Tracing lineage by phenotypic and genotypic markers in *Salmonella enterica* subsp. *enterica* serovar 1,4,[5],12:i:- and *Salmonella* Typhimurium isolated in state of São Paulo, Brazil

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Fifty-three Salmonella *1,4,[5],12:i:- and 45* Salmonella *Typhimurium strains were characterised using phage typing, plasmid profiles and pulsed-field gel electrophoresis (PFGE) for comparison. The majority of the strains were subdivided into definitive type (DT) 41 (22.6%) and DT 193 (18%) and the 60-MDa plasmid was detected in 94.3% and 84.4% of strains, respectively. Genetic diversity was observed among all strains and 90% presented a > 70% similarity through PFGE analysis. These results suggest a close relationship between* Salmonella *1,4,[5],12:i:- and* Salmonella *Typhimurium at the serotype level.*

Key words: *Salmonella* phage typing - plasmids - pulsed-field gel electrophoresis

Salmonellosis has been a public health concern in the world due to the zoonotic feature of most *Salmonella* serovars. The laboratory surveillance of salmonellosis by serotyping has taken place at the Adolfo Lutz Institute in state of São Paulo (SP), Brazil since the 1940s (Taunay et al. 1996, Tavechio et al. 1996, 2002, Fernandes et al. 2006). A five-fold increase of *Salmonella enterica* subsp. *enterica* serovar 1,4,[5],12:i:- was observed in the 1990s (Tavechio et al. 2004) since its initial isolation in the late 1970s (Calzada et al. 1984). It has been among the top five *Salmonella* serovars that have been isolated from human and non-human sources associated with foodborne outbreaks in human and extra-intestinal infections. This monophasic serovar has also been detected in several other countries and it is hypothesised that it could be *Salmonella* Typhimurium with an antigenic formula (1,4,[5],12:i:1,2), serovar Lagos (1,4,[5],12:i:1,5) without the second-phase H antigen (Popoff 2001), or possibly a new monophasic *Salmonella* serovar according to the review reported by Switt et al. (2009). This study was designed to use phenotypic and genotypic markers to compare *Salmonella* 1,4,[5],12:i:- and *Salmonella* Typhimurium strains from human and nonhuman sources from SP, from 1991-2000, to determine the lineage of these two salmonella groups.

Fifty-three *Salmonella* 1,4,[5],12:i:- strains, which were previously analysed by antimicrobial susceptibility testing (susceptible and resistant to 1-13 antimicrobials) and confirmed as *fljB*-negative (Tavechio et al. 2004), were included in this study. These strains were mostly from human sporadic cases, intestinal (n = 23) and extra-intestinal (n = 19) infections and non-human sources, such as laboratory animals (n = 6), meat (n = 3), dessert (n = 1) and environment (n = 1) from various geographic areas in SP from 1991-2000. Forty-five *Salmonella* Typhimurium strains (susceptible or resistant to two-seven antimicrobials, which were isolated from 1991-2000, were selected from a previous study (Ghilardi et al. 2006) and included in this study for comparison.

Phage typing was performed using the specific *Salmonella* Typhimurium phage-typing scheme (Anderson et al. 1977) at the Enteric Disease Program, National Microbiology Laboratory, Public Health Agency of Canada in Winnipeg, Manitoba, Canada. All strains were typeable and the lytic patterns not conforming to standard patterns were assigned as atypical (AT) or reaction does not conform (RDNC).

The distribution of phage types of *Salmonella* Typhimurium and *Salmonella* 1,4,[5],12:i:- strains is shown in Table. According to the number of *Salmonella* Typhimurium included in this study, the two most prevalent phage types were AT 04-2406 (24.5%) and definitive type (DT) 193 (18%), whereas for *Salmonella* 1,4,[5],12:i:- phage type DT41 was the prevailing one accounted for 22.6% of the strains and was followed by AT 00-0677 (13.2%). By comparing the results from both groups described herein, it was verified that 11 *Salmonella* Typhimurium strains shared four common phage types (DT193, DT104b, DT120 and DTU302) with 19 *Salmonella* 1,4,[5],12:i:- strains. Two strains from each group could not be typed by an initial phage typing scheme. These results showed that the great majority of *Salmonella* 1,4,[5],12:i:- strains were lysed by the phages composing the specific *Salmonella* Typhimurium phage typing scheme suggesting a close relationship between these two serotypes.

