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Use of AFLP and PFGE to discriminate between *Salmonella enterica* serovar Typhimurium DT126 isolates from separate food-related outbreaks in Australia

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SUMMARY

In 2001 *Salmonella enterica* serovar Typhimurium definitive phage-type (DT) 126 was isolated at higher frequency in Australia compared to other *S.* Typhimurium phage types and in comparison to previous years. Associated with this increase was the implication of this phage type in a number of food-related outbreaks. We compared fluorescent amplified fragment length polymorphism (FAFLP) to pulsed-field gel electrophoresis (PFGE), the current 'gold standard' for molecular typing of *Salmonella* for the discrimination between outbreak-associated isolates and epidemiologically unrelated DT126 strains. FAFLP showed a greater ability to discriminate between isolates than PFGE, with 16 groups of clusters or individual isolates with $<90\%$ similarity to each other compared to three groups as determined by PFGE. Both methods were able to discriminate between isolates from two separate outbreaks in South Australia and isolates associated with an outbreak at a restaurant in New South Wales. The resolving power of both methods was not sufficient to separate all epidemiologically unrelated DT126 isolates from the outbreak isolates. We conclude that amplified fragment length polymorphism is a useful tool to assist in the discrimination of *S.* Typhimurium DT126 isolates.

INTRODUCTION

Non-typhoid *Salmonella* remains one of the most common foodborne causes of bacterial gastroenteritis throughout the world. *Salmonella enterica* subsp. I serovars are generally implicated in the incidence of bacterial foodborne gastroenteritis. The most common serovars associated with gastroenteritis are Typhimurium and Enteriditis with other serovars such as Infantis being implicated but at much lower frequencies [1–5]. *Salmonella enterica* subsp. I serovar Typhimurium (*S.* Typhimurium) has been the most common *Salmonella* serovar isolated from humans by the Australian *Salmonella* Reference Centre (ASRC) in Adelaide from 1997 to 2002 [2, 6] (Table 1). In chickens it is the most common subsp. I serovar isolated by the ASRC, with only the subsp. II serovar Sofia isolated with greater frequency. Poultry-related products are not the only source of *S.* Typhimurium, it is also routinely isolated from bovine and porcine sources as well as a range of other food products [2, 6, 7].

S. Typhimurium is routinely subtyped by phage typing where resistance or sensitivity to a panel of typing phage determines phage type [8]. Phage typing provides a first level of isolate discrimination; however, it requires a specialist laboratory and can be highly subjective [9]. The predominance of one particular phage type within a serovar in a geographical and/or temporal region often reduces the effectiveness

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Table 1. *Percentage of serovar Typhimurium isolated from humans and chickens compared to other significant subsp. I serovars, 1997–2002, percentage of total subsp. I* Salmonella *isolated*

	Serovar					
	Typhimurium		Enteritidis		Bovismorbificans	
Year	Human	Chicken	Human	Chicken	Human	Chicken
2002	30·6	24·0	8·7	<1·1	10·3	1·6
2001	33·6	35·8	6·4	<1·0	11·3	4·4
2000	29·2	20·1	7·5	<0·9	8·3	1·4
1999	42·8	14·3	8·0	<1·1	5·5	<1·1
1998	22·0	15·7	8·0	<1·5	5·1	<1·5
1997	32·5	20·7	9·6	<1·0	6·1	<1·0

Source: Australian *Salmonella* Reference Centre (ASRC), 2001 and 2002 Annual Reports.

of this method in discriminating between outbreak strains and epidemiologically unrelated isolates. Furthermore, phage-type conversion due to acquisition of DNA such as plasmids or temperate phages complicates interpretation of typing results [1, 10–13]. Molecular methods have been introduced to enhance the discrimination of isolates in epidemiological studies; these include plasmid profiling, chromosomal fingerprinting, Southern hybridization and restriction fragment length polymorphism (RFLP). RFLP methods include IS*200* restriction mapping and pulsed-field gel electrophoresis (PFGE). For a recent review of classic and molecular typing methods of *Salmonella* see Winokur [14].

