Veterinaria Italiana, **41** (4), 265-279

Surveillance system and rapid tracing of primary sources in foodborne outbreaks by *Salmonella* spp. Part II: Molecular characterisation of some strains of *Salmonella enterica* serovars Enteritidis and Typhimurium

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Summary

Salmonella enterica serovars Enteritidis and Typhimurium are the serotypes most frequently isolated from human cases. Traditional surveillance systems, based on serological characterisation and epidemiology, are not able to identify these common strains that cause outbreaks in humans. Innovative techniques are therefore necessary to accurately characterise these serotypes and hence accelerate the identification of the primary sources. Within a larger study, the goal of which was to develop an active surveillance system for outbreaks of foodborne diseases, characterisation of 42 *Salmonella* strains was performed using molecular techniques (pulsed-field gel electrophoresis [PFGE] and random amplified polymorphic DNA [RAPD]), together with the Kirby-Bauer antibiotic assay. Results showed that both techniques were unable to satisfactorily characterise the Enteritidis serotype, while only PFGE identified the Typhimurium serotype.

Keywords

Antibiotic assays, Molecular typing, Pulsed-field gel electrophoresis, Random amplified polymorphic DNA, *Salmonella enterica* Enteritidis, *Salmonella enterica* Typhimurium, Salmonellosis.

Introduction

Salmonellosis is the most important foodborne disease, both in animals and in man (9), causing over 1 400 000 human cases a year in the USA (29). Food of animal origin contaminated by *Salmonella* is the main source of infection for man. Although all *Salmonella* serotypes are conventionally considered as human and animal pathogens, only a small number actually causes disease (56). Moreover, some specific clonal types of *Salmonella enterica* have become predominant in one or more animal species and are present worldwide (4, 10). Serotypes Enteritidis and Typhimurium are those most commonly isolated from man and have become a public health concern in both and developed countries (5, 14, 50).

In the past, characterisation of *Salmonella* strains within traditional surveillance systems was performed mainly by means of sero- and phagotyping; both techniques, and especially phagotyping, the ability of which to discriminate between strains is very low (6), have been generally unsuccessful in detecting widespread serotypes, such as Enteritidis and Typhimurium, in foodborne disease outbreaks (2, 5, 19, 25). Hence, there is a need for laboratory techniques that can more accurately characterise these strains (34).

Epidemiological typing systems have been in use for the past few years and are routinely used during

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outbreak investigations to identify or confirm transmission patterns of one or more epidemiological clones. These systems are also able to detect 'epidemiological markers' which are distinctive traits of isolates that belong to the same clone and are derived from the same parental cell (43). Molecular typing techniques, based on the analysis of proteins and/or nucleic acids, are being increasingly and successfully employed during epidemiological investigations in foodborne outbreaks and they appear to be particularly useful in outbreaks where common serotypes are involved (46). Moreover, when compared with traditional methods, these techniques are quicker, more specific, are able to type almost all strains and can be applied to large numbers of microbial species (24).

Several authors support the notion of using a range of techniques for the accurate identification of different strains; they showed that combining several tests actually increases their ability to discriminate between strains, thereby contributing to the epidemiological understanding of outbreaks (7, 17, 22, 55).

Since not all the techniques are equally effective, it is of the utmost importance to select the right method to obtain acceptable results (48). For instance, results provided by pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) techniques that analyse the entire microbial genome (used in this study) are not subject to the variability that usually affects laboratory techniques based on the amplification of limited genome regions (13, 30). An additional advantage of PFGE and RAPD is their effective ability to distinguish strains; moreover they are able to rapidly identify the strains involved and to detect possible correlations among the pathogens isolated from human patients, suspected foods and animal reservoirs (2).

PFGE is most useful as a confirmatory method, due to its repeatability, reproducibility and ability to discriminate between strains; it is particularly useful in cases where the digestion patterns of strains appear to differ greatly (16), thanks to the availability of accurate guidelines for the interpretation of the resulting restriction patterns (21, 23, 43).

RAPD, which amplifies DNA with no prior knowledge of the nucleotide sequence of the target DNA, is employed mainly due to its very rapid execution (11, 30).

The reproducibility of the RAPD is notoriously affected by a number of problems, but it is commonly held that the selection of an appropriate set of primers could make RAPD an alternative method for genetic typing of serotype Enteritidis offering rapidity and reproducibility (6). However, standard guidelines for pattern interpretation are not yet available and the principles usually adopted for the interpretation of other typing systems, such as PFGE, are not easily transferable to RAPD (48).

The aim of the present study was to assess the efficacy of PFGE and RAPD, associated with the Kirby-Bauer antibiotic assay, in order to characterise *Salmonella enterica* serovars Enteritidis and Typhimurium isolated in the Abruzzo region of Italy between April 2000 and October 2002. The antibiotic assay was included to monitor and assess the phenotype of bacterial populations and its possible correlation with the genetic traits of the isolated strains.

Materials and methods

Bacterial strains

A total of 42 bacterial strains were analysed. In the first part of the study, these strains were identified as belonging to the Enteritidis (total: 27) and Typhimurium serovars (total: 15) using traditional techniques (microbiology and serology) (41). Of

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the Enteritidis serotypes, 25 strains were of human origin, while the remaining two came from foods (mayonnaise, sausages, ketchup and crepes). All Typhimurium serotypes were of human origin.

Pulsed-field gel electrophoresis

Restriction enzymes *Xba* I, *Bln* I, *Spe* I, with *Salmonella* strain Braenderup 267 as the marker of molecular weights (32, 39, 52, 54), were used for the analysis of all 42 *Salmonella* isolates. Field strains isolated from the preliminary outbreaks were adopted as reference strains for profile analysis of both serotypes.

All strains were cultured in non-selective agar medium for 14-18 h at 37°C ± 1°C. A suspension was later obtained with 2 ml of cell suspension buffer (100 mM Tris HCl pH 8.0 -100 mM EDTA pH 8.0), adjusted to reach an optical density of 0.850 (wavelength 610 nm). The blocks were prepared adding the bacterial suspension with proteinase K (0.5 mg/ml) an equal volume of agarose (Seakem Gold Agarose - BioWhittaker) to which dodecil-sulphate sodium (SDS) had been added (agarose 1%: SDS 1%). Each block was lysed in cell lysis buffer (50 mM Tris HCl pH 8.0 - 50 mM EDTA pH 8.0 - 1% sarcosyl), then added to proteinase K (0.1 mg/ml) at 54°C for 2 h and agitated constantly with an orbital agitator (160 rpm) in a double boiler. Blocks were later washed twice with sterile distilled water, four times with Tris-EDTA (10mM Tris HCl pH 8.0 - 1 mM EDTA pH 8.0) and then stored in Tris-EDTA at 4°C until use. Digestion was performed by placing a 2 mm section of each block in a solution with 50 units (comb with 10 wells: 0.25 units/µl) or 30 units (comb with 15 wells: 0.3 units/µl) of *Xba* I, *Bln* I and *Spe* I.

