ORIGINAL ARTICLE

Fluorescent amplified fragment length polymorphism genotyping of *Salmonella* Enteritidis: a method suitable for rapid outbreak recognition

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Objective To perform fluorescent amplified fragment length polymorphism (FAFLP) analysis on phage type (PT) reference strains of *Salmonella enterica* subsp. *enterica* serotype Enteritidis (*S.* Enteritidis), and *S.* Enteritidis PT 6 and 6a recent clinical isolates to determine its usefulness for primary characterization of clinical *S.* Enteritidis isolates, and then to determine whether FAFLP is suitable for rapid characterization of strains in an outbreak situation.

Methods Twenty-five PT reference strains of *S.* Enteritidis and 20 *S.* Enteritidis PT 6 and 6a clinical isolates were subjected to FAFLP analysis using the selective primer combinations *Eco* + 0–*Mse* + T and *Eco* + 0–*Mse* + TA.

Results FAFLP successfully separated each one of the 25 *S.* Enteritidis PT strains into distinct profiles, while macrorestriction and PFGE using *Xba*I identified 20 pulsed-field profiles. FAFLP also resolved cases and outbreaks due to *S.* Enteritidis PTs 6 and 6a.

Conclusions The resolving power of FAFLP was higher than that of PFGE. FAFLP is a highly discriminatory genotyping method and, in conjunction with phage typing for primary subdivision of *S.* Enteritidis, provides a rapid and powerful tool for strain differentiation, both for outbreak investigation and for epidemiologic surveillance.

Keywords FAFLP, *Salmonella* Enteritidis, molecular typing

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INTRODUCTION

Salmonella enterica is a leading cause of gastrointestinal infection. In 1998, isolates from over 23 000 cases (1998 PHLS data set) were referred to the Laboratory of Enteric Pathogens (LEP), the *Salmonella* reference laboratory for England and Wales, of the Public Health Laboratory Service. *Salmonella enterica* subsp. *enterica* serotype Enteritidis (*Salmonella* Enteritidis) is the serotype most commonly responsible for human infection. It accounted for over 16 000 (69.1%) salmonella cases referred to the LEP in 1998, and nearly 11 000 (61.4%) cases in 1999. Phage typing is used for primary subdivision of *Salmonella* Enteritidis, and the published scheme differentiates 27 phage types (PTs) using 10 typing phages [1]. PT 6 has been the most commonly isolated PT of *Salmonella* Enteritidis after PT 4, and has been responsible for a number of recent outbreaks. PT 6 infection is nearly always associated with raw eggs and chicken [2–5].

Though phage typing has a role in the epidemiologic surveillance of salmonella outbreaks, there is a need to distinguish between strains within a PT, and between clones that cause outbreaks and other isolates responsible for sporadic infections. Various molecular methods have been used to resolve these isolates, including plasmid profile typing [6,7], ribotyping [7], IS*200* fingerprinting [8–10] and pulsed-field gel electrophoresis (PFGE) [7,9–11]. To date, PFGE has been found to be the most discriminatory molecular typing method applicable to *Salmonella* Enteritidis [12], although plasmid profile typing has been successfully applied for epidemiologic investigation of some outbreaks of *Salmonella* Enteritidis PT 4 [13].

Amplified fragment length polymorphism (AFLP) is a PCR-based technique that permits sampling of the entire bacterial genome. Fluorescent AFLP (FAFLP) [14] is an enhancement of the original technique, used for high-resolution genotyping of *Escherichia coli* [15], methicillin-resistant *Staphylococcus aureus* [16], *Streptococcus pyogenes* [17,18], *Mycobacterium tuberculosis* [19], *Campylobacter jejuni* and *C. coli* [20]. Though AFLP studies of *Salmonella* Enteritidis have previously been reported, they

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were not successful in discriminating between strains within PTs [21,22], which is necessary for outbreak analysis and epidemiologic surveillance.

