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New Options for Rapid Typing of *Salmonella enterica* Serovars for Outbreak Investigation

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1. Introduction

A number of different serovars of *Salmonella enterica* are often implicated in human non-typhoidal outbreaks. Globally, serovars Typhimurium and Enteritidis are often the causative agent of such outbreaks, but other serovars can also be significant (Table 1). Some serovars such as Infantis and Virchow are routinely linked to outbreaks of gastroenteritis. The consumption of raw food products or poor food handling practices and/or storage procedures is often a catalyst for such outbreaks (Behravesh et al., 2010).

Accurate monitoring and tracking of specific *Salmonella* strains is of paramount importance, especially during an outbreak scenario. Large scale outbreaks such as the multistate *S.* Saintpaul outbreak in the U.S.A. in 2008 that implicated peppers as the source of contamination (Behravesh et al., 2010) highlight the need for high resolution testing procedures to enable confident identification of the source(s) of the outbreaks. Incorrect identification of potential sources will delay controlling and limiting the spread and health impact of outbreaks. Typing methods must be in place must not only identify an outbreak strain but also distinguish that strain from closely related but genetically distinct strains of the same serovar. However, many serovars of *Salmonella enterica* can appear clonal, making differentiation of strains difficult. Consequently, a high resolution typing system is required to distinguish individual strains within a serovar.

S. enterica can be subdivided into over 2,500 serotypes and, this, while useful in initial identification does not provide any further information. Classical methodologies such as bacteriophage (phage) typing can provide further subdivision within a serovar. Bacteriophage typing is a widely used phenotypic method for differentiation of clinically significant *Salmonella* serovars including Typhimurium, Enteritidis and Virchow. However, phage typing is a specialist methodology and is often unavailable to laboratories undertaking routine surveillance of *Salmonella*. Furthermore, particular phage types may dominate I a region over a period of time. This potentially makes identification of an outbreak strain difficult. Pulsed-field gel electrophoresis (PFGE) has routinely been employed for subtyping serovars and, where applicable, subtyping phage types of serovars such as Typhimurium, Enteritidis and Virchow. More recently Multiple-Locus Variable-number tandem-repeats (VNTR) Analysis (MLVA) (Lindstedt et al., 2003) and Multiple Amplification

of Phage Locus Typing (MAPLT) (Ross & Heuzenroeder, 2005) have been developed as PCR-based methodology for rapid, high resolution subtyping of *Salmonella* serovars.

Serovar	Year(s)	Associated food product	Country
Agona	2008	Processed cereal products	U.S.A.
Anatum	2006	Herbs (basil)	Denmark
Bareilly	2010	Bean sprouts	U.K.
Bovismorbificans	2001	Fast food outlets	Australia
Braenderup	2008	Egg product	Japan
Chester	1999	Cuttlefish chips	Japan
Derby	2006	Restaurant food	Japan
Havana	1998	Alfalfa	U.S.A.
Hvittingfoss	2005	not determined	Australia
Infantis	1999	Poultry	U.S.A.
Kedougou	2008	Infant formula	Spain
Montevideo	2007–2008	Various	Japan
Ohio	2005	Pork	Belgium
Oranienburg	1999	Cuttlefish chips	Japan
Potsdam	2002	Restaurant salad dressing	Australia
Saintpaul	2008	Raw produce	U.S.A.
Saintpaul	2009	Alfalfa	U.S.A.
Schwarzengrund	2006	Dry dog food	U.S.A.
Senftenberg	2007	Herbs (basil)	U.K.
Singapore	2004	Sushi	Australia
Tennessee	2006	Peanut butter	U.S.A.
Virchow	1997-1998	Sun-dried tomatoes/garlic	Australia
Virchow	2004-2009	Various	Switzerland

Table 1. Examples of non-typhoidal or Typhimurium and Enteritidis *S. enterica* serovars implicated with foodborne gastroenteric outbreaks

MLVA targets loci harbouring short tandem repeat sequences, using PCR with the product analysed for fragment length by capillary electrophoresis (Lindstedt et al., 2003) and separates isolates based on the number of tandem repeats in each locus. For *S.* Typhimurium, five loci have been described and a protocol for analysis described by The Institut Pasteur (www.pasteur.fr/recherche/genopole/PF8/mlva/). Loci for other serovars of interest have been described including Enteritidis (Boxrud et al. 2007; Malorney et al., 2008; Ramisse et al., 2004), Typhi (Lui et al., 2003) and Infantis (Ross & Heuzenroeder, 2008) although an agreed MLVA protocol for these serovars is yet to be ratified.

MAPLT is a multiplex PCR-based approach which detects prophage loci located within the *Salmonella* genome (Ross & Heuzenroeder, 2005). The assay is a binary method and is based on the presence or absence of particular loci. Depending on the design of primers for each locus, prophage PCR products can be simply detected by agarose gel electrophoresis, or they may be detected by capillary sequencing in the same manner as MLVA, or by real-time PCR. MAPLT primers have been described for serovars Infantis (Ross & Heuzenroeder, 2008), Typhimurium (Ross et al., 2009) and Enteritidis (Ross & Heuzenroeder, 2009).

While both methods usually provide resolution equivalent to that generated by PFGE, often particular loci within an assay do not provide sufficient allelic diversity for maximum isolate separation. For example, it has been reported that a number of different sized fragments for the plasmid-based MLVA locus STTR-10 in a range of Typhimurium isolates (Lindstedt et al., 2004). Conversely, specific definitive phage types (DTs) of serovar Typhimurium were found to have little or no allelic variation for this locus (Ross & Heuzenroeder, 2005). Routine analysis of human, food and environmental isolates of a range of Typhimurium phage types including untypable and **r**eactive-**d**oes **n**ot **c**onform (RDNC) isolates suggest that both STTR-9 and STTR-3 display much reduced allelic diversity compared to the other three loci (STTR-5, STTR-6 and STTR-10) (Ross & Heuzenroeder, unpublished data). This is particularly evident within specific definitive types. MAPLT data generated by our laboratory shows a similar phenomenon where some prophage loci are found in >95% of *Salmonella* isolates or are completely absent. For example, $gtrC_{ST64T}$ is generally found in many *S.* Typhimurium tested but the $gtrA_{ST64T}$ was rarely detected in the same group of isolates (Ross & Heuzenroeder, 2005).