More recently amplified fragment length polymorphism (AFLP) has been developed to separate bacterial isolates including *Salmonella* at the genetic level [3, 7, 9, 15–18]. In this procedure, PCR primers target specific sequences ligated to either end of restriction endonuclease-digested genomic DNA rather than genomic DNA itself, generating a high number of fragments for analysis. The greater number of fragments available for analysis generated by this method compared to other molecular methods potentially increases the level of discrimination between isolates [19]. No knowledge of the organism's DNA sequence is required prior to analysis and since the primers are specific for the adaptors attached to the genomic fragments, highly stringent conditions can be applied to the amplification step(s), minimizing non-specific amplification. The use of fluorescently labelled primers has allowed the process to be automated, thus increasing the number of fragments that can be generated in an analysis without compromising band resolution. For a review of AFLP techniques see Mueller & Wolfenbarger [20].

Definitive phage-type 126 (DT126) of serovar Typhimurium has previously been reported from both human and chicken sources but usually only a maximum of 20 specimens from each source were isolated each year by the ASRC. In 2001, however, 150 were isolated from human patients while 315 were isolated from chickens [5] (Table 2). This represented a 15-fold increase in numbers for humans and a 30-fold increase in chickens making it the most common phage type isolated from both sources for that year. The increase in numbers were partly attributed to a number of outbreaks including two outbreaks in South Australia and a third outbreak in New South Wales (NSW), from which specimens were received by the ASRC in Adelaide.

Because of the increased frequency of Typhimurium DT126 isolates recovered from both humans and chickens in 2001 and the corresponding increase in the number of outbreaks attributed to this phage type it was decided to conduct a study to determine any genetic and epidemiological relationships between isolates and outbreaks. We used fluorescent amplified fragment length polymorphism (FAFLP) to discriminate between DT126 isolates and then compared those results to PFGE, the current 'gold standard' for molecular typing of *Salmonella*. We assessed the ability of both techniques to separate isolates of *S.* Typhimurium DT126 including those from three separate outbreak scenarios to determine the most discriminatory method for these types of analyses.

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Table 2. *Total numbers of* S. *Typhimurium and* S. *Typhimurium DT126 isolated from humans and chickens from 1997 to 2002*

	Human		Chicken	
Year	Total *S.*T	DT126	Total *S.*T	DT126
2002	548	54 (9·8)	527	78 (14·8)
2001	592	150 (25·3)	772	315 (40·8)
2000	402	11 (2·7)	387	11 (2·8)
1999	868	<16 (<1·8)	228	14 (6·1)
1998	390	<13 (<3·3)	473	64 (13·5)
1997	572	<13 (<2·2)	354	<9 (<2·5)

Source: Australian *Salmonella* Reference Centre (ASRC), Annual Reports 2001 and 2002.
*S.*T, *Salmonella* Typhimurium. Figures in parentheses in DT126 columns refer to percentage DT126 of total *S.* Typhimurium.

This paper represents one of the few studies so far where AFLP has been used to discriminate between *Salmonella* isolates within a phage type.

METHODS

Strains

All *S.* Typhimurium DT126 isolates were provided by the Australian *Salmonella* Reference Centre (ASRC), Institute of Medical and Veterinary Science, Adelaide, South Australia. Serotyping had previously been undertaken using the Kaufmann–White scheme and bacteriophage typing was performed using the Anderson scheme of 31 phages [8], both by the ASRC. All DT126 isolates were designated with XX-126-YYY designation, the XX indicating the year of isolation and YYY the numbering of each isolate.

The DT126 isolates included representative strains from two separate outbreaks that occurred in South Australia in 2001. Outbreak 1 (seven isolates provided) occurred in the early months of 2001 where dairy products were suspected of being the source of the outbreak. Outbreak 2 (11 isolates provided) occurred in the middle of the same year and chicken was suspected of being the primary source of infection. Eleven DT126 isolates were also provided from a 2001 outbreak that occurred in NSW (outbreak 3). The remaining isolates were obtained from epidemiologically unrelated clinical and environmental sources from around Australia.