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TABLE

Relationship between phage type and pulsed-field gel electrophoresis (PFGE) profiles of *Salmonella* Typhimurium and *Salmonella* 1,4,[5],12:i:- strains isolated during 1991-2000, in state of São Paulo, Brazil

	Salmonella Typhimurium (n = 45)		*Salmonella* 1,4,[5],12:i:- (n = 53)	
Phage type	Strains (%)	PFGE profiles[a]	Strains (%)	PFGE profiles[a]
DT 4	1 (2.2)	Xt24	0	-
DT 10	1 (2.2)	Xt10	0	-
DT 27	1 (2.2)	Xt6	0	-
DT 49	6 (13.4)	Xt1 (3), Xt20, Xt25, Xt28	0	-
DT 99	1 (2.2)	Xt11	0	-
DT 104	1 (2.2)	Xt3	0	-
DT 110b	1 (2.2)	Xt13	0	-
DT 135	2 (4.5)	Xt1, Xt17	0	-
DT 153	1 (2.2)	Xt23	0	-
AT 97-6274	1 (2.2)	Xt9	0	-
AT 97-6276	1 (2.2)	Xt8	0	-
AT 04-2406	11 (24.5)	Xt1 (4), Xt7, Xt12, Xt14, Xt16, Xt18, Xt19, Xt26	0	-
AT 04-4538	1 (2.2)	Xt21	0	-
AT 04-6846	1 (2.2)	Xt15	0	-
AT 04-6871	1 (2.2)	Xt4	0	-
AT 04-6884	1 (2.2)	Xt4	0	-
UT 7	1 (2.2)	Xt6	0	-
UT 8	1 (2.2)	Xt27	0	-
DT 104b	1 (2.2)	Xt3	6 (11.3)	X4 (3), X7 (2), X22
DT 120	1 (2.2)	Xt31	5 (9.5)	X2 (4), X30
DT 193	8 (18.0)	Xt2 (3), Xt5 (2), Xt22, Xt29, Xt32	6 (11.3)	X6 (2), X11, X23, X24, X27
DT U302	1 (2.2)	Xt30	2 (3.8)	X19, X29
DT 41	0	-	12 (22.6)	X1 (6), X14, X15, X18, X20, X25, X26
DT 180	0	-	3 (5.6)	X2 (2), X28
DT 208	0	-	2 (3.8)	X31, X32
AT 00-0677	0	-	7 (13.2)	X3 (5), X16, X17
AT 04-6814	0	-	3 (5.6)	X5 (2), X10
AT 04-6788	0	-	1 (1.9)	X9
AT 04-6811	0	-	1 (1.9)	X21
AT 00-3219	0	-	1 (1.9)	X13
AT 04-6828	0	-	1 (1.9)	X33
AT 04-6839	0	-	1 (1.9)	X12
UT	0	-	2 (3.8)	X8

a: number of strains for PFGE profile; AT: atypical; DT: definitive type; UT: untypeable.

A previous study on phage typing, antimicrobial resistance and plasmidial profiles of *Salmonella* Typhimurium isolated from children living in two Brazilian cities, Salvador and Rio de Janeiro, was described by Asensi et al. (1995). They also detected the predominance of DT193 (47.7%) among six different phage types as well as a significant percentage (31%) of strains that could not be typed, with 2% considered to be RDNC. They did not detect any DT 104 isolate.

Five monophasic strains displaying resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (pentaresistant or R-type ACSSuT) belonged to DT 193 (2), DT 208, DT 104b and DT U302. There were no monophasic strains phage typed as DT 104; however, one *Salmonella* Typhimurium DT 104 strain R-type ACSSuT was detected. Another seven *Salmonella* Typhimurium strains [phage types AT 04-2406 (3), AT 04-6846, DT 49, DT 135 and DT 104b] displayed the pentaresistant pattern. Although streptomycin was not included in the study reported by Asensi et al. (1995) in Brazil, they also found 47% of *Salmonella* Typhimurium isolates with the ACSSuT pattern.