In this study, we employed FAFLP for genotypic analysis of defined PT reference strains of *Salmonella* Enteritidis and for 20 recent isolates of *Salmonella* Enteritidis PTs 6 and 6a, PT 6 being the second most common PT after PT4 implicated in *Salmonella* Enteritidis outbreaks.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Twenty-five reference strains of *Salmonella* Enteritidis of known PFGE profile [7] were examined (Table 1). Twenty recent clinical isolates of PTs 6 and 6a (Table 2), three of which were known to be epidemiologically related, were cultured aerobically on nutrient agar plates for 16 h. Stock cultures were stored on Protect bacterial preservers (Technical Service Consultants Ltd, Heywood, Lancashire, UK) at −70 °C.

FAFLP

Genomic DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen, Crawley, UK). DNA was eluted into a final volume of 50 μL of distilled water. FAFLP analysis was done as described by Desai et al. [18]. *Mse*I and *Eco*RI were used to digest approximately 500 ng of genomic DNA for 2 h. To the digested DNA was added 5.0 μL of ×10 T4 DNA ligase buffer, 400 U of T4 DNA ligase (New England Biolabs, Hitchin, Hertfordshire, UK), *Eco* Adapter to a final concentration of 0.1 μM, *Mse* Adapter to a final concentration of 0.5 μM (both from Sigma-Genosys, Pampisford, Cambridgeshire, UK) and 16.8 μL of sterile distilled water, to give a final reaction volume of 50 μL. *Eco* adapter and *Mse* adapter were added to final concentrations of 1 nM and 5 nM, respectively, to the PT 6 and 6a isolates.

PCR was performed in a total reaction volume of 25 μL, consisting of 2.5 μL of the above digestion–ligation reaction mixture, 2.5 μL of 10 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (all from Life Technologies, Paisley, UK), 0.2 μM 6-FAM fluorescently labeled *Eco* + 0 primer, 1 μM *Mse* + T primer (both primers from Sigma-Genosys), and 0.65 U of *Taq* DNA polymerase (Life Technologies). The same PCR protocol was also used for amplification with *Eco* + 0 and *Mse* + TA primers (Sigma-Genosys). Touchdown PCR was performed [18], with the exception that the final extension step at 60 °C for 30 min was omitted.

Table 1 *Salmonella* Enteritidis phage type reference strains

Reference strain	Phage type (PT) [7]	PFGE profile [7]
E2331[a]	1	X11
E2457	2	X12
P66040	3	X13
E2187	4	X1
P99764/A	4a	X1
P70001	5	X11A
P99327	6	X1B
E2408	6a	X15
P130362	7	X12A
E2468	8	X12
E2402	9	X16
P95661	12	X27
E464	13	X22
E2387	14	X23
E2402	15	X24
E866	16	X25
P95940	17	X26
P89448	18	X14
E1949	19	X13A
P68147	20	X28
P138532	21	X1
P84357	22	X30
P88255	23	X12
P99768	24	X1
P104204	30	X1E

[a] Laboratory accession number of LEP isolates.

Fragment analysis

Gel separation and fragment analyses were performed as described previously [16]. Fragments were sized with GeneScan version 3.1 software (Applied Biosystems (AB), Warrington, Cheshire, UK). The data collected in GeneScan 3.1 were transferred to Genotyper 2.5 (AB).

In the current absence of convenient automated analysis, electropherograms were visually screened for the presence or absence of polymorphic fragments. They were scored in a binary matrix, and were recorded as a text (tab-delimited) file in Excel 98 (Microsoft). Dice coefficients of similarity [23] were calculated with in-house software. Cluster analysis was performed using an unweighted pair-group method with arithmetic averages (UPGMA) in the NEIGHBOR program of PHYLIP [24] and then displayed in TREEVIEW version 1.5 [25].

Predictive modeling

Predictive modeling of *Salmonella* Typhi R27 plasmid (GenBank accession number NC 002305) and *Salmonella* Typhimurium low copy number plasmid pSC101 (GenBank accession number NC 002056) was performed as described by Arnold et al. [15]. Briefly, the downloaded sequences were imported into Macvector (DNA*), and *Eco*RI and *Mse*I were selected from the commercial enzymes folder and applied to the sequences.

RESULTS

To examine intergel reproducibility, all PCR products were run on three gels and the results compared. The fragment sizing did not vary outside ±1 bp.

Decreased adapter concentrations were used for the 20 *Salmonella* Enteritidis PT 6 and 6a isolates, because of an occasional problem of n + 3 peaks being observed. These were due to excess *Mse* adapter being carried over into the PCR reaction and the 5′ to 3′ strand acting as an additional primer.