FAFLP

Overnight cultures of isolates were prepared as described above. Total genomic DNA was extracted from the 10 ml culture by the method of Pitcher et al. [21] with minor modifications. The lysozyme step for Gram-positive bacteria was incorporated and sarkosyl was omitted from the guanidium thiocyanate step. FAFLP of *Salmonella* was based on the *Mse*I/*Eco*RI protocol developed by Vos et al. [16]. The FAFLP restriction endonuclease digestion/ligation and preselective and selective PCR steps were undertaken by the method described in the AFLP™ Microbial Fingerprinting Protocol Handbook (PE Applied Biosystems, Foster City, CA, USA). Restriction endonucleases were obtained from New England BioLabs (Beverley, MA, USA). Core reaction mixtures for the PCR were obtained from Applied Biosystems (part no. 402005). Adaptor sequences with *Mse*I and *Eco*RI ends as well as primers for the preselective and selective PCRs including the 5′-labelled *Eco*RI selective primer are described elsewhere [7, 16]. All oligonucleotides were supplied by Geneworks (Adelaide, South Australia).

PCR fragments were analysed with an ABI Prism 377 Sequencer (PE Applied Biosystems) as per the manufacturer's protocol. Briefly, 1·8 μl of selective PCR product was added to an equal volume of GeneScan-1000 size standard and run under denaturing conditions on the sequencer. Fragments were resolved on a 36 cm, 5% long-range/6 M urea gel at 51 °C. Computer analysis of the resolved fragments was undertaken with the GeneScan Analysis version 3.1 program (PE Applied Biosystems).

Data analysis of FAFLP

Spreadsheet data generated by the GeneScan Analysis program was converted to PC format and opened in Microsoft Excel with file origin as 'DOS or OS/2 (PC-8)'. The 'Minutes' and 'Data point' columns were deleted and the files reformatted for eventual analysis by GelCompar IV software (Applied Maths, Kortrijk, Belgium). These files were then saved as 'Text (OS/2 or MS-DOS)' files. Files were then converted via the 'Molecular weight to gel' program and saved as.int files prior to analysis in GelCompar IV. Comparison or relatedness of isolates was undertaken by a dendrogram generated by 'Cluster by bands' with relatedness between isolates determined by Dice coefficient and clustering by the unweighted pair group method with arithmetic means (UPGMA). A threshold of

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similarity of $\geqslant 90\%$ as accepted in the literature was used to define genetically similar isolates [18, 22].

Chromatogram files generated by the sequencer in Mac format were converted to PC format and given a '.fsa' extension. Files were imported into Genotyper version 3.6 NT (PE Applied Biosystems) (Windows 2000 NT operating system) for detailed analysis of peaks.

PFGE

A subset of isolates representing the complete spectrum of clustering patterns determined by FAFLP were selected for analysis by PFGE. The protocol for PFGE followed that of Maslow et al. [23]. Briefly, cells grown overnight in brain heart infusion (BHI) broth (Oxoid, Thebarton, South Australia) were embedded in agarose and lysed by incubating the plug in 4 ml lysis buffer supplemented with 4 mg lysozyme (Roche, Mannheim, Germany) and 80 μg/ml RNAse. Plugs were then digested with proteinase K, washed and the DNA digested overnight with *Xba*I restriction endonuclease (New England BioLabs). The next day the plugs were placed into the wells of a 1% agarose gel prepared with PFGE-grade agarose (Bio-Rad Laboratories, Hercules, CA, USA) in 0·5× TBE buffer [24]. The PFGE was run on a Bio-Rad CHEF-DR® III system for 19 h at 6·0 V/cm at 4 °C, initial switch time 2 s, final switch time 50 s. After running the gel was stained in ethidium bromide, destained in water and photographed under UV light.

Staphylococcus aureus strain NCTC 8325 DNA digested with *Sma*I was used as a control and marker strain for normalization of gels during post-electrophoresis analysis [25]. The preparation of control plugs was virtually identical to the plug preparations for *Salmonella* except that 4 ml overnight culture was added to ice-cold PIV buffer [23] prior to centrifugation, lysostaphin (5 U/ml final concentration) was added to the lysis mixture instead of RNAse and the plugs were digested with *Sma*I at 25 °C.