Salmonella enterica serovar Virchow (*S.* Virchow) is a relatively less common serovar, showing a prevalence to certain geographic regions. In recent years *S.* Virchow has ranked among the top 10 serovars among human isolates in countries located in the African, European, Oceania, Latin American and Caribbean regions (Galanis et al., 2006). Australia is one country where *S.* Virchow has always been endemic, particularly in the Australian state of Queensland and has ranked among the ten most common serovars derived from human source since as early as 1991 (Australian Salmonella Reference Centre [ASRC], 1999-2009). *S.* Virchow is a ubiquitous organism that can be detected in various food animals and environmental sources such as chickens, pigs, horses and sewage sludge (ASRC, 1999-2009). However poultry and poultry-related products were reported to be the most prevalent reservoir in a number of countries. Over a ten year period to 2009, the majority of *S.* Virchow isolates received and serotyped by the ASRC were from poultry and eggs. In the United Kingdom *S.* Virchow has been linked to chickens and chicken-related products (Threlfell et al., 2002; Willocks et al; 1996). *S.* Virchow is a public health concern as a significant causative agent of food-borne gastroenteric outbreaks and severe extra-intestinal infections. Poultry are the main, but not exclusive reservoir of this serovar (Adak & Threlfall, 2005; Maguire et al., 2000; Semple et al., 1968). While some reported outbreaks in different countries were poultry-associated other food sources implicated in *S.* Virchow outbreaks included sun-dried tomatoes and processed milk products (Bennett et al., 2003; Taormina et al., 1999; Uresa et al., 1998). Systemic *S.* Virchow infections in young children have also been reported in Australia and the United Kingdom (Ispahani & Slack, 2000; Messer et al., 1997).

The current international phage typing scheme for *S.* Virchow was developed in 1987 and comprises 13 typing phages (Chamber et al., 1987). Fifty-seven lysis patterns or phage types have been identified (Torre et al., 1993). Phage types (PTs) 8 and 26 are the most predominant phage types in the UK consisting of 50% of isolates (Torre et al., 1993). Australia and Spain are the other two countries routinely using phage typing routinely. In Spain from 1990-1996 the most frequently isolated *S.* Virchow phage types were PTs 8, 19 and 31 (Martín et al., 2001), whereas in Australia the same period the most common phage types were PTs 8, 31 and 34 (ASRC, 1999-2009). These results demonstrate the important role of phage typing in the global surveillance of the *S.* Virchow population. It also indicates that PT8 is a global phage type predominating in endemic countries, whereas PTs 26 and 34

seem to be geographically specific to the UK and Australia respectively (Sullivan et al., 1998). In addition phage typing acts as a long-term epidemiological typing tool revealing any changes in incidence of *S.* Virchow phage types within a particular source. With respect to *S.* Virchow in Australia, no significant changes in the incidence of phage types were observed from human source in the decade to 2009 where PT8 was the most prevalent phage type (>50%) (ASRC, 1999-2009). In contrast, there were noticeable changes in the *S.* Virchow population in chickens and eggs based on the *S.* Virchow isolates received by ASRC. Even though PT8 was most commonly isolated from chickens and eggs in most years during the same period of time, the proportion of PT8 within these sources has decreased from 81.9% in 2000 to 35.5% in 2009 (ASRC, 1999-2009).

This chapter describes how specific data from MLVA and MAPLT can be combined into a single composite assay, thereby maximizing the resolving power of the assay for closely related isolates. The two classical typing methods, PGFE and phage typing provide a benchmark for determining the efficacy of MLVA and MAPLT, both individually and as a composite methodology. Previously published data for the two most significant non-typhoidal serovars, Typhimurium and Enteritidis have been re-analysed to determine the most variable loci for each protocol and single assays containing these loci have been identified for each serovar. The addition of phage typing data for serovar Enteritidis has also been taken into consideration to determine whether loci selection can be influenced by phage type. MLVA and MAPLT protocols have been developed for serovar Virchow with comparisons with PFGE. A single MLVA/MAPLT hybrid assay for *S.* Virchow has been developed and described here for the first time.

2. Materials and methods

2.1 *S.* Virchow strains and culture conditions

A total of 43 epidemiologically unrelated *S.* Virchow isolates were used for the development of MLVA and MAPLT assays. The isolates were provided by the ASRC, Institute of Medical and Veterinary Science, Adelaide, South Australia. The isolates represented a cross-section of the most commonly identified phage types submitted to the ASRC and were originally isolated throughout Australia between 2005 and 2008. Serotyping and phage typing of all *S.* Virchow isolates had previously been undertaken by the ASRC. Unless otherwise stated, all isolates were routinely cultured either on XLD agar medium or in bovine heart infusion broth (BHI) (Oxoid) at 37^{o}C.

2.2 MLVA of *S.* Virchow

MLVA was undertaken utilizing primer sets previously described for Typhi (Liu, et al., 2003), Typhimurium (Lindstedt et al., 2003; Lindstedt et al., 2004) and Enteritidis (Boxrud et al., 2007; Ramisse et al., 2004) Primer sets targeting specific MLVA loci were selected based on their ability to differentiate within particular serovars of *Salmonella*. The touchdown PCR reaction and thermal cycler conditions were the same as those previously described (Ross & Heuzenroeder, 2005). Confirmation of fragment lengths as determined by genotyping was undertaken by nucleotide sequencing of selected isolates using Big Dye Terminator, version 3-1 (Applied Biosystems, Foster City, Calif.). Both genotyping and nucleotide sequencing were performed on an Applied Biosystems 3700 DNA Analyser. Data were entered into

BIONUMERICS v4.61 software (Applied Maths, Kortrijk, Belgium) as numerical values (fragment lengths in base pairs (bp) and negative PCR results entered as '0'). Dendrograms depicting the genetic similarity of isolates as determined by their MLVA profiles were generated using the categorical multi-state coefficient with zero tolerance and clustering by UPGMA utilising BIONUMERICS v4.61software (Applied Maths).