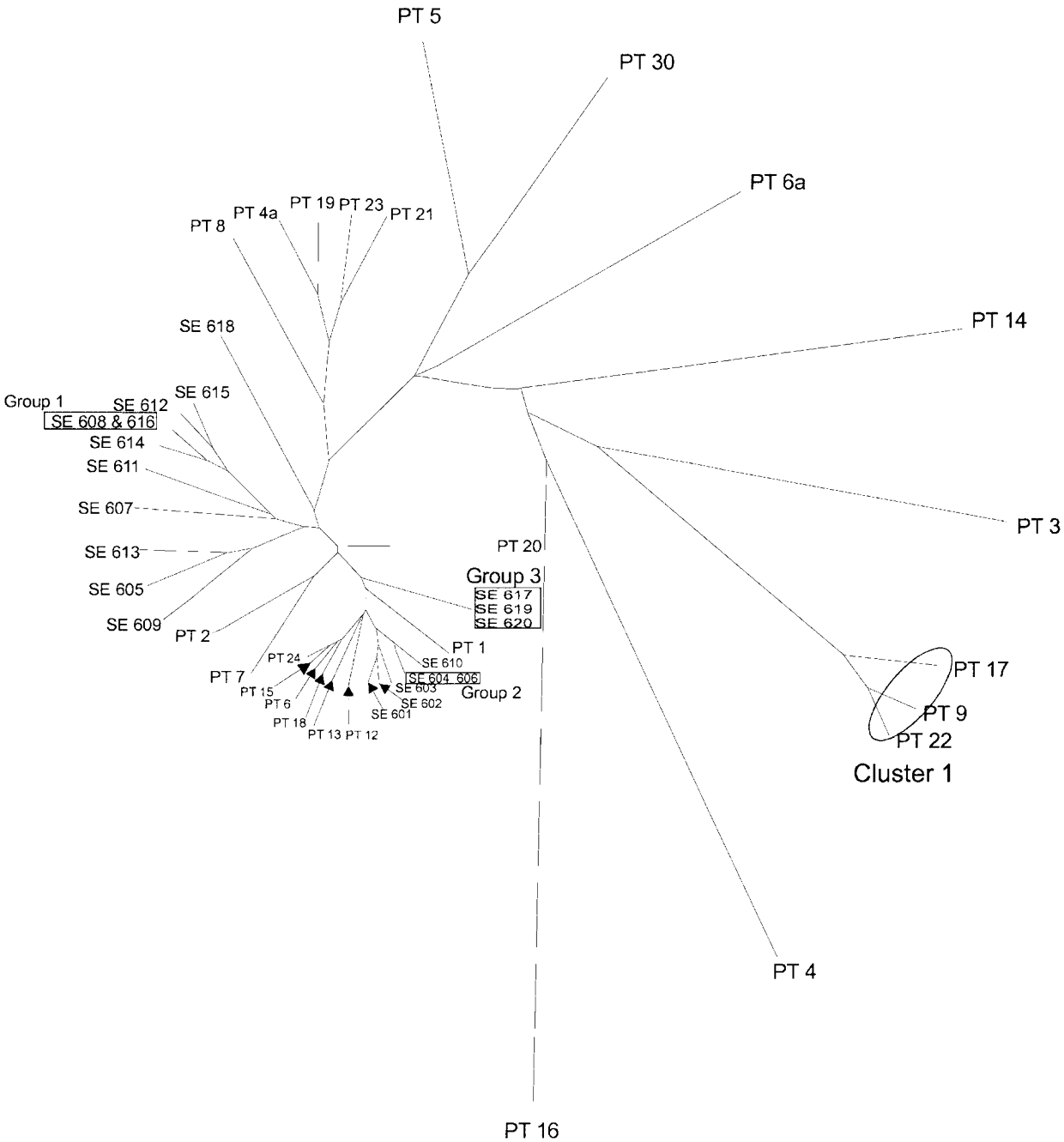

Figure 1 The dendrogram shows the relationship by FAFLP between 25 *Salmonella* Enteritidis phage type reference strains (PT series) and 20 PT 6 and 6a isolates (SE series) using the unweighted pair-group method with arithmetic averages (UPGMA).