Computer analysis of PFGE

A photograph of the gel was scanned into a computer and a .tif image generated. The image was optimized in GelCompar IV software (Applied Maths) and normalized using the *S. aureus* control as per the program guidelines. A dendrogram showing relatedness of isolates was generated with similarity by Dice coefficient and clustering determined by UPGMA as for FAFLP analysis described above. As for FAFLP analysis, a threshold of similarity of $\geqslant 90\%$ was used to define genetically similar isolates.

RESULTS

FAFLP

A total of 92 *S.* Typhimurium DT126 isolates were analysed by the *Eco*RI(A) and *Mse*I(A) FAFLP selective primer combination. FAFLP analysis of the isolates revealed one large cluster of 65 isolates with approximately 89% similarity. This cluster was divided into two subgroups, A1 and A2, based on the arbitrary $\geqslant 90\%$ threshold point for similarity (Fig. 1). The two subgroups A1 and A2 contained 34 and 31 isolates respectively. These two groups contained the isolates attributed to outbreaks 1 and 2, both of which occurred in South Australia in a 6-month period in 2001. Isolates from these two outbreaks were evenly distributed throughout the two groups. Six outbreak 1 isolates were clustered within subgroups A1 and A2 with one remaining isolate, 01-126-026, showing approximately 85% similarity to the other outbreak 1 isolates. Eight outbreak 2 isolates were found within subgroups A1 and A2 with a further three isolates (01-126-002, 01-126-006, 01-126-009) showing $>80\%$ similarity to the other outbreak 1 isolates. As well as containing most of the isolates from outbreaks 1 and 2 subgroups A1 and A2 contained a further 51 epidemiologically unrelated isolates from the most populated regions of Australia. These isolates were predominantly obtained from clinical patients or from chickens and chicken meat-related industries while a further four were from non-poultry domestic animal sources (01-126-007 feline, 01-126-011 canine, 99-126-040 and 99-126017 both equine) and one was from quail (01-126-019). It was not known whether the quail was from a commercial producer or was wild game.

The eight isolates tested from outbreak 3 which included clinical isolates as well as isolates obtained from the suspected sources of the outbreak predominantly clustered together in group B. No other DT126 isolate tested fell within this group.

Analysis of the chromatograms and comparison of spreadsheet data relating fragment length to relative fluorescence indicated that all isolates had similar major peaks based on fragment length. Differences in relative fluorescence (peak height) of corresponding peaks of individual isolates were observed. The

