazil.

Studies of strains isolated from other parts of the world also showed that these strains were resistant to nalidixic acid, trimethoprim-sulfamethoxazole and streptomycin. These resistance profiles reflect the systematic use of those drugs to treat *Salmonella* infections (Suh and Song, 2006; Hur et al., 2011;

Álvarez-Fernández et al., 2012; Nesbitt et al., 2011; Kozoderović et al., 2011; Thong et al., 2011).

Regarding the virulence genes that were analyzed, the majority of the strains (97.6%) studied harbored all of the virulence genes that were investigated. The two strains (SE75 and SE 183) that did not harbor the gene *prot6E* most likely had some mutation in this gene that did not allow the primers to anneal because this gene is a plasmidial gene and these strains were positive for the other plasmidial gene, *spvB*, that was analyzed. The high frequency of the presence of the virulence genes that were investigated highlights the pathogenic potential of the studied *S*. Enteritidis strains, which have been causing disease in humans and contaminating food for 24 years in Brazil. Similar results related high prevalence of some virulence genes in strains isolated from North America and Africa (Dione et al., 2011; Shah et al., 2011a; Zou et al., 2012).

In conclusion, the nalidixic acid resistance observed after 1996 indicates an increase in the use of quinolones to treat *Salmonella* infections in Brazil. The data suggest that the majority of the *S*. Enteritidis strains studied might descended from a common ancestor that differed little over the 24-year period and has been contaminating food, infecting humans and causing disease for more than two decades in Brazil.

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