Use of pulsed field gel electrophoresis (PFGE) and single-enzyme amplified fragment length polymorphism (SE-AFLP) to subtype isolates of *Salmonella enterica* serotype enteritidis

Caterina Mammina[1], Giovanni M. Giammanco[1], Cristina Romani[2], Antonino Nastasi[2]

[1]*Dipartimento di Igiene e Microbiologia "G. D'Alessandro", Università degli Studi di Palermo, Palermo;* [1]*Dipartimento di Sanità Pubblica "G.B. Morgagni", Università degli Studi di Firenze, Firenze*

Correspondence to: Antonino Nastasi, Dipartimento di Sanità Pubblica "G.B. Morgagni", Viale Morgagni 48, 50134 Firenze E-mail antonino.nastasi@unifi.it

This work was partially supported by a grant from the Ministry of Health

Abstract

Serotype Enteritidis is still the main serotype infecting humans and poultry worldwide. Subtyping of isolates belonging to this serotype is difficult, because of the wide clonal circulation of a few bacterial clones. This study presents the results of the characterization of 49 isolates of *S.* Enteritidis identified at the southern Italy Centre for Enteric Pathogens (CEPIM) during the years 2002-2003 by the methods of Pulsed Field Gel Electrophoresis (PFGE) and Single-Enzyme Amplified Fragment Length Polymorphism (SE-AFLP). Clustering of the strains by SE-AFLP and PFGE is very similar, but the first technique is more rapid and user-friendly and does not require sophisticated equipment. Further work is needed for a more accurate assessment of SE-AFLP, but preliminary results suggest it could be a promising support to epidemiological investigations.

Key words: Salmonella Enteritidis, SE-AFLP, PFGE, molecular epidemiology

Introduction

Enteritidis is still the main serotype infecting humans and poultry worldwide. Since the third quarter of 2002, in many European countries, including Italy, after a phase of declining incidence between the late '90s and 2002 in relation to the implementation of European Directive 92/117 for breeding flocks and improved sanitation and farming practices for egg-laying and broiler flocks, the incidence of human infection has again increased.[1,2] The most recent reports from the EnterNet european surveillance network documents that more than 75% of the human *Salmonella* strains identified by the national reference laboratories belong to the Enteritidis serotype.[3]

Phage typing has traditionally been the first-line method for characterizing Enteritidis isolates, but the predominance of a few phage types (PTs) has made it necessary to apply molecular typing approaches, such as Pulsed Field Gel Electrophoresis (PFGE) and plasmid analysis.[4-6] The range of DNA fingerprinting techniques has been progressively expanding in recent years to include several polymerase chain reaction (PCR)-based approaches, such as random amplified polymorphic DNA (RAPD) or more sophisticated applications, such as amplified fragment length polymorphism analysis (AFLP). This last technique, originally developed as a universal DNA typing method for crop plants, has been applied to the characterization of plant, animal and prokariotic DNAs. It is based upon the digestion of DNA by restriction endonucleases and selective amplification of a subset of DNA fragments achieved by the ligation of oligonucleotide adapters and subsequent annealing of primers containing adapter-defined sequences with one additional arbitrary nucleotide as the final 3' base.[7-10]

In this study, a single enzyme approach (SE-AFLP), originally developed for the analysis of *Legionella* and *Helicobacter* species, has been applied to 49 sporadic and epidemic Enteritidis isolates identified in the years 2002-2003 at the Centre for Enteric Pathogens for Southern Italy (CEPIM) and previously pheno-genotyped by phage typing and PFGE.

Methods

Bacterial strains

Forty-nine isolates of serotype Enteritidis identified at the CEPIM during the period October 2002 - May 2003 were studied: 14 isolates were from eggs and layer hens; five were human isolates from epidemiologically defined outbreaks and the remaining 30 (approximately 10.0%) were

randomly selected among apparently sporadic human isolates.