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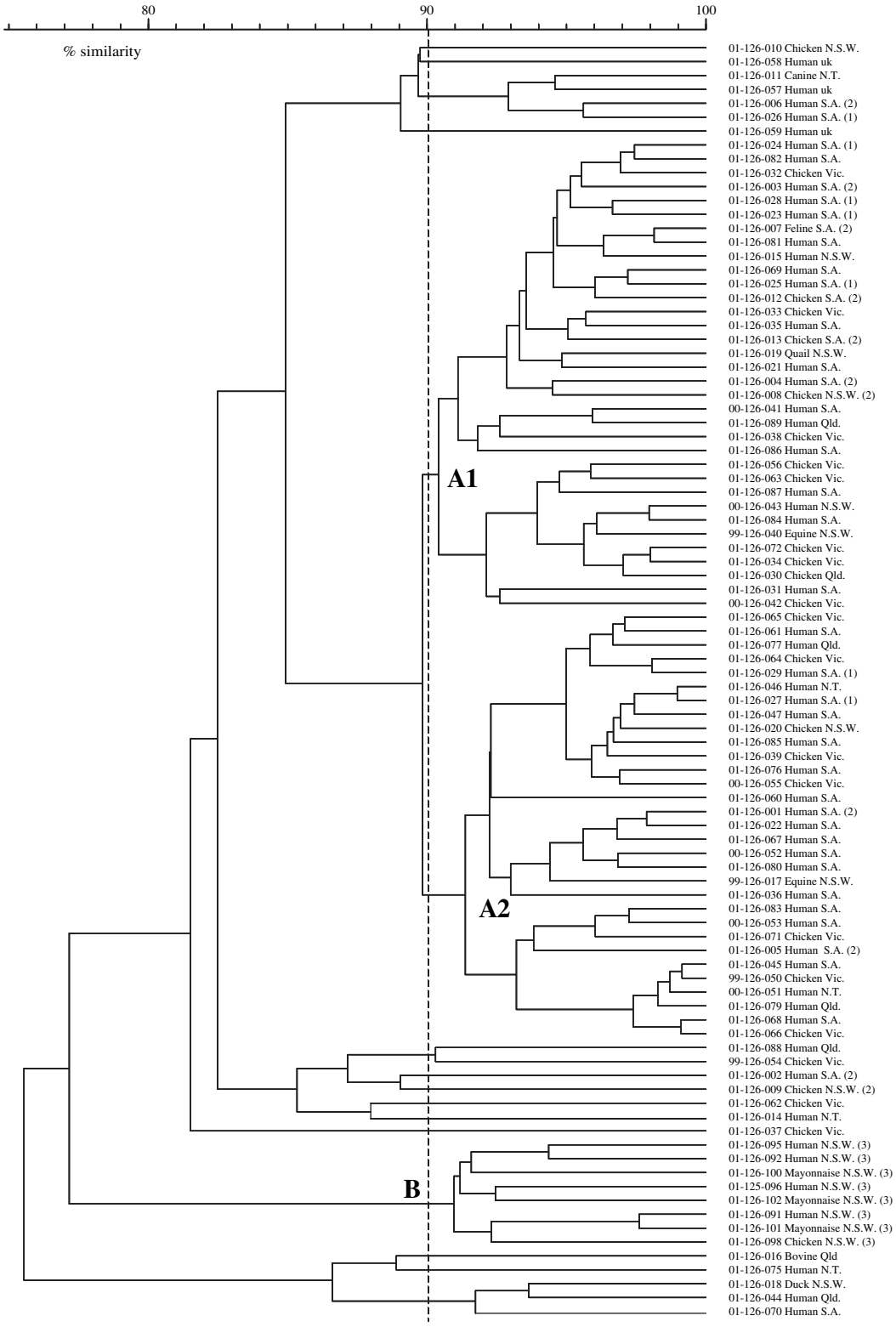

Fig. 1. Dendrogram showing similarity of DT126 isolates as determined by FAFLP. Groupings based on a threshold of $\geqslant 90\,\%$ similarity are indicated in bold. Isolates from the three outbreaks are designated (1), (2) and (3). Most isolates associated with outbreaks 1 and 2 clustered within subgroups A1 and A2. The isolates from outbreak 3, the NSW restaurant outbreak clustered in a separate group. Abbreviations for Australian states and territories are: N.S.W., New South Wales; N.T., Northern Territory; Qld., Queensland; S.A., South Australia; Vic., Victoria; W.A., Western Australia; uk, Unknown.

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chromatograms of isolates from outbreaks 1 and 2 were very similar, with minor differences, predominantly in peak height, being noted. The variations observed appeared to be similar to peak height variations of epidemiologically unrelated isolates. The chromatograms of the outbreak 3 isolates differed from the other profiles. Major differences included a missing 145-bp peak, and unique peaks at 265, 339, 379 and 404 bp. Variation in height of other major peaks of outbreak 3 isolates appeared to be similar to the variation in peaks for isolates from outbreaks 1 and 2 as well as other isolates described above.

PFGE

A subset comprising 70 isolates from the FAFLP analysis including isolates from the three outbreaks were tested by PFGE. All isolates selected for PFGE analysis were typable by this method. Two main groups comprising isolates of $\geqslant 90\%$ similarity were generated by PFGE (Fig. 2). Group A comprised 54 isolates and included all test isolates attributed to the two South Australian outbreaks (outbreaks 1 and 2). Group C contained 12 isolates, all were associated with outbreak 3. A third group (group B) comprised only four epidemiologically unrelated isolates. Interestingly, all four isolates in this group were from diverse sources, in host and geographical location and in the case of the two NSW isolates, isolated over a 2-year time period.