Table 2 Recent *Salmonella* Enteritidis PT 6 and 6a isolates examined

Laboratory code	Isolate number	Phage type	Resistance type	Travel associated	Date of isolation	Sporadic/Outbreak
SE 601	P5229560	6	$[a]	Spain	June 2000	S
SE 602	P5238980	6	$	Spain	June 2000	S
SE 603	P5245640	6	$	Spain	NK	S
SE 604	P5235741	6	$	No	June 2000	S
SE 605	P5239780	6	$	No	July 2000	S
SE 606	P5248370	6	$	No	July 2000	S
SE 607	P5242140	6	A[b]	Spain	July 2000	S
SE 608	P5259210	6	A	Spain	June 2000	S
SE 609	P5255850	6	A	No	NK	S
SE 610	P5204760	6a	$	Malta	March 2000	S
SE 611	P5239370	6a	$	Malta	June 2000	S
SE 612	P5239020	6a	A	Majorca	June 2000	S
SE 613	P5242070	6a	A	Majorca	June 2000	S
SE 614	P5241030	6a	ANxCp[c]	Spain	June 2000	S
SE 615	P5248680	6a	ANxCp	Spain	July 2000	S
SE 616	P5250320	6a	ANxCp	Spain	July 2000	S
SE 617	P5257720	6a	T[d]	No	NK	O
SE 618	P5258341	6a	T	No	NK	S
SE 619	P5257720	6a	T	No	NK	O
SE 620	P5258400	6a	T	No	NK	O

[a]Sensitive.
[b]Ampicillin resistant.
[c]Ampicillin, nalidixic acid and ciprofloxacin resistant.
[d]Tetracycline resistant.
NK, not known.

The 25 *Salmonella* Enteritidis PT reference strains were resolved by FAFLP as 25 distinct profiles. Sixteen FAFLP profiles were identified among the 20 *Salmonella* Enteritidis PT 6 and 6a isolates using the selective primer combinations *Eco* + 0–*Mse* + T and *Eco* + 0–*Mse* + TA. Forty-six polymorphic *Eco* + 0–*Mse* + T fragments were identified in the size range 95–317 bp, with 55 polymorphic *Eco* + 0–*Mse* + TA fragments being used to further subdivide strains. (These data were combined in a single spreadsheet).

The type strains of PT 4 and PT 16 had very different FAFLP profiles from the remainder of the collection. (There was a 48 FAFLP fragment difference between PT 4 and PT 22, the third most divergent FAFLP profile, and a 50 FAFLP fragment difference between PT 16 and PT 22.) These findings were identical on re-extraction and processing.

Dendrograms of combined *Eco* + 0–*Mse* + T and *Eco* + 0–*Mse* + TA FAFLP data created using UPGMA (Figure 1) and neighbor joining (data not shown) had similar topologies. One cluster of type strains similar by FAFLP was observed (Figure 1). This consisted of three strains, PTs 9, 17 and 22, that differed by no more than nine FAFLP bands. Three groups of *Salmonella* Enteritidis PT 6 and 6a isolates were identical by FAFLP using these primer combinations, group 1 (SE 604 and SE 606), group 2 (SE 608 and SE 616) and group 3 (SE 617, SE 619 and SE 620). SE 617, SE 619 and SE 620 had been isolated from three members of the same family (family M). Though these three isolates had the same phage type and antibiotic resistance profile as another isolate, SE 618, SE 618 differed from them by nine FALP fragments. SE 617 and SE 618 also had the same plasmid profile and *Xba*I pulsed-field profile (PFP) (data not shown). Among the 20 isolates of PTs 6 and 6a, no clustering was observed between strains with the same antibiotic resistance profiles or between isolates from individuals who had recently returned from the same region abroad.

Figure 2 compares the FAFLP profiles of the PT reference strain of PT 6a with SE 611, a PT 6a isolate, using both selective primer combinations.