Samples were incubated at 37°C ± 0.1°C for 2 h (*Xba* I enzyme) or 4 h (*Bln* I and *Spe* I enzymes). Digested sections were included in agarose (Seakem Gold Agarose - BioWhittaker), 1% in TBE (6.5 mM Tris HCl - 2.25 mM borate - 0.125 mM EDTA) until the gel solidified. Electrophoresis of the final product was performed in TBE in a Cheff mapper machine (BioRad); the temperature was maintained at 14°C ± 0.1°C for 18 h (electrophoresis parameters are reported in Table I). The gel was stained with ethidium-bromide (1 µg/ml), visualised in the transilluminator and photographed with a Polaroid camera.

Table I
Parameters of the pulsed-field gel electrophoresis

Parameters	*Xba* I	*Bln* I	*Spe* I
Low molecular weight	30 kb	30 kb	20 kb
High molecular weight	700 kb	700 kb	400 kb
Initial swith time	2.16 sec	2.16 sec	2.98 sec
Final swith time	63.8 sec	63.8 sec	35.38 sec

Images were retrieved in 'tif' format through a UVP photodocumentation system (Alpha Innotech). Images were analysed with BioNumerics 4.0 (Applied Maths, Kortrijk Belgium) which automatically calculates the strain similarity matrix according to the Dice coefficient, with the optimisation parameter set at 1.5%, with a tolerance of 1.5% (16, 22, 24). The dendrogram was calculated according to these values, using the unweighted pair group method using arithmetic averages (UPGMA). Starting from the single similarity matrixes for each enzyme, the software created a single combined matrix, obtained from the average of the values of the single tests; each matrix was considered of equal importance, regardless of restriction enzyme.

The criteria developed by Tenover to interpret DNA restriction patterns were used to evaluate

possible epidemiological correlations and to define the strains investigated (47).

The ability to discriminate between strains was assessed using Simpsons diversity index (18, 24, 37):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} nj\,(nj - 1)$$

Where

N = total number of strains tested

s = total number of different types identified

n_j = number of j type identical strains.

Random amplified polymorphic DNA

All strains were also characterised using the RAPD, in accordance with the guidelines of the European Reference Laboratory for Zoonoses in Berlin (31).

All strains were cultured overnight in Circle Grow broth (BIO 101), and then centrifuged (14 000 rpm for 6 min at 4°C). The pellet was reconstituted in Tris-EDTA (50 mM Tris HCl pH 8.0 - 1 mM EDTA pH 8.0) and then brought to 100°C for 10 min to cause cell lysis.

1254 (CCG CAG CCA A) and 1283 (GCC ATC CCC A) were used as primers; DNA molecular weight marker X (Roche) was used as the molecular weights marker. Reactions developed in a 20 µl solution containing GeneAmp PCR buffer (11.7 mM Tris HCl pH 8.3 - 58.4 mM KCl), 3.5 mM $MgCl_2$, 200 µM dNTP (Roche), 0.12 µg/µl bovine seroalbumin (BSA), 2.4 pmol primer (Eurobio), 1.6 U AmpliTaq DNA polymerase (Roche). To achieve 100-175 ng of bacterial DNA in the final solution, 5 µl of sample were added.

Amplification was performed in a thermal cycler (Perkin-Elmer) as follows: one cycle consisting of 94°C for 1 min, 6 cycles consisting of 94°C for 30 s, 20°C for 40 s, and 72°C for 1 min, 40 cycles consisting of 94°C for 30 s, 30°C for 40s, and 72°C for 2 min, one cycle consisting of 72°C for 4 min and storing at 4°C.

Amplification products were analysed in agarose (Agarose MP - Roche) 2% including ethidium bromide (0.5 µl/ml). Electrophoresis patterns were compared to evaluate identical bands corresponding to fragments of the same mobility. Strains were classified according to the different number of bands. Excessively luminous bands were considered as double fragments (44).

When two or more of the major bands differed, they were classified as belonging to different types.

When patterns of only one major band of a strain differed, or when one or two minor bands differed, these were considered subtypes of a single strain (8, 30). Patterns corresponding to each type were identified with letters and subtypes with a numerical suffix.

Kirby-Bauer antibiotic assay

Sensitivity to antimicrobial drugs was tested by diffusion in Mueller Hinton agar according to Kirby-Bauer (20), using the following antimicrobial set:

- nalidixic acid: 30 µg
- sulfisoxazole: 25 µg
- ampicillin: 10 µg
- tetracycline: 30 µg
- cefazolin: 30 µg
- cephalothin: 30 µg
- ciprofloxacin: 5 µg
- enrofloxacin: 5 µg
- chloramphenicol: 30 µg
- colistin: 10 µg
- gentamicin: 10 µg
- cefotaxime: 30 µg
- kanamycin: 30 µg
- trimethoprim + sulphamethoxazole: 1.25/23.75 µg
- streptomycin: 10 µg
- amoxicillin + clavulanic acid: 20/10 µg

Results were read according to the criteria set by the National Committee for Clinical Laboratory Standards (15, 35, 36). Strains were classified as follows:

a) sensitive (inhibited by all the above antimicrobials)

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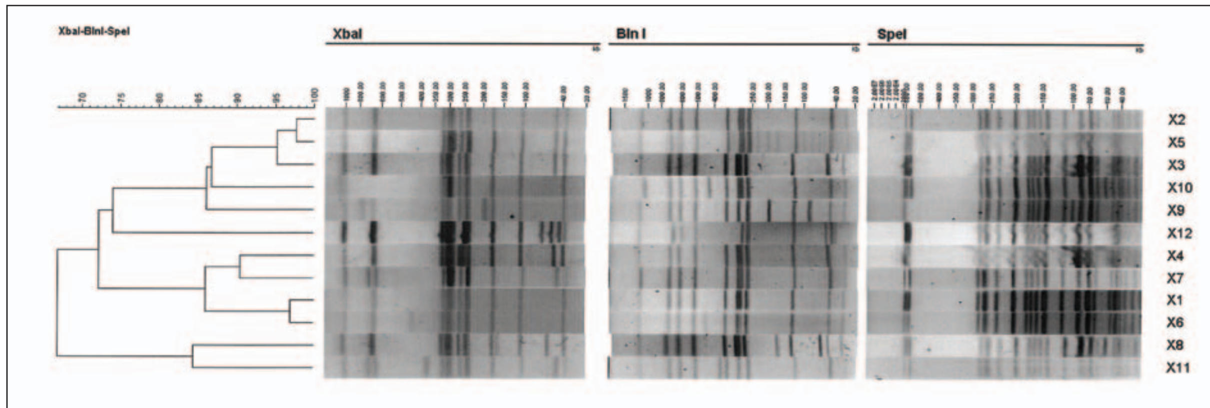

Figure 1
PFGE patterns of *Salmonella enterica* serotype Enteritidis strains (*Xba* I, *Bln* I and *Spe* I enzymes)

b) resistant (not inhibited by up to three antimicrobials)

c) multi-resistant (inhibition failure by at least four antimicrobials).