In Spain, Echeita et al. (1999) found that all 288 *Salmonella* 4,5,12:i:- strains isolated from humans and pork products in 1998 and 1999, which were analysed by phage

typing and antimicrobial susceptibility, belonged to phage type DT U302 and most of them presented R-type ACSSuT. Afterwards, de la Torre et al. (2003), also in Spain, compared 23 *Salmonella* 4,5,12:i:- to 24 *Salmonella* Typhimurium isolates from 1998-2000 by pulsed-field gel electrophoresis (PFGE) and plasmid profiling. These belonged to four and six phage type patterns respectively, with most of them being pentaresistant . The authors found subtypes that were closely related (> 80% similarity) and possibly related (> 65% and < 80% similarity) and because 70% of the *Salmonella* 4,5,12:i:- isolates were phage type DT U302, the authors suggested that those monophasic isolates originated from *Salmonella* Typhimurium DT U302.

In a recent study from Luxembourg, Mossong et al. (2007) reported the occurrence of outbreaks due to *Salmonella* 1,4,[5],12:i:- phage type DT 193 with the R-type ASSuT. The phage typing results of our monophasic strains showed that DT 193 ranked third (6 strains) along with DT 104b and included two pentaresistant (ACSSuT) strains with eight additional markers.

The plasmid profiles were determined using the alkaline extraction method for small volumes of cell cultures