2.3 MAPLT of *S.* Virchow

Phages were induced from *S.* Virchow isolates as previously described (Ross & Heuzenroeder, 2008). Ten microlitres of each phage suspension were spotted onto lawns of epidemiologically distinct *S.* Virchow indicator isolates, allowed to dry and incubated at 37^{o}C until plaquing could be observed. Phages that generated different lysis profiles (Fig. 1.) were selected for DOP-PCR to detect different phage sequences. DNA was extracted from phage and DOP-PCRs were undertaken as previously described (Ross & Heuzenroeder, 2009). Unique bands (Fig. 2.) were extracted from agarose gels and cloned into the vector PCRs 4-TOPO and transformed into TOPO One Shots TOP10 chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Amplification of cell lysates using the TOPO primers was followed by sequencing PCR, undertaken with Big Dye Terminator v3-1 (Applied Biosystems, Foster City, CA). Characterization of sequence data was subsequently performed with KODON v3.5 (Applied Maths) and sequences compared with genomic library data for phage identification.

MAPLT analysis was undertaken with the primer combinations derived from prophages ST64B and P22 as published previously (Ross & Heuzenroeder, 2005), as well as loci identified by DOP-PCR from S. Virchow-derived prophages (Table 2). Amplification conditions using touchdown PCR and subsequent analysis were carried out as described previously (Ross & Heuzenroeder, 2005). MAPLT profiles for the *S.* Virchow isolates were determined based on the presence or absence of PCR product for all loci tested.

Fig. 1. Detection of different *S.* Virchow-derived bacteriophages by comparing plaquing patterns on lawns of *S.* Virchow isolates V15, V11 and V09 (as examples). By detecting differences in these patterns, potentially genetically different phages can then be isolated and identified by DOP-PCR and sequencing. This method results in a range of MAPLT primers that can detect a broad range of phage sequences in *S.* Virchow.

2.4 PFGE of *S.* Virchow

The protocol for PFGE was based on that of Maslow et al., (1993) as modified by Ross & Heuzenroeder (2005). Agarose-embedded *Salmonella* DNA and the *Staphylococcus aureus* strain NCTC 8325 marker DNA (Tenover et al., 1995) were digested overnight with the restriction endonucleases XbaI and SmaI, respectively (New England BioLabs Beverley, MA). The PFGE running conditions in the BIO-RAD CHEF-DR III System and subsequent comparisons of band profiles were undertaken as described previously (Ross & Heuzenroeder, 2005) using the GELCOMPAR II program (Applied Maths).

2.5 Data analysis

Comparison of the discriminatory power of all typing methods was undertaken using Simpson's index of diversity (Hunter & Gaston, 1988).

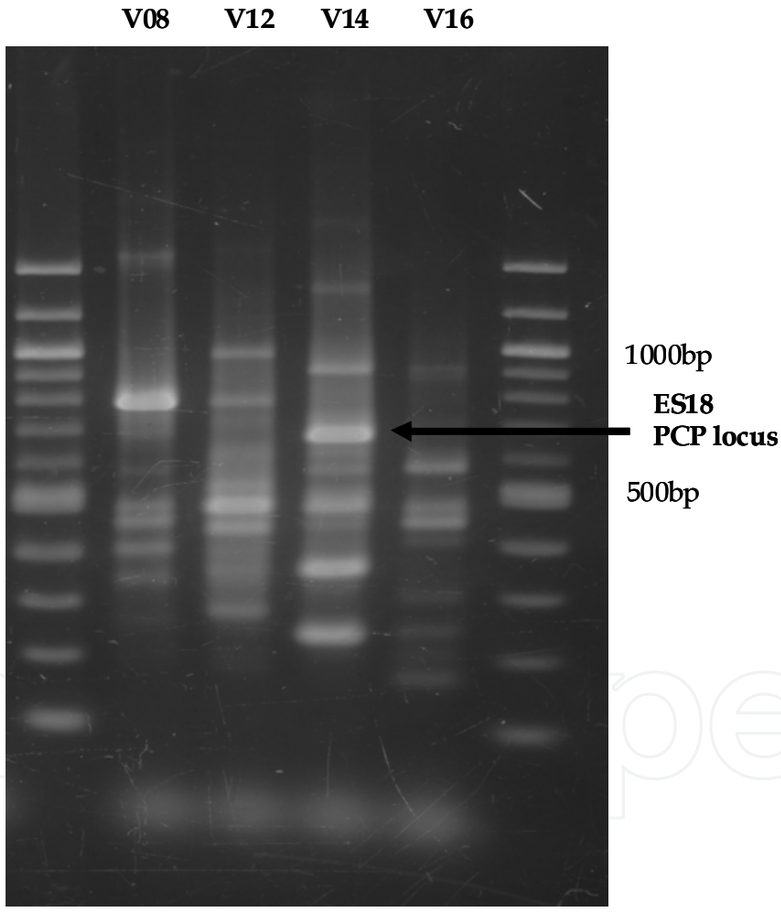


Fig. 2. DOP-PCR amplified phage DNA from *S*. Virchow isolates (V08, V12, V14 and V16). Individual bands were excised, cloned and sequenced to identify phage (see text for details). Phage from *S*. Virchow isolate V08 contained Fels2 sequences, V14 contained sequences from phage ES18 and V16 contained phage sequences from P186. The band containing the ES18 portal capsid protein sequence (PCP) is indicated as an example. No phage sequence was analysed from isolate V12 at time of publication. Molecular weight marker (first and last lanes) is a 100kb ladder.

Table 2. Primers for MAPLT analysis of *S.* Virchow

Phage	Gene or locus	Encoded proteins	Primers
P22	*nin*B	ninB protein	ninBF1: AACCTTTGAAATTCGATCTCCAGC ninBR1: CTTCGTCTGACCACTTAACGC
ES18	*gene 9*	putative coat protein	PCPF: TGGAACGCACAGCATGATGC PCPR: GGACTGCACCTGAATATTCGG
Fels2	STM2736	CII protein	Fels2cIIF: TGTATGGAAACGGCAGCCAG Fels2cIIR: GTCACAACATGGCGAAGCTG
Gifsy-1	STM2608	Terminase large subunit	Gifsy1AF: GATCACGCATCCATTATGTTCAC Gifsy1AR: TATTCCCGTACCGCTTACCAC
186	*cII*	CII protein	P186cIIF: GACATAGCGGGATTAGTCTGC Fels2cIIR: GTCACAACATGGCGAAGCTG
18b	gene P	Endolysin	P186PF: TCACCGATTACAGCGACCAC P186PR: TGGTGACCAGCTTTTCGAGAC
ST64B	SB04	Putative portal protein	SB04F: TGTCATACGACACCTATACCG SB04R: TGTTCTGCACCATGTGCAATG
P7	*sit*	Putative injection transglyosylase	P7sitF: TGACCTTGATCGCGTACTCAC P7sitR: TAGCCACCAGGAGACATCTG
V16[(i)]	DOP13.7	Possible tail fibre protein	13.7F: CGGTTAGCTCCGTGGTTAAG 13.7R: TAGCCACCAGGAGACATCTG