Results

Nine electrotypes from the original 27 strains of serotype Enteritidis were identified by the *Xba* I – PFGE: type A (reference strain) and 8 closely related subtypes (A1-A8), with a maximum of three different bands (Fig. 1; Table II). The ability to discriminate (D) was 0.82.

Bln I – PFGE identified 7 electrotypes: type A (reference strain), 5 closely related subtypes (A1-A5), and 1 subtype (A6) potentially related to the reference strain, with up to 5 different bands (Fig. 1; Table II). The ability to discriminate (D) was 0.56.

Spe I – PFGE also identified 7 electrotypes: type A (reference strain) and 6 closely related subtypes (A1-A6), with a maximum of two different bands (Fig. 1; Table II). The ability to discriminate (D) was 0.70.

The identified patterns, and subsequently the ability to discriminate, were increased when all the results of the PFGE enzymes were combined, with D increasing to 0.85 (*Xba* I + *Bln* I, 10 patterns) and to 0.91 with all the three enzymes (12 patterns).

All the electrophoretic patterns were closely correlated with the *Xba* I and *Spe* I enzymes, with minimal differences among patterns (no more than three different bands), and closely or potentially correlated with the *Bln* I enzyme (up to five different bands), although epidemiological data did not support the correlation.

RAPD, performed on strains of serotype Enteritidis, produced 22 identical patterns when using the 1254 primer (Figs 2, 3 and 4; Table II) and 23 identical patterns when using the 1283 primer (Figs 5 and 6; Table II). No readable patterns were

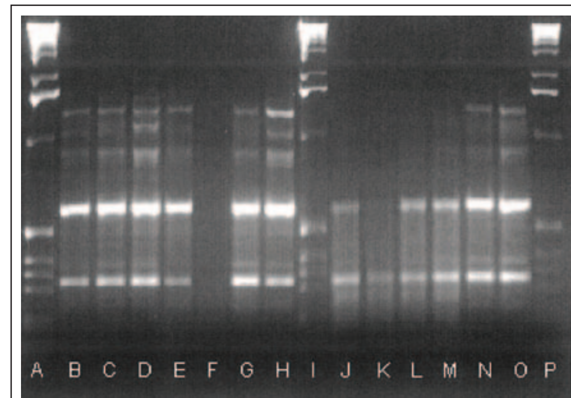

Figure 2
RAPD (primer 1254) of 13 strains of *Salmonella enterica* serotype Enteritidis
Columns A, I and P: standard; B: 13, C: 14; D: 15; E: 8; F: 9; G: 10; H: 11; J: 12; K: 21; L: 22; M: 23; N: 24; O: 25

Table II
Summary of epidemiological investigations, results of molecular analysis and antibiotic assays on 27 strains of *Salmonella enterica* serotype Enteritidis, isolated from human cases or from food in some districts of the Abruzzo region of Italy (2000-2002)

Epidemiological data			PFGE Enzymes				RAPD primers		
Strain ID	Isolation data	Origin of strain	*Xba* I Pattern	*Bln* I Pattern	*Spe* I Pattern	PFGE pattern	1254 Pattern	1283 Pattern	Sensitivity to antimicrobial drugs Pattern
1	AV-2000	Human	A (Rif)[a]	A (Rif)[a]	A (Rif)[a]	X1	A (Rif)[a]	A (Rif)[a]	Sensitive
2	AV-2000	Human	A	A	A	X1	A	A	Sensitive
3	AV-2000	Human	A	A	A	X1	A	A	Sensitive
4	AV-2000	Human	A2	A1	A	X6	A	A	Sensitive
5	AV-2000	Human	A5	A5	A6	X9	A	A	Sensitive
6	AV-2000	Human	A6	A1	A1	X10	A	A	Sensitive
7	AV-2000	Food	A	A	A	X1	A	A	Sensitive
8	LN-2001	Human[b]	A1	A	A	X2	A	A	Sensitive
9	LN-2001	Human[b]	A3	A	A	X7	[c]	[c]	Sensitive
10	LN-2001	Human[b]	A1	A	A	X2	A	[c]	Sensitive
11	VA-2001	Human	A3	A	A	X7	A	A	Sensitive
12	VA-2001	Human[b]	A3	A	A	X7	[c]	A	Sensitive
13	TE-2001	Food	A3	A	A	X7	A	A	Sensitive
14	TE-2001	Human	A3	A	A	X7	A	A	Sensitive
15	TE-2001	Human	A7	A4	A2	X11	A	A	Sensitive
16	TE-2002	Human[b]	A1	A	A	X2	A	A	Resistant (Nx-A-S-Su-T-SXT-Amc)[d]
17	TE-2002	Human	A1	A	A3	X3	[c]	A	Sensitive
18	TE-2002	Human	A1	A	A	X2	A	A	Sensitive
19	TE-2002	Human	A1	A2	A3	X5	A	A	Sensitive
20	TE-2002	Human[b]	A1	A	A5	X4	A	[c]	Resistant(S-Su)[d]
21	GI-2002	Human	A1	A	A3	X3	[c]	A	Resistant (K-S)[d]
22	GI-2002	Human	A4	A3	A1	X8	[c]	[c]	Resistant (K-S)[d]
23	GI-2002	Human	A1	A	A3	X3	A	A	Resistant (K-S)[d]
24	GI-2002	Human	A4	A3	A1	X8	A	A	Resistant (K-S)[d]
25	GI-2002	Human	A1	A	A3	X3	A	A	Resistant (K-S)[d]
26	CH-2002	Human	A8	A6	A4	X12	A	A	Sensitive
27	CH-2002	Human	A8	A6	A4	X12	A	A	Sensitive

a) reference strain
b) strain from sporadic cases
c) pattern could not be read
d) A ampicillin
Amc amoxicillin + clavulanic acid
K kanamycin
NX nalidixic acid
S streptomycin
Su sulfisoxazole
SXT trimethoprim + sulfamethoxazole
T tetracyline