Phage typing

All strains were phage typed according to the method described by Ward et al. [11]

Molecular typing

PFGE: Preparation of cells and endonuclease digestion of the DNA with *Bln*I and *Xba*I were done as previously described with minor modifications.[12] In brief, bacteria were grown in Brain Hearth Infusion broth at 37°C for 18 hours. After centrifugation and washing, the sediments were resuspended in cell suspension buffer (100 mM Tris HCl, 100 mM EDTA [pH 8.0]) and adjusted to an optical density of 0.38 to 0.44 at 450 nm.The cell suspensions (500 µl) were mixed with equal volumes of melted 1.2% chromosomal-grade agarose (Bio-Rad Laboratories, Hercules, CA, USA) and dispensed into wells of reusable plug moulds (Bio-Rad). After solidification, the plugs were transferred into tubes containing 5 ml of lysis buffer (50 mM Tris HCl, 50 mM EDTA [pH 8.0], 1% sarcosyl) and 0.1 mg of proteinase K per ml and lysed overnight in a shaking water bath at 54°C. Then, they were washed twice with deionized distilled water for 5 minutes per wash and four times with TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 8.0]) for 30 minutes per wash at room temperature. Agarose-embedded DNA was digested with 50 U of *Xba*I or *Bln*I (Promega, Madison, WI, USA) overnight in a water bath at 37°C. Restriction fragments were separated by electrophoresis in 0.5x Tris-borate-EDTA buffer at 14°C for 21 hours by using a CHEF Mapper (Bio-Rad) with pulse times of 2 to 40 seconds. The gel was stained with ethidium bromide, and DNA bands were visualized with a UV transilluminator. The interpretation of the PFGE patterns was aided by the use of Molecular Analyst Fingerprinting Plus software, version 1.6 (Bio-Rad). DNA fragment profiles were visually assessed and one or more mismatched bands were assigned to distinct pulsotypes.

SE-AFLP: Bacterial DNA was extracted by a nucleic acid extraction kit (Promega) submitted to a simultaneous reaction of digestion with the restriction endonuclease *Hin*dIII and ligation at 37°C for 3-4 hours. Adapters H1, 5'-ACGGTATGCCACAG-3', and H2, 5'-AGCTCTGTCGCATACCGT-GAG-3' (MWG Biotech, Ebersberg, Germany) were used. Digested-ligated DNA was heated to 80°C for 10 minutes to inactivate the ligase, and subsequent PCR was carried out in an amplification mixture of 50 ml containing 200 µM each deoxynucleoside triphosphate (dNTP), 2.5 mM MgCl2, 150 ng of primer and 1 U of *Taq* polymerase. The primer used for PCR reactions had the sequence 5'-GGTATGCGACAG-AGCTTX-3', where X was either A, T, G or C. Use of a mix of the four primers was also tested. PCR amplification consisted of 1 cycle at 94°C for 4 minutes, followed by 33 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2.5 minutes. PCR products were detected on a 1.5% agarose gel run alongside a 100-bp ladder at 100 V for 3 h, followed by staining in ethidium bromide and visualized under UV light.[10]

Determination of molecular size of fragments and interpretation of banding patterns

DNA fragment profiles were detected by the RestrictoScan software (Taxotron package, Taxolab, Institut Pasteur, Paris, France) and the molecular weight of the bands was calculated by comparing migration values to the average of the two nearest molecular weight standard lanes. Both PFGE and SE-AFLP banding patterns were analyzed by the Taxotron software (Taxolab). A distance coefficient was calculated using the complement of the Dice index with an allowed error limit of 1% and dendrograms were generated by the Unweighted Pair Group Method of Averages (UPGMA) algorithm.

Results

Phage typing differentiated the 49 isolates into nine types: the most common were phage type (PT) 6 and PT4, which were found in 17 (34.0%) and 15 (30.0%) isolates, respectively. PT8 and PT6A were found in four and three human isolates, while the remaining types were identified in one or two strains. Three strains were untypeable (Table 1).