Comparison of all DT126 isolates tested by PFGE indicated a high level of genetic relatedness. More than 50% of isolates (41 isolates) clustered in group A showed 99% similarity (Fig. 2) and 10 of the remaining 13 group A isolates with $\geqslant 90\%$ similarity clustered in two subgroups of 100% homology in each subgroup.

Comparison of similarity between FAFLP and PFGE

A comparison was made to see whether the same isolates which clustered in one group as determined by FAFLP clustered together when analysed by PFGE. The isolates from outbreaks 1 and 2 which clustered in group A when analysed by PFGE tended to fall predominantly into FAFLP subgroup A1 (four and six isolates respectively) with some falling into group A2 (two and four isolates respectively). In total, nearly 80% of PFGE group A isolates clustered in either FAFLP subgroup A1 or A2. Ten PFGE group A isolates separated out as 'unrelated' strains when analysed by FAFLP. The isolates from outbreak 3 formed a distinct cluster by both PFGE and FAFLP. Using a threshold of similarity of $\geqslant 90\%$ for discrimination described above, PFGE generated three groups each containing at least four isolates while AFLP generated 16 groups, 10 of which comprised individual isolates only.

DISCUSSION

Prior to 2001 *S.* Typhimurium DT126 was only isolated infrequently compared to many other phage types of this serovar. However, during 2001, there was a sudden upsurge in the number of DT126 isolates received by the ASRC in Adelaide. Associated with this increase were a number of food-related outbreaks attributed to DT126 strains. These included two local outbreaks in South Australia and a third outbreak linked to a restaurant in NSW. The increase in numbers of DT126 isolates also included strains isolated from epidemiologically unrelated sources throughout Australia. Fluorescent AFLP and PFGE were employed to determine the relatedness of the DT126 isolates and results compared to determine which method provided the greater level of discrimination between strains.

PFGE data indicated that most isolates including those from the two outbreaks in South Australia are clonal (Fig. 2, group A). A small number of isolates from various Australian states were found to be distantly related to the main PFGE cluster. Conversely the isolates from the restaurant outbreak in NSW formed a more distinct group (C), separate from other DT126 strains examined in this study. This confirms that the NSW restaurant outbreak was caused by a distinct DT126 strain as there were no epidemiologically unrelated isolates detected in this study showing similarity to the restaurant outbreak strains.

By comparison, FAFLP divided the DT126 isolates into 16 groups comprising either clusters or individual isolates (Fig. 1). Like PFGE, analysis of the FAFLP data suggests a close relatedness between the isolates involved in the two South Australian outbreaks. FAFLP chromatogram analysis revealed very few major differences between peaks corresponding to base-pair length of the South Australian outbreak isolates and other closely related but epidemiologically distinct isolates (data not shown). Variation between isolates appeared to be due more to fluctuations in peak intensity which is a function of PCR efficiency [19]. However, there was variation observed in the