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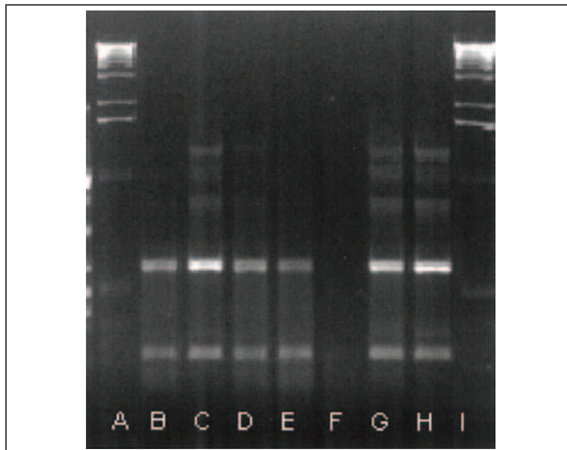

Figure 3
RAPD (primer 1254) of 7 strains of *Salmonella enterica* serotype Enteritidis
Columns A and I: standard; B: 26; C: 27; D: 16; E: 20; F: 17; G: 18; H: 19

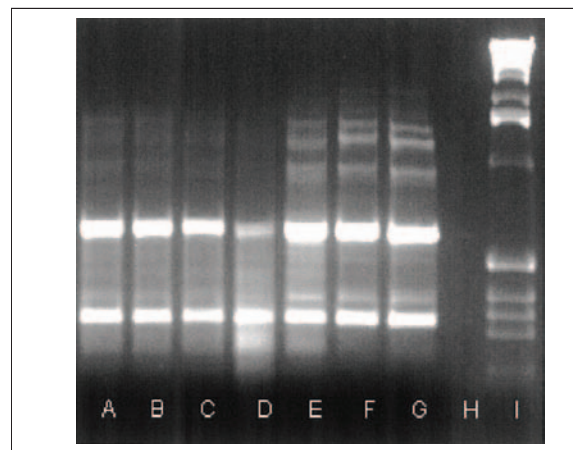

Figure 4
RAPD (primer 1254) of 7 strains of *Salmonella enterica* serotype Enteritidis
Column I: standard; A: 1; B: 2; C: 3; D: 4; E: 5; F:7; G: 6

obtained for the remaining strains (five of those tested with the 1254 primer, and 4 of those tested with the 1283 primer).

The molecular identity of all the readable patterns, also involving strains that were not epidemiologically correlated, provided proof of the low ability of this technique to discriminate, both when used alone or associated with PFGE.

Antibiotic assay results revealed four different patterns with 7 strains (25.9%), showing resistance to one or more of the 16 antimicrobial drugs used in the test (Table II). This test was characterised by a low ability to discriminate (D = 0.43), but when the test was associated with the three enzymes-PFGE, the number of patterns, and therefore the ability to discriminate (D) of the PFGE itself improved, increasing from 0.91 to 0.93.

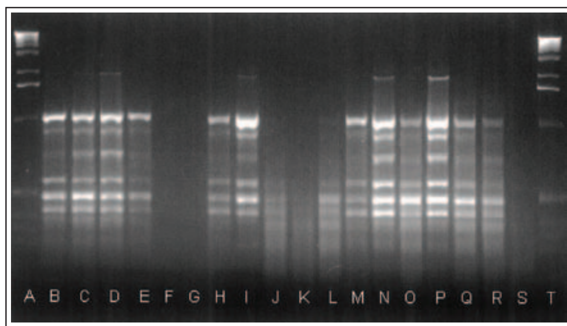

Figure 5
RAPD (primer 1283) of 18 strains of *Salmonella enterica* serotype Enteritidis
Columns A and T: standard; B: 13; C: 14; D: 15; E: 8; F: 9; G: 10; H: 11; I: 12; J: 21; K: 22; L: 23; M: 24; N: 25; O: 26; P: 27; Q: 16; R: 20; S: 17

Nine different electrophoretic patterns from the original 15 strains of serotype Typhimurium were generated by the *Xba* I – PFGE (Fig. 7; Table III): type A with two closely related patterns (A1 and A2), 4 potentially related profiles (A3-A6) and 2 unrelated patterns (B and C), with a maximum of seven different bands; the ability to discriminate (D) was 0.90.

Bln I identified 11 different patterns (Fig. 7; Table III); 2 related patterns characterised by only three different bands (A and A1). All the other patterns (B-J) did not correlate with the A reference strain, with up to 13 different bands. The ability to discriminate (D) was 0.95.

Spe I identified 13 different patterns (Fig. 7; Table III): type A (reference strain), 3 subtypes closely related to A (A1-A3); the 9 remaining subtypes were only

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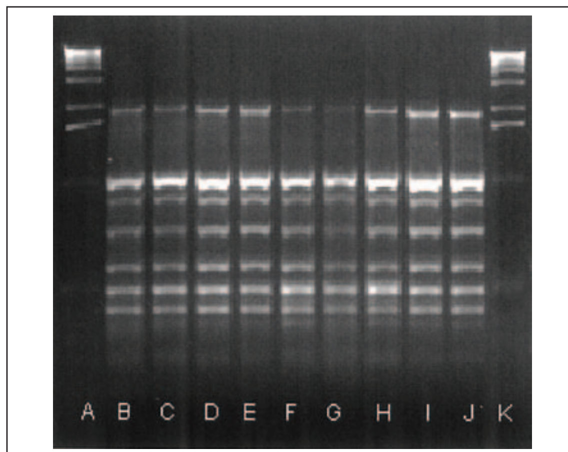

Figure 6
RAPD (primer 1283) of 9 strains of *Salmonella enterica* serotype Enteritidis
Columns A and K: standard; B: 18; C: 19; D: 1; E: 2; F: 3; G: 4; H: 5; I:7; J: 6

potentially related to A with up to six different bands from the reference strain (A4-A12). The ability to discriminate (D) was 0.98.

The ability to discriminate was increased when all results of the PFGE enzymes were combined; in particular, D increased to 0.95 (*Xba* I + *Bln* I, 11 patterns) and to 0.99 with all the three enzymes (14 patterns).