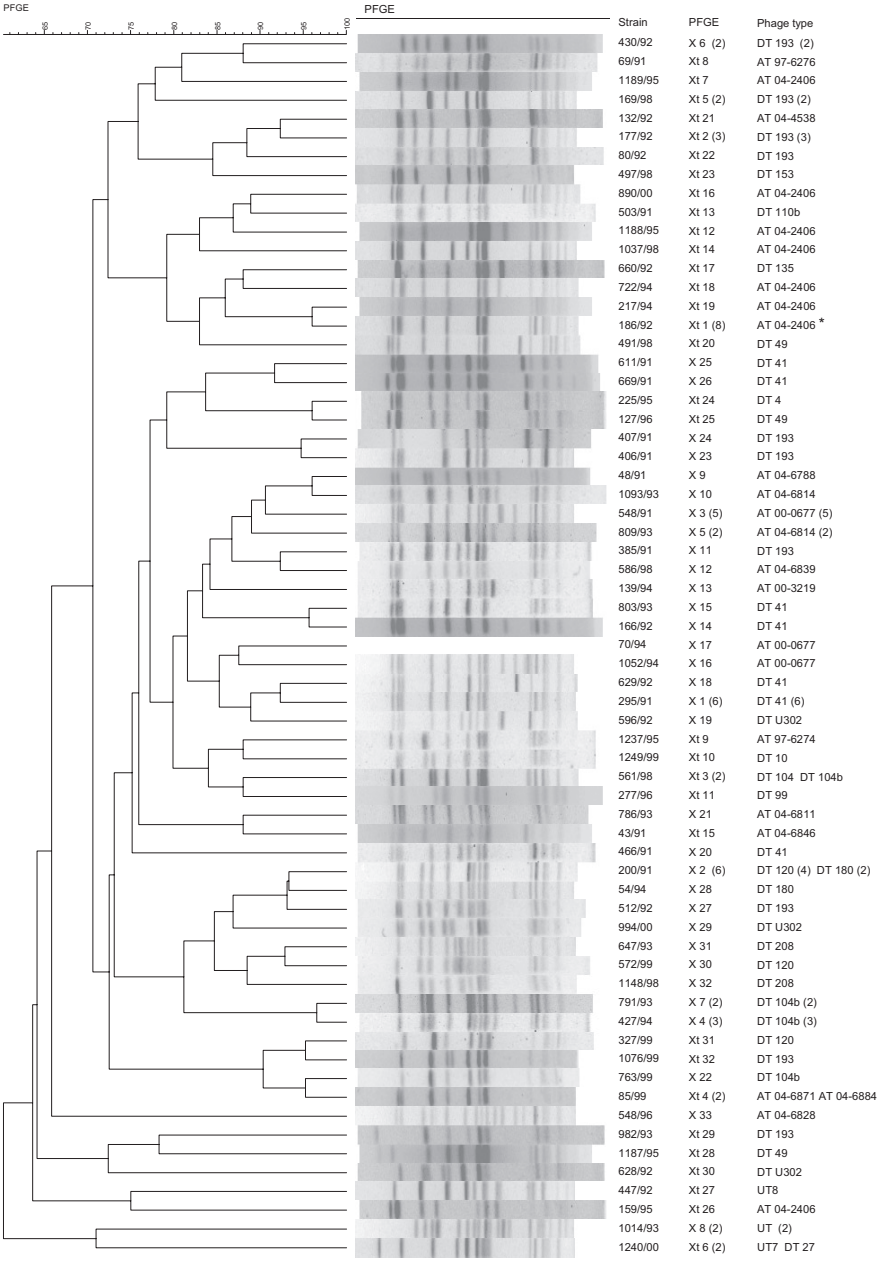

Fig. 1: dendrogram generated by BioNumerics software showing the relationship between *Salmonella* 1,4,[5],12:i:- (X) and *Salmonella* Typhimurium (Xt) serovars obtained by pulsed-field gel electrophoresis (PFGE) after XbaI restriction. Number of strains displaying each PFGE or phage type is between parenthesis. Assigned as atipical (AT) 04-2406 (4); definitive type (DT) 49 (3); DT 135 (1).

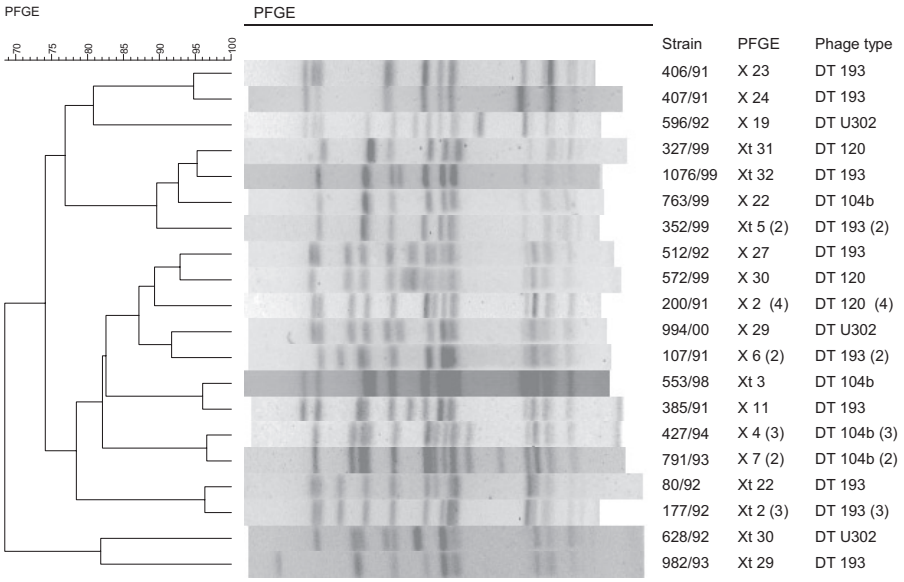

Fig. 2: dendrogram generated by BioNumerics software showing the relationship among strains belonging to the four phage types found in common with *Salmonella* 1,4,[5],12:i:- (X) and *Salmonella* Typhimurium (Xt) serovars, obtained by pulsed-field gel electrophoresis (PFGE) after XbaI restriction. Number of strains displaying each PFGE or phage type is between parenthesis. Dice (opt: 1.50%) (tol: 1.5% - 1.5%) (H > 0% S > 0%) [0% - 100%]. DT: definitive type.

as described by Birnboim and Doly (1979). The approximate molecular masses of the plasmids are determined by comparing plasmids of known molecular mass.