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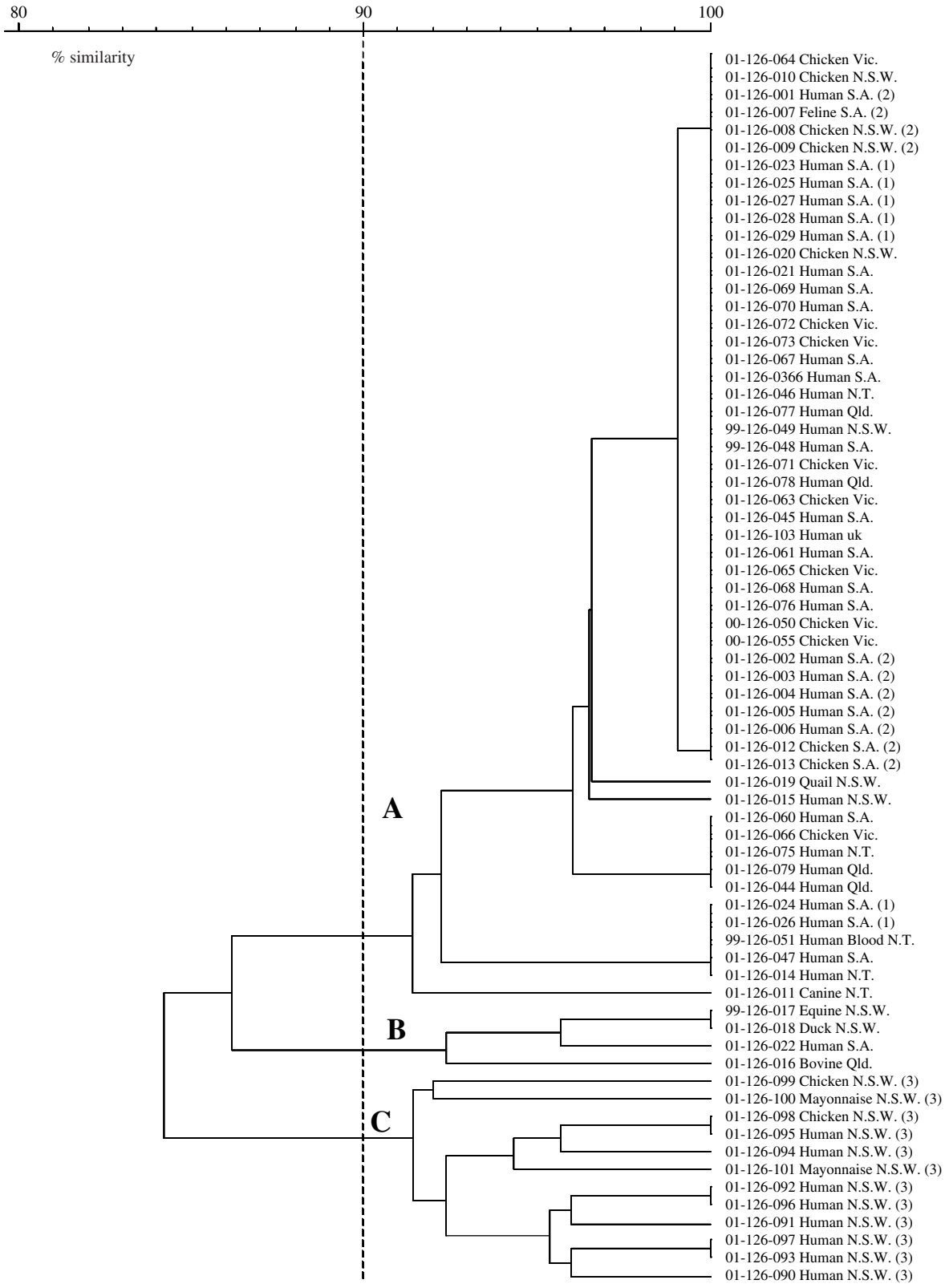

Fig. 2. Dendrogram showing similarity of DT126 isolates as determined by PFGE. All isolates associated with outbreaks 1 and 2 clustered in group A and the NSW restaurant outbreak isolates clustered together as a unique group in group C. Isolates from the three outbreaks are designated (1), (2) and (3). Abbreviations for Australian states and territories are: N.S.W., New South Wales; N.T., Northern Territory; Qld., Queensland; S.A., South Australia; Vic., Victoria; W.A., Western Australia; uk, Unknown.

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presence or absence of other peaks in individual chromatograms (and accompanying spreadsheets) which accounted for the separation of isolates observed.

Isolates from the two South Australian outbreaks did separate out into different groups when analysed by AFLP. In fact, four isolates (01-126-026 from outbreak 1 and 01-126-002, 01-126-006 and 01-126-009 from outbreak 2) were found to have $<90\%$ similarity when analysed. It is possible that particular isolates had acquired or lost DNA in the form of plasmid or phage DNA which may account for the variation observed. However, variation due to peak intensity may mean that the FAFLP similarity index of $\geqslant 90\%$ is set too high. Reducing the similarity index threshold to 88% would cluster nearly all the isolates from outbreaks 1 and 2 together in one group (except isolates 01-126-002 and 01-126-009). This would also reduce the number of clusters determined by FAFLP from 16 to nine (compared to three by PFGE). The threshold of similarity would need to be further reduced to 82% to include all isolates from outbreaks 1 and 2 into one cluster, leaving four FAFLP clusters. As it is possible that these isolates have acquired or lost DNA prior to analysis and that the separation observed is genuine it is felt that this threshold would be artificially low and not a true reflection of the genetic similarity of these isolates.