RAPD produced 2 identical patterns, when using the 1254 primer (Fig. 8; Table III). The ability to discriminate was low (D = 0.13). All strains were classified as belonging to pattern A, with one exception that presented two different bands (pattern B).

The 1283 primer did not discriminate for this serotype, giving a single pattern for all the strains tested (Fig 8; Table III).

The antibiotic assay detected 7 different patterns with 9 resistant strains (60%) (Table III).

In regard to serotype Typhimurium, the technique was characterised by a moderate ability to discriminate (D = 0.80). When PFGE and the antibiotic assay were associated, the ability to discriminate increased from 0.99 to 1.

Discussion

Conventional surveillance systems are able to detect correlations among outbreaks caused by common serotypes of *Salmonella*, such as Enteritidis and Typhimurium, only when incidence increases abruptly. These systems have low sensitivity when the incidence of foodborne disease cases is low over periods exceeding six months (5). The present study showed how a surveillance system supported by distinguishing typing techniques can provide satisfactory results, especially where the serotype

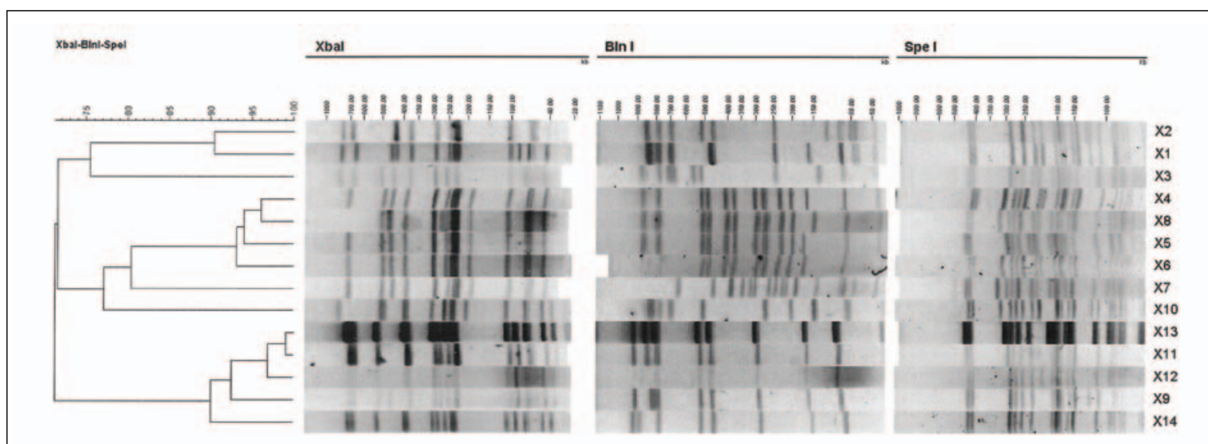

Figure 7
PFGE patterns of *Salmonella enterica* serotype Typhimurium strains (*Xba* I, *Bln* I and *Spe* I enzymes)

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Typhimurium is concerned.

The characterisation of *Salmonella* using one or several molecular techniques (such as PFGE and RAPD), is useful when the discriminatory index (D) exceeds 0.95 (26, 28, 43).

In regard to the Enteritidis strains, our study showed that the ability to discriminate of the PFGE does not reach the utility threshold (D = 0.91), even when the three enzymes (*Xba* I, *Bln* I and *Spe* I) are used together. Strains tested were not epidemiologically correlated (different geographic and temporal settings), but nevertheless a close molecular relationship between them was detected, due to the greater genetic homogeneity of the Enteritidis strain, when compared with other serotypes.

The results obtained here do not support the notion, however, of PFGE being the most effective genetic discrimination technique for the characterisation of the Enteritidis strains (12, 22, 23, 50, 51).

Enteritidis RAPD results (Figs 2, 3, 4, 5 and 6) support findings in the literature, with special reference to the unsatisfactory performance of this technique, especially due to its low ability to discriminate (12), which is greatly affected by the choice of primers (27). Other disadvantages are its low reproducibility and repeatability (38, 43, 53), affected by a number of parameters (pH, buffer ionic strength, reaction temperature, primer-DNA interaction, DNA secondary structure, efficiency of the thermal cycler heating block, Taq – polymerase quality, inadequate DNA preparation, RNA contamination) (30, 48, 53). In regard to the reproducibility of the RAPD, our study provided evidence that conflicted with reports from other authors (27, 42).

The RAPD did not provide, in a single test, the patterns of all the strains examined. Therefore, the comparison had to be made among patterns amplified and analysed in the course of different tests; this procedure was advised against by some authors, who indicated that the better results are obtained when all the bacterial strains to be analysed are subjected to a single amplification reaction and run in the same electrophoretic gel (48, 53). Furthermore, this technique generated patterns that were not easy to read, with subsequent difficulties in the interpretation stage.

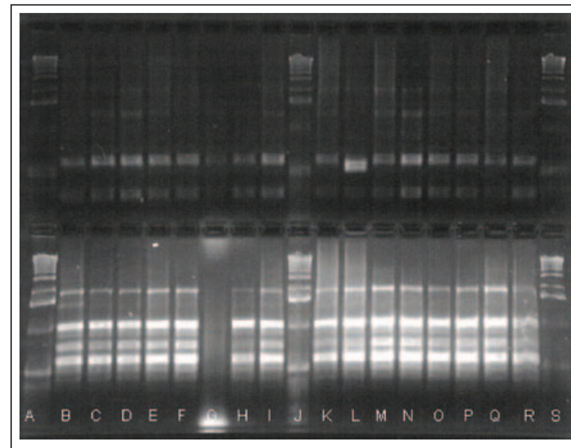

Figure 8
RAPD of strains of *Salmonella enterica* serotype Typhimurium
Columns A-J and S: standard; B: 14; C: 2; D: 1; E: 3; F: 4; G: 15; H: strain not belonging to the investigated cases; I: 9; K: 5; L: 6; M: 7; N: 8; O: 10; P: 12; Q: 11; R: 13

Therefore, RAPD was considered not sufficiently reliable to trace epidemiological correlations among strains belonging to the Enteritidis serotype.

Antibiotic assay results (Table I) were in disagreement with other studies (1, 49), especially in regard to the high resistance of the Enteritidis strains to streptomycin and kanamycin. Moreover, the limited value of this technique for epidemiological purposes was demonstrated, whether it was performed alone or associated with others.