[(i)] Gene or locus accession numbers as follows:
P22: GeneBank accession no: AF217253
ES18: GenBank accession number AY736146
Fels2: GenBank accession number AE006468 (Prophage sequence of *Salmonella* Typhimurium strain LT2 fr
Gifsy-1: GenBank accession number AE006468 (Prophage sequence of *Salmonella* Typhimurium strain LT2
186: GenBank accession number U32222.1
ST64B: Genbank accession number AY055382
P7: GenBank accession number AF503408
[(ii)] Unidentified prophage loci in *S.* Virchow isolate V16

3. Results

3.1 Composite data for *S.* Typhimurium

Ten loci comprising seven MAPLT and three MLVA sites were selected for analysis in the development of a combined MAPLT/MLVA protocol; $c1_{ST64B}$ $SB06_{ST64B}$, $SB26_{ST64B}$, $SB28_{ST64B}$, $SB46_{ST64B}$, gene 9_{ST64T}, $gtrC_{ST64T}$, STTR-5, STTR-6 and STTR-10. A dendrogram was generated reflecting analysis by this method (Figure 3). A total of 29 different profiles were generated. As previously observed, *S*. Typhimurium DT126 isolates were distinct from DT108, DT12 and DT12a isolates. The overall Simpson's Index of Diversity (DI) value for all non-DT126 isolates was 0.91, compared with previously published values of 0.83 for MLVA and 0.41 for MAPLT (Ross, et al., 2009). The Simpson's Index of Diversity (DI) value for the DT126 isolates was not calculated as most of these isolates were derived from two outbreaks and therefore would have skewed any statistical analysis due to their clonality.

3.2 Composite data for *S.* Enteritidis

Based on previously published data (Ross & Heuzenroeder, 2009), a combined MAPLT/MLVA was devised based on the most variable loci from each assay. Consequently a universal protocol targeting the following ten loci was devised; $SB40_{ST64B}$, $SB21_{ST64B}$, $SB28_{ST64B}$, $SB46_{ST64B}$, $gtrA_{ST64T}$, $gtrB_{ST64T}$, STTR-3, STTR-5, SE-1 and SE-2. These ten loci can be initially used where no phage typing data is available. Where phage typing data is available, improved separation within a phage type can be achieved. For example, our data shows that, instead of locus $SB21_{ST64B}$, the substitution of the ST64T gene *9* locus at the 5′ end (g*9*:5′) (Ross & Heuzenroeder, 2005) improves separation of phage type 26 isolates (Figure 4a) while the composite assay for the phage type 4 isolates indicated that the ten universal loci described above were suitable for this phage type (Figure 4b). The addition of ST64B *immC* gene *c1* improved separation of the *S*. Enteritidis RDNC isolates and isolates unable to be typed (ut) by phage typing (isolate designations RDNC- and Eut- respectively) (Figure 4c). Simpson's Index figures for the combined MAPLT/MLVA assay and comparisons to the previously published data for individual assays are provided in Table 3.

PT	MAPLT	MLVA	Composite	PFGE
26	0.87 (14)	0.89 (17)	0.99 (21)	0.66 (6)
4	0.83 (10)	0.85 (10)	0.99 (19)	0.48 (4)
ut/RDNC	0.98 (23)	0.96 (20)	0.99 (25)	0.89 (11)

Table 3. Comparative Simpson's Index values for *S*. Enteritidis phage types

Simpson's Index data for separate PFGE, MLVA and MAPLT analyses previously published (Ross and Heuzenroeder, 2009) Figures in brackets are the number of different profiles generated by each assay.