PFGE restriction analysis produced patterns of seven to 10 bands ranging in size from 59 to 667 kilobases. Only those bands having a molecular size higher than 50 kilobases have been considered for comparative analysis. Molecular characterization of the 49 isolates by *Bln*I PFGE analysis identified five pulsotypes (A to E; Figure 1): a predominant type A was found in 37 (75.5%) isolates, while type D was found in eight (16.3%) isolates and the remaining types only in one or two isolates (Table 1). No further differentiation was achieved by the analysis of *Xba*I PFGE profiles.

The specificity of the four primers used in the SE-AFLP protocol was first tested by comparing their PCR amplification products. Different banding patterns were produced by each primer

Table 1. Distribution of isolates of *S.* Enteritidis by phage type and genotype obtained by PFGE and SE-AFLP.

Phage type	PFGE genotype		Four primer SE-AFLP genotype	Number of isolates by source		
	*Bln*I	*Xba*I		human		egg / layer hen
				outbreak	sporadic	
PT4	A	A	A	1	9	4
	E	A	A		1	
PT6	A	A	A	3	4	9
	C	A	A		1	
PT8	D	B	C		2	
	D	B	E		2	
PT6a	B	A	D		2	
	D	A	B		1	
PT1B	A	A	A		2	
PT1	A	A	A		1	
PT2	D	B	C		2	
PT4B	A	A	A		1	
PT30	A	A	A		2	1
NT*	D	A	C	1		

* NT = untypeable

on a single strain. However, analysis of a set of ten strains by using a single primer in the PCR reaction showed a poor discriminatory power, because of the low number of bands. Moreover, primer G was unable to produce amplification products from all, but one, of the isolates. When the same set of strains was submitted to PCR by using a mix of the four primers, a more complex electrophoretic pattern, including five to seven bands, was obtained (Figure 2). These patterns were reproducible in individual experiments. By this modified SE-AFLP method, five types (A to E) were obtained: 36 (73.5%) of the 49 isolates were included into the most frequent type A, whereas five (10.2%) were attributed to the type C and one or two to the remaining three types (Figure 3).

Table 1 summarizes the results obtained by applying the two typing methods. SE-AFLP seems to be more discriminative on the strains belonging to the group PT 2/8, that are divided into types C and E, whereas PFGE includes them into a single pulsotype, D. On the contrary, two strains that PFGE attributes to pulsotypes C and E, respectively, are grouped by SE-AFLP into the major cluster A. The three isolates PT6A are more consistently classified by SE-AFLP than PFGE: indeed, isolates 13 and 23, SE-AFLP type D, proved to be indistinguishable from each other also by plasmid profile (data not shown), but strain 50 was assigned to a different type B, whilst PFGE included this last one into a pulsotype seemingly associated with the group PT2/8.

Discussion

Enteritidis is the most frequently identified serotype in Europe, so it is essential to undertake subtyping to investigate and confirm outbreaks, accurately evaluate the trends in infection cases and identify any emerging issues. Unfortunately, Enteritidis has proved to be difficult to subtype since only a few bacterial clones are circulating worldwide.[13]

Phage typing has been traditionally applied to this pathogen successfully and has proved invaluable, especially for long term or wide geographic scale epidemiological studies. The prominent feature of the present epidemiological phase of the serotype Enteritidis history is a shift in the phage types identified in Europe, with a decreasing frequency of PT4 while other phage types are increasing both in numbers and in proportion.[1] Data from the EnterNet *Salmonella* database show that PT4 which accounted for 61.8% of isolates in 1998, has fallen to 32.1% in 2003.[2] The efficiency of phage typing might be positively influenced by this widening heterogeneity, but a number of both technical and theoretical issues hamper a larger application of this subtyping method: among the more relevant ones, unavailability of phage suspensions except for a limited number of reference laboratories, difficulties of standardization in performing and reading, negative effects on specificity by phage conversion or adaptation of surface bacteriophage receptors and consequent phage type shift.[14,15]

Figure 1. Unweighted group method using arithmetic averages (UPGMA) phylogenetic dendrogram showing the genetic distances obtained by PFGE using restriction endonuclease *Bln*I from 49 *Salmonella* Enteritidis isolates.