All three techniques (PFGE, RAPD and antibiotic assay) adopted for the characterisation of the serotype Enteritidis were unsatisfactory for epidemiological studies, because they were unable to adequately differentiate strains which are not

Table III
Summary of epidemiological investigations findings, molecular analysis and antibiotic assay results on 15 strains of *Salmonella enterica* serotype Typhimurium, isolated from human cases in some districts of the Abruzzo region (2000-2002)

Epidemiological data			PFGE Enzymes				RAPD primers		
Strain ID	Isolation data	Origin of strain	*Xba* I Pattern	*Bln* I Pattern	*Spe* I Pattern	PFGE pattern	1254 Pattern	1283 Pattern	Sensitivity to antimicrobial drugs Pattern
1	LN-2001	Human	A (Rif)[a]	A (Rif)[a]	A (Rif)[a]	X1	A (Rif)[a]	A (Rif)[a]	Resistant (A-C-S-Su-T-Amc)[b]
2	LN-2001	Human	A6	B	A2	X9	A	A	Sensitive
3	LN-2001	Human	C	D	A8	X13	A	A	Sensitive
4	LN-2001	Human	C	D	A4	X12	A	A	Sensitive
5	LN-2001	Human	C	F	A6	X14	A	A	Resistant (Su-T-SXT)[b]
6	LN-2002	Human	B	C	A5	X10	B	A	Sensitive
7	LN-2002	Human	A3	I	A12	X6	A	A	Resistant (A-S-Su-T)[b]
8	LN-2002	Human	A3	H	A7	X4	A	A	Resistant (A-S-Su-T)[b]
9	VA-2001	Human	A	A	A	X1	A	A	Resistant (A-S-Su-T -Col)[b]
10	TE-2002	Human	A4	G	A10	X7	A	A	Resistant (A-S-Su)[b]
11	TE-2002	Human	A5	J	A11	X8	A	A	Resistant (A-S-Su-T)[b]
12	TE-2002	Human	A3	H	A9	X5	A	A	Resistant (A-S-Su-T)[b]
13	TE-2002	Human	A2	E	A9	X3	A	A	Sensitive
14	CH-2001	Human	A1	A1	A1	X2	A	A	Resistant (A-Su-T-SXT)[b]
15	CH-2001	Human	C	D	A1	X11	A	[c]	Sensitive

a) reference strain
b) A ampicillin
Amc amoxicillin + clavulanic acid
C chloramphenicol
Col colistin
S streptomycin
Su sulfisoxazole
SXT trimethoprim + sulfamethoxazole
T tetracycline
c) pattern could not be read

epidemiologically correlated.
As far as the serotype Typhimurium was concerned, PFGE showed a high power of discrimination, especially using the *Bln* I enzyme (D > 0.95), and was in close agrement with epidemiological data; however, this observation does not concur with other reports (19, 30).
Xba I and *Spe* I PFGE also detected molecular

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identities in strains that were not correlated epidemiologically, even if these enzymes provided PFGE with a very good ability to discriminate (D = 0.90 and 0.98, respectively), so that the correlation between molecular typing results and epidemiological data is low.

The lack of firm epidemiological evidence as far as strain correlation is concerned, makes it impossible to support other findings that describe PFGE as one of the most effective techniques for the detection of common genetic traits among different isolates (5, 40).

The ability to discriminate of the RAPD for the Typhimurium serotype was negligible (D = 0.00 and 0.13 for the primers used) (Fig. 8); these results agree with some findings (30) and disagree with others (42).

With reference to phenotype characterisation using the antibiotic assay (Table II), Typhimurium serotype results confirmed the high percentage of multiresistant human strains (3, 4, 5, 30) and demonstrated the lack of value of this technique for epidemiological purposes.

In brief, of the three techniques used to characterise the Typhimurium serotype, only the *Bln* I, PFGE was able to differentiate the strains examined in this study, supporting the epidemiological evidence of the sporadic nature of these outbreaks. This result is confirmed by the well-known wide genetic variability of serotype Typhimurium (33). Consequently, the identification of closely related strains in different geographical areas could suggest both a worldwide spread of the Typhimurium serotype or the occurrence of closely related emerging clonal groups (45).

The other techniques were scarcely useful as epidemiological tools, since RAPD detected an almost perfect homogeneity among strains and the antibiotic assay scarcely differentiated among different antimicrobial resistance patterns.

In conclusion, PFGE, RAPD and antibiotic assay were of little value for the epidemiological characterisation of *Salmonella enterica* serotype Enteritidis. As far as the Typhimurium serotype was concerned, PFGE was the only technique that was able to characterise the isolates epidemiologically.

References

1. Adesiyun A., Carson A., McAdoo K. & Bailey C. 2000. Molecular analysis of *Salmonella enteritidis* isolates from the Caribbean by pulsed-field gel electrophoresis. *Pan Am J Publ Health*, **8** (5), 342-347.
2. Ahmed R., Soule G., Demczuk W.H., Clark C., Khakhria R., Ratnam S., Marshall S., Ng L-K., Woodward D.L., Johnson W.M. & Rodgers F.G. 2000. Epidemiologic typing of *Salmonella enterica* serotype enteritidis in a Canada-wide outbreak of gastroenteritis due to contamined cheese. *J Clin Microbiol*, **38** (6), 2403-2406.
3. Baggesen D.L. & Aarestrup F.M. 1998. Characterization of recently emerged multiple antibiotic-resistant *Salmonella enterica* serovar *typhimurium* DT104 and other multiresistant phage types from Danish pig herds. *Vet Rec*, **143**, 95-97.
4. Baggesen D.L., Sandvang D. & Aarestrup F.M. 2000. Characterization of *Salmonella enterica* serovar Typhimurium DT104 isolated from Denmark and comparison with isolates from Europe and the United States. *J Clin Microbiol*, **38** (4), 1581-1586.
5. Bender J.B., Hedberg C.W., Bouxrud D.J., Bessser J.M., Wicklund J.H., Smith K.E. & Osterholm M.T. 2001. Use of molecular subtyping in surveillance for *Salmonella enterica* serotype Typhimurium. *N Engl J Med*, **344** (3), 189-195.
6. Betancor L., Schelotto F., Martinez A., Pereira M.,