Predictive modeling performed on two *Salmonella* plasmids revealed the presence of forty *Eco*RI restriction sites flanked by *Mse* restriction sites in the *S.* Typhi R27 plasmid, and no *Eco*RI restriction sites in the *S.* Typhimurium low copy number plasmid pSC101. Therefore, using the selective primer combinations *Eco* + 0–*Mse* + T and *Eco* + 0–*Mse* + TA, 18 possible FAFLP fragments would be generated from the *S.* Typhi R27 plasmid. However, only two fragments of a corresponding size were observed in FAFLP profiles, one in PT4 and the other in PT16.

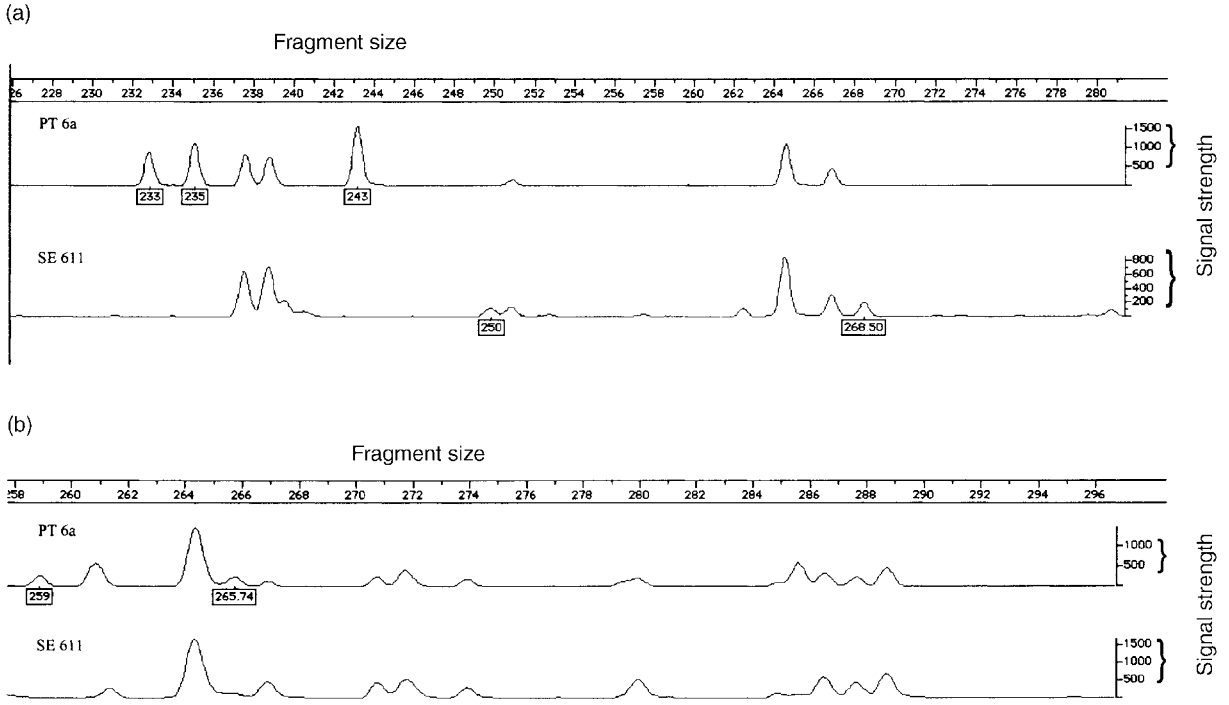

Figure 2 FAFLP profile comparison of PT 6a and SE 611: (a) primer combination *Eco* + 0–*Mse* +TA, fragments from 226 to 280 bp in size; (b) primer combination *Eco* + 0–*Mse* +T, fragments from 258 to 296 bp in size. Genotyper v 2.5 electropherograms show that FAFLP has been successfully used to resolve these two strains of PT 6a. Polymorphic amplified fragments included in the study are labeled. (a) There are five amplified fragment differences between PT 6a and SE 611 in the size range 226–280 bp using the selective primer combination *Eco* + 0–*Mse* +TA. PT 6a has three amplified fragments not present in SE 611 (sized 232.82 bp, 235.08 bp and 243.16 bp). SE 611 has two amplified fragments not present in PT 4 (sized 249.84 bp and 268.50 bp). (b) There are two amplified fragment differences between PT 6a and SE 611 in the size range 260–296 bp using the selective primer combination *Eco* + 0–*Mse* +T. PT 6a has three amplified fragment differences not present in SE 611 (sized 259 bp and 265.74 bp).