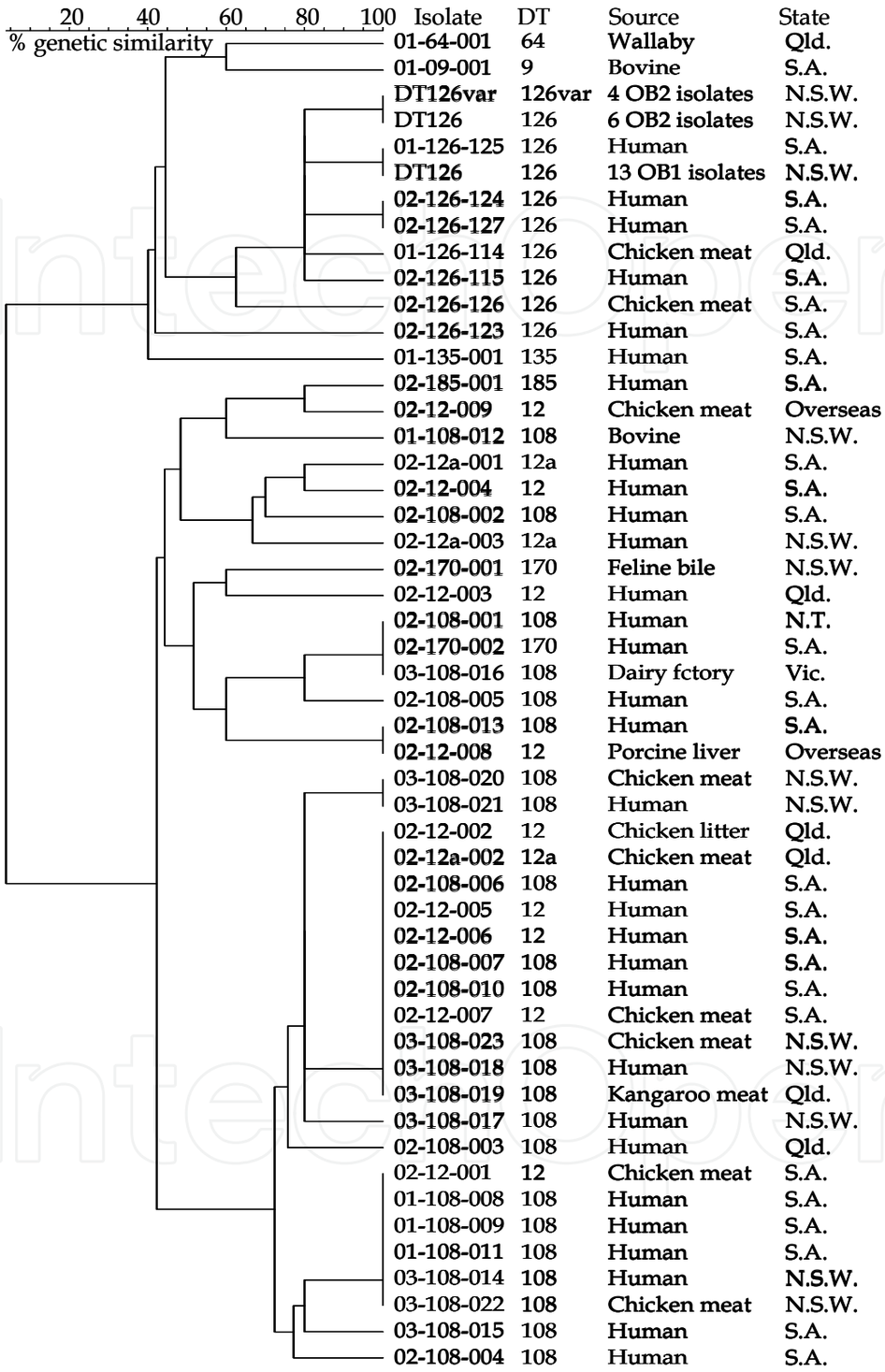


Fig. 3. Dendrogram showing genetic similarity of *S.* Typhimurium isolates. Abbreviations for states are: N.S.W. New South Wales, N.T. Northern Territory, Qld. Queensland S.A. South Australia, Vic. Victoria, W.A. Western Australia.

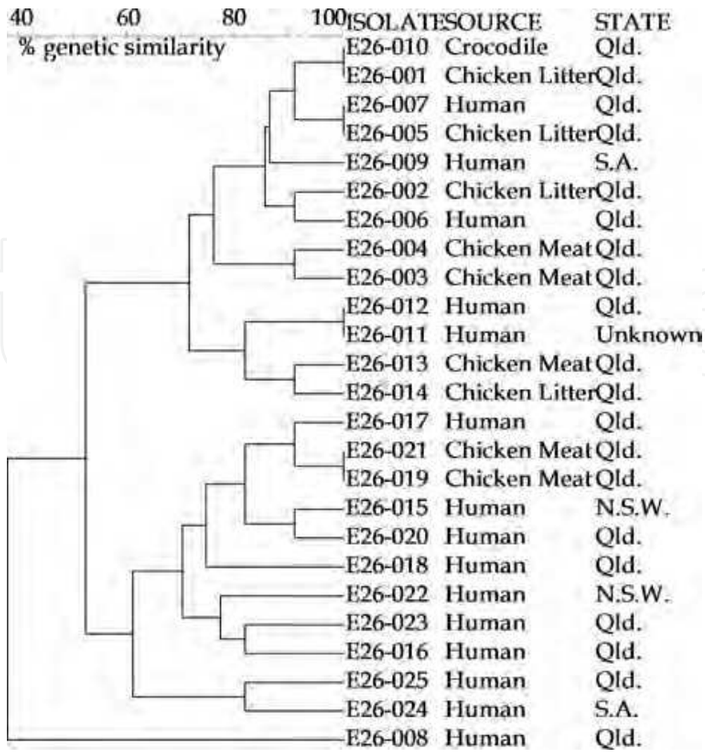


Fig. 4a. Dendrogram of *S. Enteritidis* PT26 analysed with composite MAPLT/MLVA data. No further information available for isolate E26-11

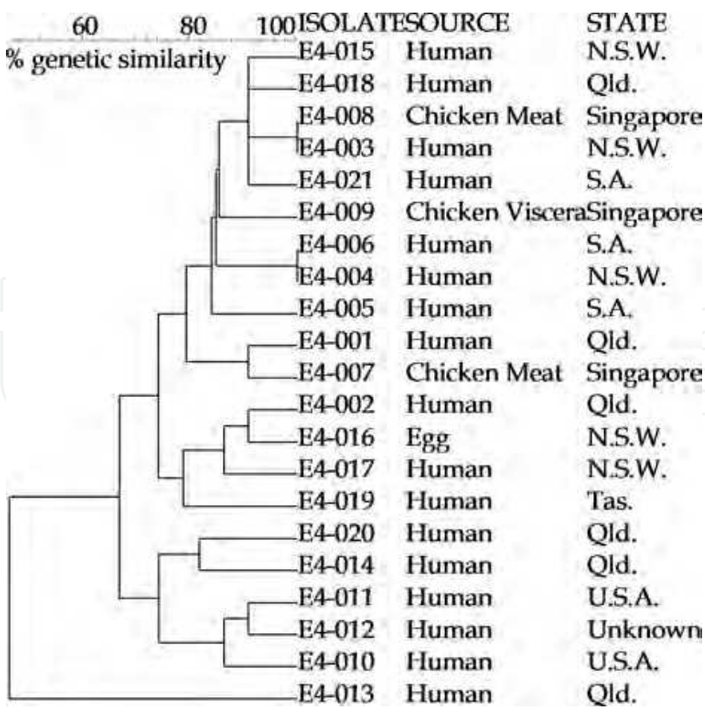


Fig. 4b. Dendrogram of *S. Enteritidis* PT4 analysed with composite MAPLT/MLVA data. All Australian states except where indicated.