Algorta G., Rodriguez M.A., Vignoli R. & Chabalgoity J.A. 2004. Random Amplified Polimorphic DNA and phenotyping analysis of *Salmonella enterica* serovar Enteritidis isolates collected from humans and poultry in Uruguay from 1995 to 2002. *J Clin Microbiol*, **42** (3), 1155-1162.
7. Biendo M., Thomas D., Dechepy O., Laurans G. & Eb F. 2003. Molecular epidemiology of ampiciclin-resistant clinical isolates of *Salmonella enterica* serovar Typhimurium. *Int J Med Microbiol*, **293**, 219-223.
8. Bingen E., Bonacorsi S., Rohrlich P., Duval M., Lhopital S., Brahimi N., Vilmer E. & Goering R.V. 1996. Molecular epidemiology provides evidence of genotypic heterogeneity of multidrug-resistant *Pseudomonas aeruginosa* serotype O: 12 outbreak isolates from a pediatric hospital. *J Clin Microbiol*, **34** (12), 3226-3229.
9. Brenner F. W., Villar R.G., Angulo F. J., Tauxe R. & Swaminathan B. 2000. *Salmonella* nomenclature. *J Clin Microbiol*, **38**, 2465-2467.
10. Centers for Disease Control (CDC) 2001. PHLIS Surveillance Data. *Salmonella*. Annual Summaries: *Salmonella*, 2001. CDC, Atlanta (cdc.gov/ncidod/dbmd/phlisdata/salmonella.htm)
11. Chansiripornchai N., Ramasoota P., Bangtrakulnonth A., Sasipreeyajan J. & Svenson S.B. 2000. Application of randomly amplified polymorphic DNA (RAPD) analysis for typing Avian *Salmonella enterica* subsp. Enterica. FEMS *Immunol Med Microbiol*, **29**, 221-225.
12. Clark C.G., Kruk T.M.A.C., Bryden L., Hirvi Y., Ahmed R. & Rodgers F.G. 2003. Subtyping of *Salmonella enterica* serotype enteritidis strains by manual and automated PstI-SphI ribotyping. *J Clin Microbiol*, **41** (1), 27-33.
13. Clewley J.P. 1998. A user's guide to producing and interpreting tree diagrams in taxonomy and phylogenetics. Part 3. Using restriction fragment length polymorphism patterns of bacterial genomes to draw trees. *Commun Dis Publ Health*, **1**, 208-210.
14. Fernandes S. A., Ghilardi A. C. R., Tavechio A.T., Machado A. M. O. & Pignatari A.C.C. 2003. Phenotypic and molecular characterization of *Salmonella enteritidis* strains isolated in Sao Paulo, Brazil. Rev *Inst Med Trop San Paulo*, **45** (2), 59-63.
15. Gorman R., Adley C.C. 2004. Characterization of *Salmonella enterica* serotype typhimurium isolates from human, food and animal sources in the Republic of Ireland. *J Clin Microbiol*, **42** (5), 2314-2316.
16. Grothues D. & Tümmler B. 1991. New approaches in genome analysis by pulsed-field gel electrophoresis: application to the analysis of *Pseudomonas* species. *Molec Microbiol*, **5** (11), 2763-2776.
17. Helmuth R. & Schroeter A. 1994. Molecular typing methods for *S. enteritidis*. *Int J Food Microbiol*, **21**, 69-77.
18. Hunter P.R. & Gaston M.A. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol*, **26**, 2465-2466.
19. Jeoffreys N.J., James G.S., Chiew R. & Gilbert G.L. 2001. Practical evaluation of molecular subtyping and phage typing in outbreaks of infection due *Salmonella enterica* serotype *Typhimurium*. *Pathol*, **33**, 66-72.
20. Kirby J., Bauer A. W., Sherris C. & Turck M..1966. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol*, **45**, 493-496.
21. Koort J.M.K., Lukinmaa S., Rantala M., Unkila E. & Siitonen A. 2002. Technical improvement to prevent DNA degradation of enteric pathogens in Pulsed-Field Gel Electrophoresis. *J Clin Microbiol*, **40** (9), 3497-3498.
22. Laconcha I., Lòpez-Molina N., Rementeria N., Audicana A., Perales I. & Garaizar J. 1998. Phage typing combined with Pulsed-Field Gel Electrophoresis and Random Amplified

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Polymorphic DNA increases discrimination in the epidemiological analysis of *Salmonella enteritidis* strains. *Int J Food Microbiol,* **40**, 27-34.

23. Laconcha I., Baggesen D. L., Rementeria A. & Garaizar J. 2000. Genotypic characterisation by PFGE of *Salmonella enterica* serotype Enteritidis phage types 1, 4, 6 and 8 isolated from animal and human sources in three European countries. *Vet Microbiol,* **75**, 155-165.
24. Landeras E., Gonzàles-Hevia M.A. & Mendoza M.C. 1998. Molecular epidemiology of *Salmonella* serotype Enteritidis. Relationships between food, water and pathogenic strains. *Int J Food Microbiol,* **43**, 81-90.
25. Liebana E., Garcia-Migura L., Breslin M.F., Davies R.H. & Woodward M.J. 2001. Diversity of strains of *Salmonella enterica* serotype enteritidis from English poultry farms assessed by multiple genetic fingerprinting. *J Clin Microbiol,* **39** (1), 154-161.
26. Liebana E., Guns D., Garcia-Migura L., Woodward M.J., Clifton-Hadley F.A. & Davies R.H. 2001. Molecular typing of *Salmonella* serotypes prevalent in animals in England: assessment of methodology. *J Clin Microbiol*, **39** (10), 3609-3616.
27. Lin A.W., Usera M.A., Barrett T.J. & Goldsby R.A. 1996. Application of Random Amplified Polymorphic DNA Analysis to differentiate strains of *Salmonella enteritidis*. *J Clin Microbiol,* **34** (4), 870-876.
28. Ling J.M., Koo I.C., Kam K.M.& Cheng A.F. 1998. Antimicrobial susceptibilities and molecular epidemiology of *Salmonella enterica* serotype Enteritidis strains isolated in Hong Kong from 1986 to 1996. *J Clin Microbiol*, **36** (6), 1693-1699.
29. Mead P. S., Slutsker L., Dietz V., McCaig L. F., Bresee J. S. & Shapiro C. 1999. Food-related illness and death in the United States. *Emerg Infect Dis,* **5**, 607-625.
30. Mhand R.A., Brahimi N., Moustaoui N., El Mdaghri N., Amorouch H., Grimont F., Bingen E. & Benbachir M. 1999. Characterization of extended-spectrum B-Lactamase-producing *Salmonella typhimurium* by phenotypic and genotypic typing methods. *J Clin Microbiol,* **37** (11), 3769-3773.
31. Miyamoto T., Tian H.Z., Okabe T., Trevanich S., Asoh K., Tomoda S., Honjoh K.-I. & Hatano S. 1998. Application of Random Amplified Polymorphic DNA Analysis for detection of *Salmonella* spp. in foods. *J Food Prot,* **61** (7), 785-791.
32. Murase T., Nakamura A., Matsushima A. & Yamai S. 1996. An epidemiological study of *Salmonella enteritidis* by Pulsed-Field Gel Electrophoresis (PFGE): Several PFGE patterns observed in isolates from a food Poisoning outbreak. *Microbiol Immunol,* **40**, 873-875.
33. Nastasi A. & Mammina C. 1995. Epidemiological evaluation by PCR ribotyping of sporadic and outbreak-associated strains of *Salmonella enterica* serotype Typhimurium. *Res Microbiol,* **146**, 99-106.
34. Nastasi A. & Mammina C. 1996. Epidemiology of *Salmonella enterica* serotype enteritidis infections in southern Italy during the years 1980–1994. *Res Microbiol,* **147**, 393-403.
35. National Committee for Clinical Laboratory Standards. 2000. Performance standards for antimicrobial disk susceptibility test; approved standard-seventh edition. M2-A7. National Committee for Clinical Laboratory Standards, Wayne, Pa. 26 pp.
36. National Committee for Clinical Laboratory Standards. 2002. Performance standards for antimicrobial susceptibility testing; twelfth informational supplement. Vol. 22, n.1. M100-S12. National Committee for Clinical Laboratory Standards, Wayne, Pa, 136 pp.