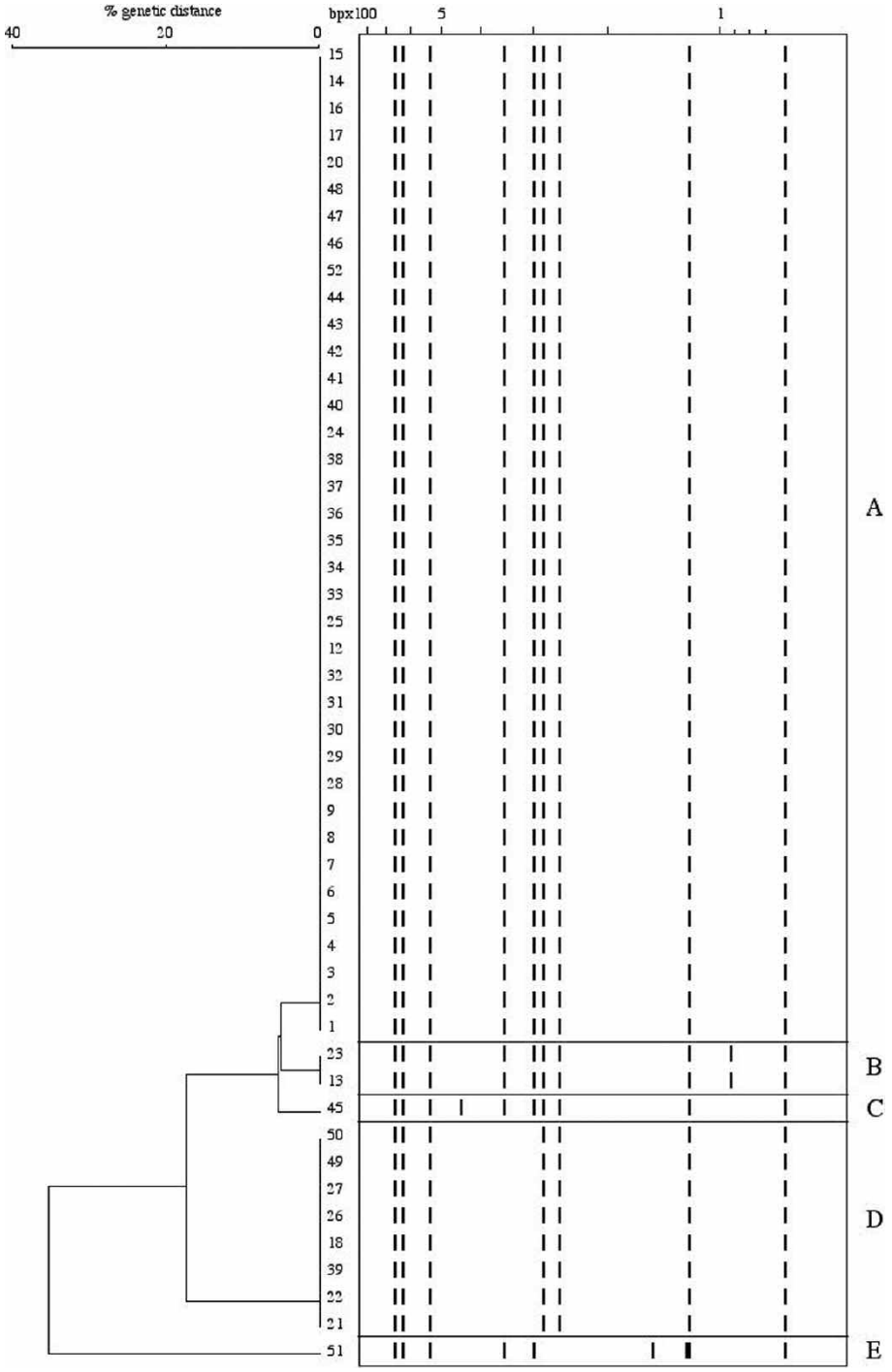