While both analyses suggest that the two outbreaks in South Australia appeared to be caused by the same clone although there is no epidemiological evidence to suggest there was any link between the two outbreaks. The bakery outbreak (outbreak 1), first notified in early March 2001, identified the consumption of custard-filled pastries as a common feature of presenting cases. Subsequent investigations traced the source to a bakery which distributes its products to 59 outlets in the Adelaide metropolitan area, as well as supplying a number of commercial caterers. While poor hygiene and food-handling practices were identified at the source, the original source of the DT126 contamination remains unknown. The other South Australian outbreak (outbreak 2), involving a total of 93 cases, was linked to the consumption of contaminated chicken meat and was subsequently traced to a local chicken meat processor. Data obtained from by the processor indicated that contamination occurred between May and September, just prior to and during the outbreak. Action to remedy this contamination was implemented and as a result, the number of reported cases declined [26]. Further information regarding these two outbreaks, including investigation reports and follow-up actions, can be obtained from the Australian Government [27].

Data generated by PFGE and FAFLP suggest that there is generally a high degree of similarity between of *S.* Typhimurium DT126 isolates in Australia. Similar studies of *Salmonella* serovars and phage types show that there is variation in levels of relatedness of any given serovar and/or phage type [28]. As would be expected, there is greater diversity between serovars and less diversity within phage types. Lindstedt et al. [17] found that different serovars of *Salmonella* could be readily be separated by AFLP, but successful separation of epidemiologically unrelated isolates within each serovar by AFLP varied. By contrast Gebreyes & Altier [29] successfully employed AFLP to separate isolates of *S.* Typhimurium partially based on phage-type (seven DTs) and multidrug-resistance patterns.

This study has demonstrated that, in general, clustering patterns generated by FAFLP match those patterns obtained by PFGE. This is confirmed by the separation of the NSW restaurant outbreak isolates from the main clusters as well as most PFGE group A isolates, including the two South Australian outbreak isolates, were clustered in the two FAFLP group A subgroups A1 and A2. This clustering may have been assisted in part to the nature of the collection being examined as all isolates were isolated in Australia and most were obtained during a single calendar year. This may also explain the apparent high level of genetic similarity of the DT126 isolates observed.

Employment of AFLP has increased in recent years for the discrimination of closely related strains of bacteria (as well as other organisms). AFLP has been used for a variety of analyses of *Salmonella*, from separation of serotypes and serovar phage types [30] to the determination of genetic variability of multidrug-resistant isolates [29] and separation of serovar Typhimurium phage types and detection of potential markers for phage typing by molecular methods [3, 9]. AFLP offers a level of discrimination at least equal to that of PFGE (or any other typing methods) and often provides a greater level of separation of individual isolates (although there are some exceptions to this rule). Furthermore the method is highly reproducible. Previous papers have demonstrated that results of AFLP are reproducible despite the greater number of DNA preparatory steps required compared to PFGE [18, 19, 31]. Previous work in our laboratory with *S.* Sofia, a serovar that is not always amenable to

PFGE analysis has shown that the technique gives reproducible results [7].

In conclusion we have demonstrated the practical use of AFLP for the discrimination of closely related strains of a single phage-type of *S.* Typhimurium. AFLP potentially provides a level of discrimination of isolates of DT126 equal to or greater than PFGE. However, because the sensitivity of AFLP in detecting minor genetic events increases the level of separation compared to other molecular methods, data must be assessed in combination with epidemiological information to determine the status of particular isolates. We intend to continue to use both methods to analyse strains received from the community and combine this data with classical serotyping and phage typing along with epidemiological information to monitor *Salmonella* population diversity and the incidence of outbreaks in Australia.

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