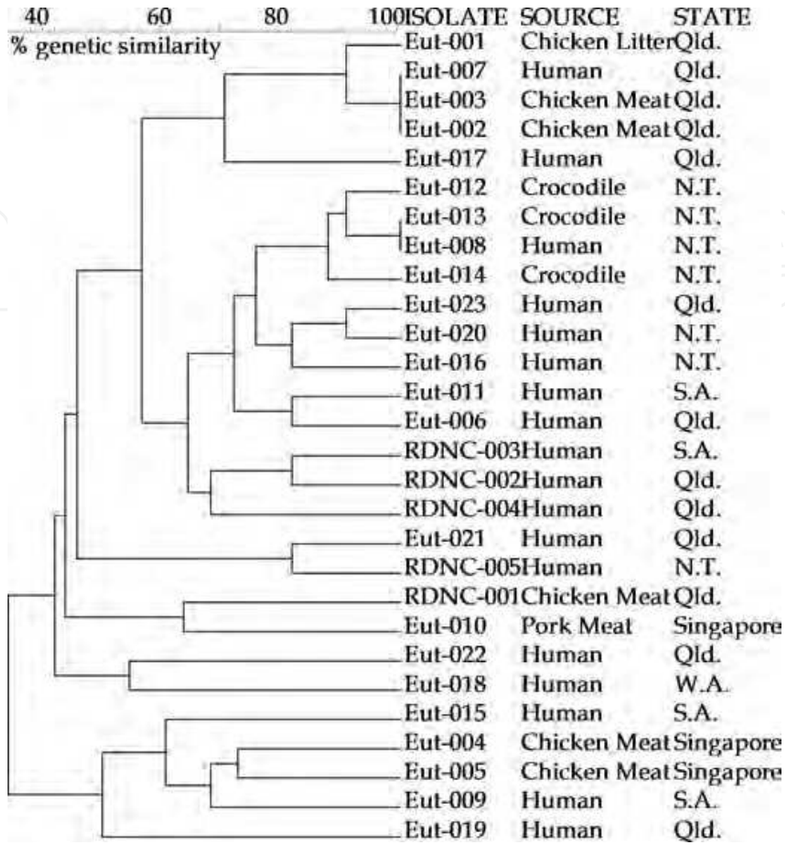


Fig. 4c. Dendogram of untypable and RDNC *S.* Enteritidis isolates analysed with composite MAPLT/MLVA data.

3.3 *S.* Virchow

PFGE analysis of *S.* Virchow divided the 43 isolates into 17 different profiles (Fig. 5). There was no distinct correlation between PFGE profile and phage type. For examples PFGE profiles 1, 3, 9 and 10 were generated from isolates with different phage types. Similarly, isolates of some phage types (17, 19, 31 and 36var1) produced PFGE profiles with 2 to 6 band differences between isolates, indicating that isolates within these phage types could exhibit an extensive genetic diversity. This study included a large proportion of PT8 isolates due to its predominance among all phage types seen in Australia. From the twenty-five PT8 isolates 15 (60%) generated PFGE profile 2. Nearly all PT8 isolates (14 out of 15) had the same MAPLT profile.

MAPLT analysis identified a number of loci derived from various bacteriophages which were useful in distinguishing between *S.* Virchow isolates. Nine MAPLT loci were subsequently chosen for *S.* Virchow differentiation based on the variability of frequency of these loci across the 43 isolates.

Using 15 MLVA primer sets previously described for a range of *S. enterica* serovars, only MLVA locus STTR-5 provided any allelic variation in the 43 *S*. Virchow isolates. The range of fragment sizes for this locus (based on the primer sequences of Lindstedt, et al., 2003) was 217bp (Fig. 6) to 271bp. There was no observed correlation between STTR-5 fragment size and phage type and in particular for PT8 the predominant type.

A composite MAPLT/MLVA dendrogram based on 9 MAPLT loci and the MLVA locus STTR-5 was generated (Fig. 6). This combination significantly improved the separation of the 43 *S*. Virchow isolates both in terms of diversity and number of different profiles generated (Table 4). More importantly, the differentiation of PT8 isolates was improved considerably using the combined method (DI = 0.88) in comparison to PFGE (DI = 0.59).

	MAPLT	MLVA	Composite	PFGE
Number of primers	9	13	10	na
Number of profiles	14	8	23	17
Simpson's DI	0.81	0.79	0.94	0.84

na not applicable

Table 4. Diversity of 43 *S*. Virchow isolates as determined by each method. Composite data based on combined MAPLT and MLVA primers; see Fig. 6 for details.

4. Discussion

The adoption of rapid, high resolution PCR-based typing assays such as MLVA and MAPLT for fine discrimination of closely related isolates of *Salmonella* may provide an alternative to phenotypic assays and current molecular methods such as PFGE. As more data is obtained it is obvious that there are sufficient differences in bacterial genome structure and prophage populations between different serovars of *Salmonella enterica* to necessitate development of such assays on a serovar by serovar basis. While PFGE is not limited by this issue, the development of PCR-based assays for specific serovars of interest is worthwhile due to the likelihood of improved discrimination of isolates and the ease of sharing data between interested laboratories and health authorities.

The combination of separate MAPLT and MLVA data into a single composite assay can provide superior discrimination of isolates than that obtained by either assay alone, as well as by PFGE. In the case of serovar Typhimurium, one of the most significant causative agents of non-typhoidal *Salmonella*-induced gastroenteritis, we have demonstrated that closely related phage types such as DT108 and DT12 can be separated by either PCR-based method, but combining the most variable loci into a single assay provides what may be the optimal separation of isolates. Furthermore, it should be noted that there was no correlation between phage type and clustering by MAPLT and/or MLVA. As mentioned previously the index of diversity for the DT126 isolates was not determined due to the clonality of the outbreak isolates clustering more tightly than would be seen with a group of epidemiologically-unrelated isolates. This however, demonstrates the ability of these PCR-based assays for discriminating outbreak isolates from closely related but epidemiologically distinct strains.