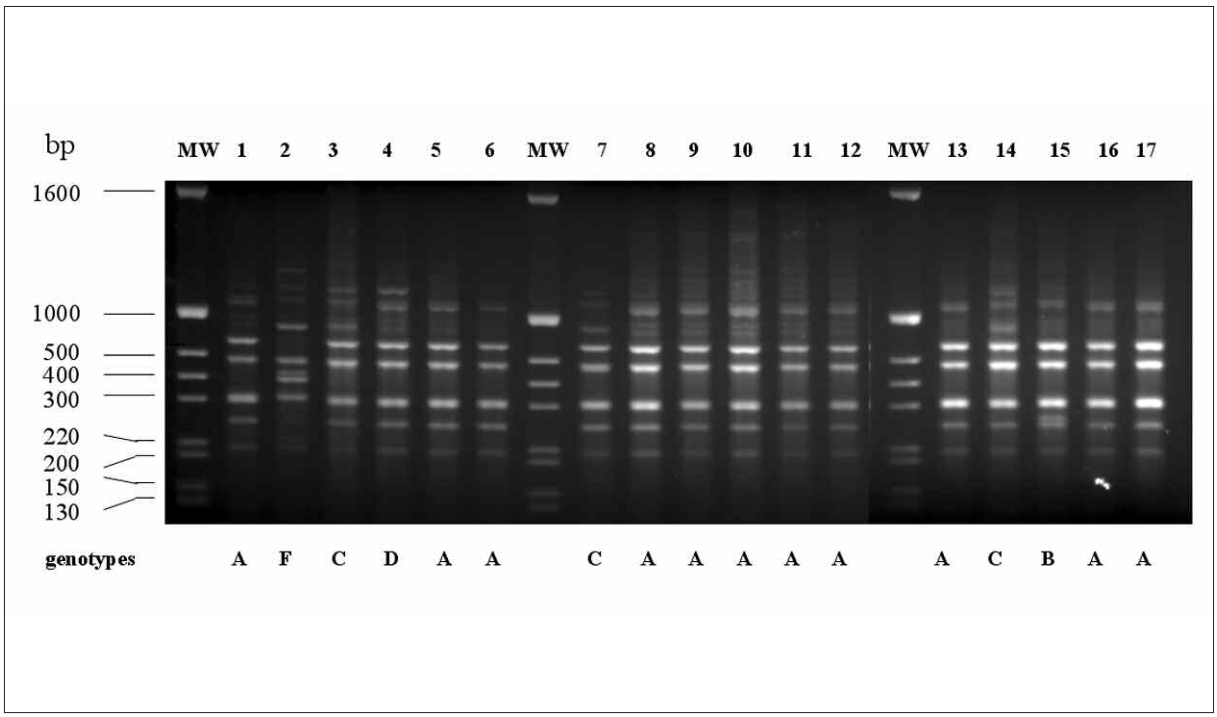


