Extended phage locus typing of *Salmonella enterica* serovar Typhimurium, using multiplex PCR-based reverse line blot hybridization

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Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is the commonest pathogen causing food-borne disease among humans and animals in Australia. A multiplex PCR-based reverse line blot (mPCR/RLB) system was developed to rapidly identify *S. Typhimurium* phage types and strains within them. The system comprised 32 biotin-labelled primer sets and 38 amino-labelled probes, based on sequences that were either phage-type-related or derived from temperate phages ST64B, P22, Gifsy-1 or Gifsy-2. The system was developed and evaluated using 168 *S. Typhimurium* isolates, representing 46 phage types. RLB patterns, based on a combination of positive hybridization and grading of signal intensities, validated by sequencing, differentiated *S. Typhimurium* isolates into 102 types. Some clusters contained isolates belonging to a single phage type while others contained isolates belonging to more than one. Most phage types exhibited at least two RLB profiles. The feasibility of this system was evaluated during investigations of three outbreaks, due to two different phage types. Within each outbreak, isolates showed identical RLB patterns, whereas sporadic isolates of corresponding phage types showed various patterns. The mPCR/RLB system was compared with multilocus variable-number tandem-repeat analysis (MLVA). The two methods demonstrated similar discriminatory abilities. Based on these preliminary results, the mPCR/RLB system is a promising tool for molecular identification of most common *S. Typhimurium* phage types. It could be used as an alternative to, or in conjunction with, MLVA for rapid strain typing during outbreaks.

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INTRODUCTION

Salmonella enterica serovar Typhimurium (*S. Typhimurium*), one of more than 2500 serovars recognized in the genus *Salmonella* (Popoff *et al.*, 2004), is a major bacterial pathogen that causes food-borne infection (salmonellosis) in humans and animals worldwide. For many years it has been the most common serovar isolated from humans and animals in Australia. In 2006 it accounted for 25.7% of human and 16.2–30.4% of chicken and bovine isolates, albeit with considerable geographical and temporal variations (Davos, 2006).

S. Typhimurium is a common and diverse serovar that requires further subtyping for surveillance or outbreak investigations. Phage typing (Anderson *et al.*, 1977) is widely used in Australia and Europe to differentiate individual strains of *S. Typhimurium*. However, it is restricted to a few reference laboratories, which causes delays and limits its epidemiological value. Interpretation

of results, even by experienced staff, may vary between laboratories. Untypable or ‘reaction-does-not-conform’ (RDNC) isolates are not uncommon. Often a single phage type (PT) predominates for long periods in a geographical area, making recognition of outbreaks difficult.

Molecular typing methods have been used for further discrimination of *Salmonella* strains, including PFGE (Guerra *et al.*, 2000), random amplified polymorphic DNA typing (De Cesare *et al.*, 2001), multilocus sequence typing (Fakhr *et al.*, 2005), IS200 typing (Jeoffreys *et al.*, 2001; Soria *et al.*, 1994), ribotyping (Nastasi & Mammina, 1995), plasmid profiling (Millemann *et al.*, 1995) and amplified fragment length polymorphism (AFLP) analysis (Hu *et al.*, 2002; Lindstedt *et al.*, 2000). Some are relatively difficult to perform, slow and expensive whilst others have limited reproducibility and discriminatory powers. Multilocus variable-number tandem-repeat analysis (MLVA) has been developed for various *Salmonella* serovars (Cho *et al.*, 2007; Lindstedt *et al.*, 2004; Liu *et al.*, 2003; Witonski *et al.*, 2006). It has allowed a high level of resolution of variant strains of the relatively homogeneous *S. Typhimurium* definitive type 104 (Lindstedt *et al.*, 2003).

Abbreviations: AFLP, amplified fragment length polymorphism; MLVA, multilocus variable-number tandem-repeat analysis; mPCR/RLB, multiplex PCR-based reverse line blot; PT, phage type; RDNC, reaction-does-not-conform.

A number of phage type-specific sequences have been identified in *S. Typhimurium* and its phages and used to distinguish phenotypically related definitive types of *S. Typhimurium* by PCR and sequencing (Hu *et al.*, 2002; Lan *et al.*, 2007; Mikasova *et al.*, 2005; Ross & Heuzenroeder, 2005). As this involves multiple single PCRs and sequencing, it is time-consuming and unsuitable for routine identification and typing. In this study, we developed a multiplex PCR-based reverse line blot (mPCR/RLB) assay to rapidly identify most of the phage types currently prevalent in our region and compared the results with those of MLVA.

METHODS

Bacterial isolates and DNA preparation. One hundred and sixty-eight *S. Typhimurium* isolates, representing 46 phage types, were tested. They had been isolated, mainly from human stool samples, between 2000 and 2005 and referred for serotyping to the New South Wales Enteric Reference Laboratory at the Centre for Infectious Diseases and Microbiology. Phage typing was performed, using the Anderson scheme (Anderson *et al.*, 1977), by the Microbiological Diagnostic Unit, University of Melbourne, or the Australian Salmonella Reference Centre, Institute of Medical and Veterinary Science, Adelaide (Fig. 1).