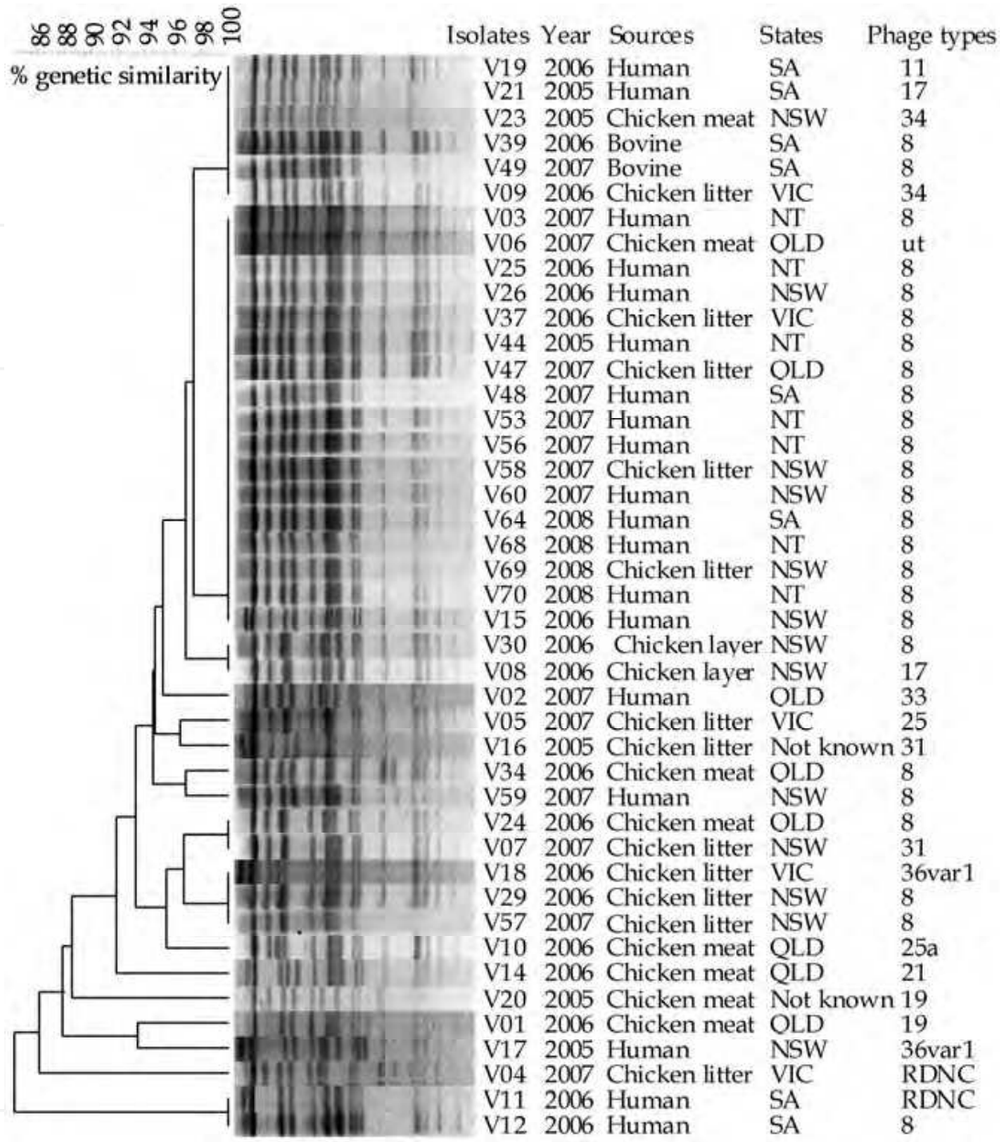
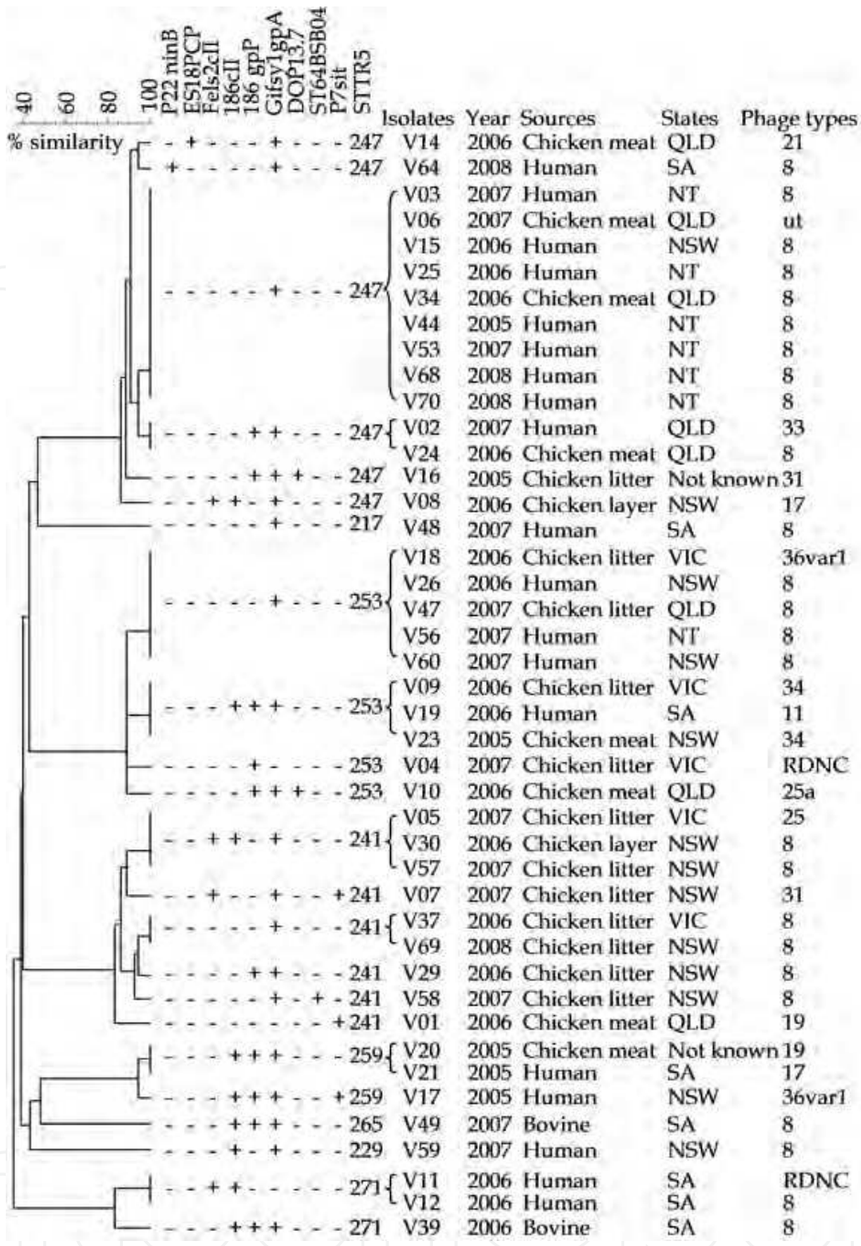


Fig. 5. Pulsed-field gel electrophoresis of 43 *S.* Virchow isloates.

While separate assays may need to be developed for different serovars with unique sets of primers, it is possible that individual loci may provide extra discrimination for particular phage types within a serovar. It has previously been reported that MLVA locus SENTR2 (locus STTR-7 as previously described by Lindstedt et al., 2003) may be useful for improved detection of differences within sample groups of both *S.* Enteritidis PT4 and PT8 isolates (Malorney et al., 2008). The data for *S.* Enteritidis presented here further supports this concept. While 10 primers sets formed the basis of a composite MAPLT/MLVA assay for this serovar (as demonstrated for the PT4 isolates), different MAPLT-derived loci proved useful for maximising isolate discrimination (see Fig. 4). This information is more relevant where phage type data is available and pre-selection of primers can be ascertained. However, even in the absence of the phage typing data, the assay may include primers for these extra loci as a matter of course.