37. Old D.C., Chisholm S.A. & Crichton P.B. 1999. Molecular fingerprinting of *Salmonella* serotype Glostrup. *Lett Appl Microbiol,* **29**, 156-159.
38. Olive D.M. & Bean P. 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol,* **37** (6), 1661-1669.
39. Peters T.M., Maguire C., Threlfall E.J., Fisher I.S.T., Gill N. & Gatto A.J. 2003. The Salm-gene project – a European collaboration for DNA fingerprinting for food-related salmonellosis. *Eurosurveillance,* **8** (2), 46-50.
40. Refsum T., Heir E., Kapperud G., Vardund T. & Holstad G. 2002. Molecular epidemiology of *Salmonella enterica* serovar typhimurium isolates determined by Pulsed-Field Gel Electrophoresis: comparison of isolates from avian wildlife, domestic animals and the environment in Norway. *Appl Environ Microbiol*, **68** (11), 5600-5606.
41. V. Rizzi, G. Migliorati, V. Acciari, V. Prencipe, E. Di Giannatale, P. Calistri & O. Matteucci 2005. Sistema di sorveglianza e rintraccio rapido delle fonti primarie di infezione nelle tossinfezioni alimentari da *Salmonella* spp. *Vet Ita*, **41** (4), 239-248.
42. Soto S.M., Guerra B., Gonzalez-Hevia M.A. & Mendoza M.C. 1999. Potential of three-way Randomly Amplified Polymorphic DNA Analysis as s typing method for twelve *Salmonella* serotypes. *Appl Environ Microbiol,* **65** (11), 4830-4836.
43. Struelens M.J. 1998. Molecular epidemiologic typing systems of bacterial pathogens: current issues and perspectives. *Mem Inst Oswaldo Cruz*, **93** (5), 581-585.
44. Struelens M.J., Schwam V., Deplano A. & Baran D. 1993. Genome macrorestriction analysis of diversity and variability of *Pseudomonas aeruginosa* strains infecting cystic fibrosis patients. *J Clin Microbiol*, **31** (9), 2320-2326.
45. Sulakvelidze A., Kekelidze M., Turabelidze D., Tsanava S., Tevsadze L., Devdariani L., Gautom R., Myers R., Morris J.G. & Imnadze P. 2000. Salmonellosis in the Republic of Georgia: Using molecular typing to identify the outbreak-causing strain. *Emerg Infect Dis,* **6** (1), 70-73.
46. Swaminathan B., Barrett T.J., Hunter S.B. & Tauxe R.V., CDC PulseNet Task Force, 2001. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. *Emerg Infect Dis,* **7** (3), 382-389.
47. Tenover F.C., Arbeit R.D., Goering R.V., Mickelsen P.A., Murray B.E., Persing D.H. & Swaminathan B. 1995. Interpreting chromosomal DNA restriction patterns produced by Pulsed-Field Gel Electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol,* **33** (9), 2233-2239.
48. Tenover F.C., Arbeit R.D. & Goering R.V. 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. *Infect Control Hosp Epidemiol*, **18** (6), 426-439.
49. Threlfall E.J., Fisher I.S.T., Berghold C., Gerner-Smidt P., Tschape H., Cormican M., Luzzi I., Schnieder F., Wannet W., Machado J. & Edwards G. 2003. Antimicrobial drug resistance in isolates of *Salmonella enterica* from cases of salmonellosis in humans in Europe in 2000: results of international multi-centre surveillance. *Eurosurveillance*, **8** (2), 41-45.
50. Thong K-L., Ngeow Y-F., Altwegg M., Navaratnan P. & Pang T. 1995. Molecular analysis of *Salmonella enteritidis* by Pulsed-Filed Gel Electrophoresis and ribotyping. *J Clin Microbiol*, **33**: 1070-1074.
51. Tsen H.Y. 2002. Molecular typing of *Salmonella enterica* serovars Typhimurium, Typhi and Enteritidis isolated in Taiwan. *J Food Drug Anal*, **10** (4), 242-251.
52. Tsen H.Y., Lin J.S., Hu H.H. & Wang T.K. 1999.

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Use of Pulsed-Field Gel Electrophoresis as an epidemiological tool for analysis of sporadic associated strains of *Salmonella typhi* isolated in Taiwan. *J Appl Microbiol,* **86**, 761-768.

53. Tyler K.D., Wang G., Tyler S.D. & Johnson W.M. 1997. Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. *J Clin Microbiol,* **35** (2), 339-346.
54. Weigel R.M., Qiao B., Teferedegne B., Suh D.K., Barber D.A., Isaacson R.E. & White B.A. 2004. Comparison of pulsed field gel electrophoresis and repetitive sequence polymerase chian reactions genotyping methods for detection of genetic diversity and inferring transmission of *Salmonella*. *Vet Microbiol*, **100,** 205-217.
55. Wright J.M., Brett M. & Bennett J. 1998. Laboratory investigation and comparation of *Salmonella* Brandenburg case in New Zealand. *Epidemiol Infect*, **121**, 49-55.
56. Yan S.S., Pendrak M.L., Abela-Ridder B., Punderson J.W., Fedorko D.P. & Foley S.L.. 2003. An overview of *Salmonella* typing public health perspectives. *Clin Appl Immunol Rev*, **4**, 189-204.