DISCUSSION

PFGE has previously been applied to these 25 PT strains of *Salmonella* Enteritidis, using both *Xba*I [7] and *Not*I [9] macrorestriction. *Xba*I macrorestriction was more discriminatory than *Not*I, generating 20 PFPs. The results from this study indicate that FAFLP is more discriminatory than standard macrorestriction (PFGE) for strains of *Salmonella* Enteritidis. In particular, FAFLP resolved reference strains of PTs 4, 4a, 21 and 24, which were identical by *Xba*I macrorestriction and PFGE (PFP X1). Reference strains of PTs 2, 8 and 23, also identical by *Xba*I macrorestriction and PFGE (PFP X12), were also resolved by FAFLP. The higher discriminatory power of FAFLP compared to PFGE is mainly attributed to the double restriction enzyme digestion employed by FAFLP as opposed to the single enzyme digestion used in PFGE, which results in more fragments being analyzed by FAFLP [26].

PT 16 appeared different to the other reference strains by both FAFLP and PFGE typing. PT 16 contains multiple copies of IS*200* (16), whereas the majority of the other strains contain only two copies [9]. PT 6a also appeared quite different from the other strains by both PFGE and FAFLP. This PT strain had three copies of IS*200*, the extra copy being found on a plasmid [9]. The presence of these extra copies of IS*200* could alter the FAFLP profile in several ways; for example, insertion of IS*200* could disrupt existing or add new *Eco*RI or *Mse*I restriction sites which will be detected by FAFLP analysis. Or if they were found within an existing FAFLP, they could alter the size of the observed FAFLP fragment.

There was no clear clustering among the other isolates of PTs 6 and 6a examined, suggesting that no predominant disease-causing clones of this PT circulate. There were three groups of isolates (Figure 1) that were identical by FAFLP using the particular primer combination. The pair in group 1 were both PT 6, and had the same antibiotic resistance profile, but differed slightly in their *Xba*I pulsed-field and plasmid profiles (data not shown). The second group consisted of a pair of isolates from people who had recently returned from Spain, both with different PTs and antibiotic resistance profiles. These strains also had different *Xba*I PFPs, but had the same plasmid profile. Both strains were resistant to ampicillin, but one was also resistant to nalidixic acid and also exhibited decreased susceptibility to

ciprofloxacin. Ampicillin resistance is plasmid-encoded, whereas resistance to nalidixic acid/ciprofloxacin is chromosomally encoded. Although some plasmid DNA may be isolated during the genomic DNA extraction procedure, the predicted fragments from the *S.* Typhi R27 plasmid were not observed in the majority of FAFLP profiles. Therefore, it is unlikely that the selective primer combinations *Eco* + 0–*Mse* + T and *Eco* + 0–*Mse* + TA would provide susceptibility information.

Furthermore, the *gyr*A genes of *Salmonella* Typhimurium have been sequenced, and the point mutation that causes quinolone drug resistance identified [27,28]. In silico FAFLP analysis has revealed that there are no *Mse*I, *Eco*RI or *Xba*I restriction sites within these sequences, and therefore quinolone resistance will not be detected by FAFLP using these primer combinations or *Xba*I macrorestriction and PFGE.

The three isolates of FAFLP group 3 were isolated from the same family, M. The family may have contracted the infection from the same contaminated food source, or one family member may have become infected and then passed on the infection to the rest.

From receipt of bacterial culture, PFGE takes 5 days. FAFLP results, on the other hand, can be obtained and analyzed within 4 days, and analysis can be expedited by automation of a number of steps in the protocol, e.g. digestion–ligation and PCR reactions, and by separating the fragments on a capillary sequencer. An additional advantage of FAFLP is that data can be easily exported from the analysis programs into database programs such as Microsoft Excel or BioNumerics, where large data sets can be compiled, manipulated and interrogated.

In conclusion, FAFLP is an accurate and reproducible typing method capable of resolving all of the 25 PT strains of *Salmonella* Enteritidis. It could be used in conjunction with phage typing to maximize strain resolution for various epidemiologic applications, including rapid recognition of outbreaks.

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