\+ MAPLT locus detected by PCR, - MAPLT locus not detected.
Fragment sizes for MLVA locus STTR-5 based on primer locations described by Lindstedt et al., (2003).

Fig. 6. A dendrogram based on composite MAPLT/MLVA data as described in section 3.3. All abbreviations for Australian states as per Fig. 3.

Development of MAPLT and MLVA as well as a composite assay for serovar Virchow has identified the importance of total genomic data being available in genome libraries such as Genbank (www.ncbi.nlm.nih.gov). While a number of suitable MAPLT loci were identified from a range of different prophages isolated from the *S*. Virchow strains with the exception of locus STTR-5, previously described MLVA loci were found to be either homologous in terms of fragment length or not detected by PCR and thus do not provide allelic variation

within this serovar. Access to total genomic data on different serovars and strains would facilitate searches for tandem repeat loci that may be unique to that serovar. It is also likely that more than one total genome per serovar may need to be sequenced to enhance the likelihood that most or all MLVA loci present in that serovar are detected. For example, many *S*. Typhimurium strains do not harbour the plasmid-based STTR-10 locus (Ross & Heuzenroeder, unpublished data). Development of a MLVA assay based solely on the sequence data of one of these isolates may have resulted in one of the most variable MLVA loci being excluded from any devised MLVA protocol. In the case of the development of a MLVA assay for *S*. Enteritidis, the genomes of two separate isolates of this serovar, LK5 and a phage type 4 isolate (as well as S. Typhimurium LT2) were analysed (Boxrud et al., 2007). Consequently, we conclude that the genomes of suitable *S*. Virchow isolates may need to be completely sequenced to identify unique tandem repeat loci that provide suitable allelic variation for a MLVA assay. In the interim however, MAPLT loci has provided excellent separation of the *S*. Virchow isolates while the inclusion of STTR-5 into a composite assay enhanced separation of the isolates, in particular the PT 8 isolates.

The use of PCR-based methodology can be quite useful in outbreak situations where the source of the outbreak must be quickly identified to stop or restrict the spread of the pathogen in the community or environment. Their usefulness is based on the high resolution capabilities, the relatively short time frame required for obtaining data and the simplicity for data sharing. It has been noted that because some MLVA loci of a strain can exhibit subtle mutations in tandem repeat number during the course of an outbreak, some subjective interpretation of data in conjunction with other epidemiological data may be necessary for accurate identification. Boxrud et al., (2007) has suggested that "interpretive criteria that account for genetic variability of MLVA patterns analogous to the Tenover criteria used for PFGE may need to be developed". In Australia, laboratories collaborating in MLVA of *Salmonella* have agreed that minor variations such as one tandem repeat change at two separate loci may not be significant, especially if epidemiological information supports the conclusion. A study on *S*. Typhimurium DT9 isolates involved in an outbreak in South Australia in 2007 revealed MLVA allelic variability in human-derived isolates that were linked to the outbreak (Ross et al., 2011). Local outbreaks of both DT9 and DT108 during 2011 have also indicated that variability in the three loci STTR-5, STTR-6 and STTR-10 can appear during the course of the outbreak (Ross et al., unpublished data). These variations however, did not prevent the rapid identification of the likely food source of the outbreak. As yet, a comparison with the stability of MAPLT loci in these isolates has yet to be determined.

With the development of PCR-based protocols being undertaken there is a need to ensure consistency of loci identification and nomenclature as well as clear guidelines for data generation. It has been noted that a single locus may be given more than one designation by different laboratories, leading to potential confusion. One example of this has been alluded to previously in this discussion; the naming of the MLVA locus as either STTR-7 or SENTR2 by different laboratories. As the name STTR-7 was documented first we have adopted this description and suggest all subsequent references to this locus be made in accordance with this nomenclature. A different example is where the sequence of a tandem repeat has been published in either direction by two different laboratories. *S*. Enteritidis MLVA locus SE-2 described by Boxrud et al., (2007), was later described as SENTR6 and published in the reverse direction. In both cases, using different nomenclature for identical loci can generate

confusion and unnecessary work for researchers during assay development and/or surveillance programmes.

Standardised guidelines for data generation and interpretation also need to be developed. We have already mentioned previously in the Introduction, guidelines for MLVA of *S.* Typhimurium published by The Institut Pasteur. Even so, there is still a lack of concordance in what constitutes an agreed tandem repeat sequence and whether single nucleotide polymorphisms in flanking tandem repeats disqualify them as being included in a tandem repeat analysis. This laboratory currently reports all *S.* Typhimurium MLVA patterns in terms of total sequence length of the five loci in base pairs in accordance with the primer sequences published by Lindstedt et al., (2003, 2004) and adopted and described in The Institut Pasteur website. This reporting method, lacking tandem repeat numbers, prevents any subsequent misinterpretation of data.

5. Conclusions

Both MAPLT and MLVA offer rapid PCR-based approaches for rapid, high resolution discrimination of phenotypically closely related but epidemiologically distinct *Salmonella* isolates. This level of discrimination is often at least equal to that offered by PFGE. Objective data generated by either PCR method can be easily shared between laboratories and appropriate jurisdictional health authorities for general pathogen surveillance purposes as well as the investigation and control of outbreaks. As either MAPLT or MLVA may be more suited for a particular serovar or, where applicable, phage type, a composite assay comprising multiplex primers from both individual assays targeting the most variable loci in a particular strain can provide the maximum level of isolate separation. This data in the form of universally agreed nomenclature, in combination with epidemiological information, would prove invaluable for detecting sources of outbreaks and thereby restricting their effects.

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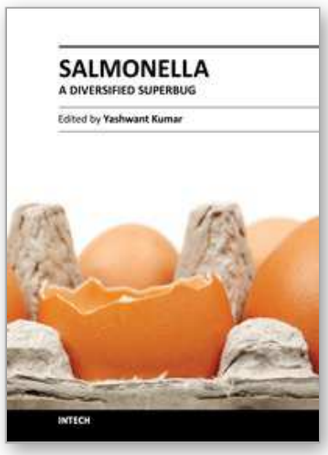

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