Plasmid analysis detected 14 different profiles with 1-5 plasmids among the *Salmonella* 1,4,[5],12:i:- strains and the great majority of those (94.3%) carried a 60-MDa plasmid alone (62.3%) or in combination with other plasmids of different sizes (32.1%). Among the *Salmonella* Typhimurium strains, there were 10 different plasmid profiles with zero-4 plasmids and 38 (84.4%) strains carried the 60-MDa plasmid alone or in combination with others of different sizes. In addition, the 60-MDa plasmid extracted from two *Salmonella* 1,4,[5],12:i:- and two *Salmonella* Typhimurium strains, which underwent plasmid restriction analysis with *Eco*RI and *Hin*dIII restriction enzymes, displayed the same restriction pattern. Asensi et al. (1995) reported that 41% of all *Salmonella* Typhimurium isolates analysed harboured a 61-MDa plasmid in a total of 33 different profiles. Because the 60-MDa plasmid is known as the *Salmonella* Typhimurium serovar-associated plasmid (Helmuth et al. 1985), which is dominant in *Salmonella* Typhimurium strains (Liebana et al. 2002), our results suggest a close relationship between these monophasic strains and *Salmonella* Typhimurium.

PFGE analysis was performed according to the CDC PulseNet Protocols (CDC 2004) using a CHEF-DR II system (BioRad Laboratories, Hercules, California). An XbaI digestion of the *S*. Braenderup H9812 control strain (Hunter et al. 2005) was used for DNA size markers. The PFGE gel was stained with ethidium bromide and the gel image was captured on the GEL DOC EQ system (Quantity One version 4.5 - Universal Hood II, Bio-Rad Laboratories, Milan, Italy) and analysed with BioNumerics software version 5.0 for Windows (Applied Maths, Belgium) to generate a dendrogram. The dice coefficient (1.5% tolerance) and the unweighted pair group method using arithmetic averages were used to perform the clustering analysis. Each PFGE profile that differed at least by one fragment was assigned a type number with X1-X^n for *Salmonella* 1,4,[5],12:i:- and Xt1- Xt^n for *Salmonella* Typhimurium.

PFGE analysis generated 9-18 fragments, with sizes between 33.3-1,135 kb per strain. There were 33 different profiles (X1 - X33) detected among 53 *Salmonella* 1,4,[5],12:i:- and 32 (Xt1 - Xt32) among 45 *Salmonella* Typhimurium strains with X1, X2 (11.3%) and Xt1 (18%) as the most prevalent profiles, respectively. A dendrogram combining the two serovar genetic profiles resulted in 65 different PFGE profiles and most of these (90%) showed similarity above 70% (Fig. 1). The distribution of PFGE profiles in relation to the phage types is shown in Table. Fig. 2 shows the relationship among the strains belonging to the four phage types found in common with *Salmonella* Typhimurium and *Salmonella* 1,4,[5],12:i:-.

It has been demonstrated that PFGE analysis can separate different *Salmonella* serotypes into distinct clusters (Liebana et al. 2001, Peters et al. 2003), also known as an inter-serotype discrimination. For strains belonging to one particular serotype, PFGE discriminates the ones associated with foodborne outbreaks from the ones isolated from sporadic cases of intestinal or extraintestinal infections denoting intra-serotype discrimination (Bender et al. 2001). The results obtained in this study showed a genetic diversity among all studied strains because they were isolated in a 10-year period and they were not associated with foodborne outbreaks. Besides, *Salmonella* Typhimurium and *Salmonella* 1,4,5,12:i:- strains were distributed in high similarity PFGE clusters, suggesting a close relationship at the serotype level.

Other studies describing the genetic similarity among strains from these two serotypes have been recently reported in Thailand (Pornruangwong et al. 2008), showing a close clonal relationship (≥ 85% similarity) by PFGE between the studied isolates and in the USA (Zamperini et al. 2007), where the results led to the conclusion that monophasic *Salmonella* 4,5,12:i:- isolates are genotypically *Salmonella* Typhimurium and should be treated in the same way as this serotype, concerning to export or import restrictions of poultry with *Salmonella* Typhimurium.

In conclusion, the results obtained in this study suggest a close relationship between *Salmonella* 4,5,12:i:- and *Salmonella* Typhimurium, although further genetic characterisation should be done in order to better trace the origin of the monophasic strains.

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