Figure 2. Electrophoretic profiles obtained by SE-AFLP of representative isolates of S. Enteritidis.

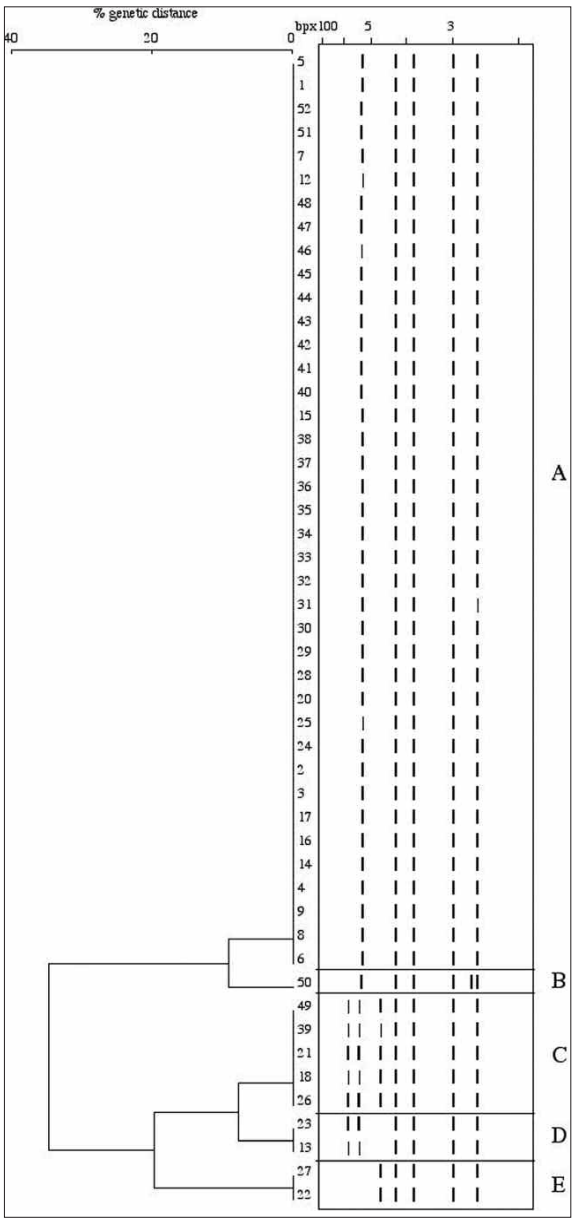


The use of molecular typing has become increasingly important, but serious limitations have been found when applied on a clonal serotype of *Salmonella*, such as Enteritidis. Indeed, PFGE, a well established method that has been largely applied to *S. enterica,* recognizes only a few profiles, by attributing the majority of circulating strains to two main lineages, the first one containing PT1, PT4 and PT6 and the second one including PT2 and PT8.[14] Moreover, PFGE is a labour-intensive and technically demanding method and requires specific electrophoresis apparatus that is likely to be restricted to research and reference laboratories.

The purpose of our study was to perform a preliminary evaluation of the discriminative power and user-friendliness of SE-AFLP in comparison to PFGE, by testing an epidemiologically characterized set of Enteritidis isolates. In our experience, these two methods have exhibited approximately equal utility, but SE-AFLP is less labour intensive, does not require specialized equipment and minimises the time taken to obtain results.To our best knowledge, the application of these two techniques has been previously applied as an intra-serotype typing approach on only multiresistant Typhimurium isolates.[16] The study by Sood et al.[16] describes the combined employment of PFGE and SAFLP for the differentiation of 19 strains of *S.* Typhimurium identified from humans and cattle in the USA in 1998. The results obtained are apparently very different from our findings: indeed, SE-AFLP proved to be poorly discriminative in comparison with PFGE, though able to recognize broader genetic clusters. Hence, the authors propose its use as a first step screening method to be integrated with the more sensitive PFGE as the second step.[16] This conclusion, although reasonable, deserves to be confirmed by a much larger investigation on a sample of isolates representative of the widest pheno-genotypic heterogeneity of the Typhimurium serotype. Moreover, in-series or in-parallel typing approaches may obtain very different results, when applied on largely differentiated serotypes with a wide host range, like Typhimurium, or on very homogeneous and almost host-specific serotypes, like Enteritidis.

The limited number of strains tested does not allow for generalisation of the study results. However, the advantages in terms of turnaround, ease of use and equipment cost suggest that SE-AFLP could be an attractive alternative to PFGE, mainly for the epidemiological investigation of food-bourne outbreaks and especially when real-time results are needed to support timely control measures. However, further work is required to produce a more accurate assessment of its reliability, reproducibility, discriminative power and consistency with epidemiological data.