Isolates from three separate outbreaks were investigated. (i) Outbreak 1: 23 isolates of PT 135 from a suspected outbreak in a drug and alcohol rehabilitation centre in 2004 were compared with two PT 126 isolates and 15 non-outbreak PT 135 isolates (collected between 2001 and 2003). (ii) Outbreak 2: 15 isolates (14 from humans, one from food) of PT 170 from three apparently related restaurant outbreaks in 2006 were compared with 22 sporadic PT 170 isolates (collected between 2001 and 2005). (iii) Outbreak 3: 17 isolates of unknown phage type from an outbreak in November 2006, involving students from a catering college, and six *Salmonella* isolates of unknown serovar from three types of food were investigated and compared with controls, which were isolates belonging to four common *S. Typhimurium* phage types, namely PT 126, 135, 170 and U290.

All isolates were grown on horse blood agar overnight at 37 °C. Single colonies of each were suspended in 200 µl molecular biology grade water and boiled for 10 min. The suspension was centrifuged and the supernatant was used for PCR.

Primers. Thirty-two sets of primers were designed, based on previously published primers and *S. Typhimurium* phage-type-related AFLP fragment sequences. Of these, 14 sets were based on bacteriophage ST64B (Ross & Heuzenroeder, 2005), 12 on bacteriophages P22, Gifsy-1 and Gifsy-2 (Mikasova *et al.*, 2005), and six on the AFLP fragments (Hu *et al.*, 2002). All primers were designed or modified so that they were 18–28 bp in length, with melting temperatures between 59 and 71 °C and product sizes in the range 90–249 bp (Table 2). The nucleotide–nucleotide BLAST program (National Center for Biotechnology Information) was used to compare primer sequences with sequences in the GenBank database, to minimize cross-priming with other regions of phage genomes or unrelated genes. All primers were 5' and 3' biotin labelled and supplied by Sigma-Genosys.

Probes. Thirty-eight probes were designed, based on amplicon sequences of each primer set: two for each of the six primer sets based on AFLP fragment sequences and one for each of the other primer sets. All probes were designed with lengths between 18 and 27 bp and melting temperatures of 61–66 °C (Table 3). BLAST searches of the

GenBank database were performed to minimize cross-hybridization with other genes. Probes were 5' amino-labelled and supplied by Sigma-Genosys.

Multiplex PCR system. A 30 µl multiplex PCR mixture containing 32 primer sets was prepared using the Qiagen Hotstar *Taq* PCR system as follows: 3.0 µl of 10 × PCR buffer, 4.5 µM MgCl₂, 0.2 mM dNTP, 0.5 µM each forward and reverse primer, 2 µl template DNA, 0.2 µl Qiagen Hotstar *Taq* polymerase (5 U µl⁻¹) and water added to a total volume of 30 µl. A touchdown PCR was performed according to the protocol described previously (Ross & Heuzenroeder, 2005) with decreasing primer-annealing temperatures from 59 to 52 °C. One representative isolate from each of 46 phage types was also tested by single PCR using each primer set to validate the mPCR results.

RLB hybridization. The RLB hybridization assay was performed based on a well-established protocol developed in this laboratory (Kong & Gilbert, 2006) with modifications. Probes were attached to a Biotyne Nylon 6.6 membrane (PALL; Gelman Laboratory). The hybridization temperature was 60 °C. Streptavidin–peroxidase (Roche Diagnostics) was used as conjugate and diluted 1:4000 in 2 × SSPE (0.18 M NaCl, 10 mM NaH₂PO₄ and 1 mM EDTA, pH 7.7) containing 0.5% SDS. The X-ray film (Hyperfilm; Amersham) exposure time was 7 min.

Sequencing. Sequencing was performed, using single PCR amplicons, to compare sequences of the corresponding targets from different isolates that gave strong or weak signals in RLB. The sequencing was conducted in both directions with BigDye Terminator version 3.7 (Applied Biosystems) on an ABI 3100 DNA sequencer.

MLVA. The MLVA typing method was used as described by Lindstedt *et al.* (2004) with the following modifications. A 30 µl multiplex PCR mix was prepared using the Qiagen HotStar *Taq* PCR system containing 3.0 µl of 10 × PCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTP, 0.1 µM each forward and reverse primer, 2 µl template DNA, 0.2 µl Hotstar *Taq* polymerase (5 U µl⁻¹) and water added to a total volume of 30 µl. PCR products were run on a 1.6% normal agarose gel prior to the capillary electrophoresis. A single target PCR was run for any isolates with missing mPCR amplicons, to confirm absence of amplification. The capillary electrophoresis solution was prepared as follows: 0.5 µl of the PCR products was mixed with 0.5 µl of the ROX1000 (Bio-Ventures) internal size marker and 9 µl formamide. The mixed solution was then subjected to capillary electrophoresis on an ABI 3100 Genetic Analyzer (Applied Biosystems).