Figure 3. UPGMA phylogenetic dendrogram showing the genetic distances obtained by the four primers SE-AFLP from 49 *Salmonella* Enteritidis isolates. Narrow bands are bands having low absorbance values.



References

1) Fisher IS. Dramatic shift in the epidemiology of *Salmonella enterica* serotype Enteritidis phage types in western Europe, 1998-2003 - results from the Enter-net international salmonella database. Euro Surveill 2004;9(11).

2) O'Brien S, Ward L. *Salmonella enteritidis* in England and Wales: increases in unusual phage types in 2002. Eurosurveillance Weekly 2002;6: 021107.
http://www.eurosurv.org/2002/021107.html

3) Quarterly reports on *Salmonella* isolates identification. International surveillance network for the enteric infections *Salmonella*, VTEC O157 and *Campylobacter.*
http://www.hpa.org.uk/hpa/inter/enter-net_menu.html

4) Boonmar S, Bangtrakulnonth A, Pornrunangwong S. Epidemiological analysis of *Salmonella enteritidis* isolates from humans and broiler chickens in Thailand by phage typing and pulsed-field gel electrophoresis. J Clin Microbiol 1998;36(4):971-4.

5) Fernandez J, Fica A, Ebensperger G. Analysis of molecular epidemiology of Chilean *Salmonella enterica* serotype Enteritidis isolates by pulsed-field gel electrophoresis and bacteriophage typing. J Clin Microbiol 2003;41(4):1617-22.

6) Mammina C, Di Noto AM, Ricci A, Nastasi A. Phenogenotyping of *Salmonella enterica* serotype Enteritidis isolates identified in Sicily during a reemergence period. Foodborne Pathogens Dis 2004;1:195-9.

7) Desai M, Threlfall EJ, Stanley J. Fluorescent amplified-fragment length polymorphism subtyping of the *Salmonella enterica* serovar Enteritidis phage type 4 clone complex. J Clin Microbiol 2001;39(1):201-6.

8) Fry NK, Bangsborg JM, Bergmans A, et al. Designation of the European Working Group on Legionella Infection (EWGLI) amplified fragment length polymorphism types of *Legionella pneumophila* serogroup 1 and results of intercentre proficiency testing using a standard protocol. Eur J Clin Microbiol Infect Dis 2002;21(10):722-8.

9) Gibson JR, Slater E, Xerry J, Tompkins DS, Owen RJ. Use of an amplified-fragment length polymorphism technique to fingerprint and differentiate isolates of *Helicobacter pylori*. J Clin Microbiol 1998;36(9):2580-5.

10) Peters TM, Threlfall EJ. Single-enzyme amplified fragment length polymorphism and its applicability for *Salmonella* epidemiology. Syst Appl Microbiol 2001;24(3):400-4.

11) Ward LR, De Sa JH, Rowe B. A phage typing scheme for *Salmonella enteritidis*. Epidemiol Infect 1987;99(2):291-304.

12) Foodborne and Diarrheal Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Diseases Control and Prevention. Standardized molecular subtyping of *Escherichia coli* O157:H7 by pulsed-field gel electrophoresis: a training manual. Atlanta, USA: Centers for Diseases Control and Prevention, 1996.

13) Ward LR, Threlfall J, Smith HR, O'Brien SJ. *Salmonella enteritidis* epidemics. Science 2000;287(5459):1753-4.

14) Laconcha I, Baggesen DL, Rementeria A, Garaizar J. Genotypic characterization 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 2000;75(2).155-65.

15) Rankin S, Platt DJ. Phage conversion in *Salmonella enterica* serotype Enteritidis: implication for epidemiology. Epidemiol Infect 1995;114(2):227-36.

16) Sood S, Peters T, Ward LR, Threlfall EJ. Combination of pulsed-field gel electrophoresis (PFGE) and single-enzyme amplified fragment length polymorphism (SAFLP) for differentiation of multiresistant *Salmonella enterica* serotype Typhimurium. Clin Microbiol Infect 2002;8(3):154-61.