Data analysis. For each probe, RLB hybridization signal intensities on the membrane were scored as 2 for strong (gene present), 1 for weak (gene present with minor sequence variation) and 0 for absent (gene absent or present with major sequence variations) signals. These were combined into a 38-digit string for each isolate representing the RLB pattern. The string was divided into seven gene groups (G1–G7) each containing six gene markers, except for G7, which comprised only two. A single digit difference in the string was regarded as a different RLB type. The data were analysed using BioNumerics (Applied Maths) software and each unique pattern was given a number. A dendrogram was generated using the categorical coefficient and Ward's algorithm.

MLVA peaks were identified according to colours and sizes using GENESCAN software provided by Applied Biosystems and were binned into alleles. The allele numbers were entered into BioNumerics as character values. The number of repeats at each locus was calculated by subtracting the known length of the flanking sequence from the amplicon length and dividing the result by the repeat sequence length for all loci, except STTR3, which contains variable numbers of either 27 or 33 bp repeats. The lengths of flanking sequences were

determined from the *S. Typhimurium* LT2 complete genome sequence from GenBank (accession no. NC_003197). An allele string for each isolate, in the order STTR9–STTR5–STTR6–STTR10pl–STTR3, was constructed by combining allele numbers, corresponding with the number of repeats (0 if there was no amplicon; 1 for flanking sequence only; 2 for one repeat; and 3 for two repeats and so on), for the first four loci. For STTR3, the allele numbers were based on eight amplicon size variants among all isolates tested ranging from 370 bp (allele 1) to 523 bp (allele 8). Different MLVA types were numbered consecutively as they were identified, based on differences in combinations of allele numbers.

Polymorphism indices were calculated using Simpson's index of diversity, as described by Hunter & Gaston (1988).

RESULTS

mPCR/RLB

The 168 *S. Typhimurium* isolates were classified into 102 RLB patterns or types, which were numbered in order as shown in Fig. 1. The polymorphism index for RLB typing was 0.9866. Fig. 2 shows a representative RLB profile of selected phage types of *S. Typhimurium*. Of the 168 isolates, 99 were clustered into 33 RLB types, each of which contained multiple isolates with identical patterns and, in most cases, corresponded with different phage types. Each of the remaining 69 isolates gave a unique RLB pattern. All RLB types could be further clustered into five groups (A–E; Fig. 1).

Of 33 RLB types with multiple isolates, one (RLB 92) contained isolates belonging to four phage types; two (RLB 41 and 95) contained three phage types; eleven (RLB 1, 45, 50, 50, 57, 59, 65, 66, 69, 78, 81 and 87) contained two phage types; and 19 RLB types each contained a single phage type, some of which were shared between RLB types (Fig. 1).

All phage types with multiple isolates, except PT 99 and PT U290, produced two or more RLB patterns. For some phage types, the majority of isolates had the same RLB patterns. For example, four of five PT 101 isolates shared RLB 87, four of six PT 126 isolates belonged to RLB 3 and three of four untypable isolates shared RLB 61. Most other phage types showed significant differences in RLB patterns between isolates and some RLB patterns were shared between phage types. For example, six PT 1 isolates had five different RLB patterns spread among cluster groups B, C and E, namely RLB 25, 26, 33, 47 and 95 (the latter was shared with PT 5 and 136). Six PT 6 var1 isolates produced five RLB patterns spread between groups A, C and E, namely RLB 11, 42, 81, 82 and 83 (the latter was shared with PT 140 var1). Four PT 141 isolates each belonged to a different RLB type, namely RLB 18, 24, 92 and 94 (RLB 92 was shared with PT 4, 193 and 195). Similarly, each of five PT U302 isolates gave a different RLB pattern, namely RLB 12, 62, 64, 68 and 69 (RLB 69 was shared with PT 186) (Fig. 1).

Of the 46 phage types tested, 21 gave distinct RLB patterns, i.e. not shared by any other phage types, including 14 with multiple isolates (PT 12a, 20 var1, 30, 43, 44, 64, 104L, 126, 126 var4, 160, 197, 208, U290 and untypable) and seven

with single isolates (PT 1 var, 4 var2, 29, 164, 165, 177 and 194). The number of distinct RLB patterns for each of these phage types varied between one and five. For two phage types, PT 126 var4 and PT 197, each of four and five isolates, respectively, gave different RLB patterns.

The numbers of gene target differences, between different RLB patterns for the same phage type, varied between 1 and 26 of the 38 probe targets. Of the isolates tested, PT 1 (six isolates) and PT 195 (four isolates) were the most variable, with 26 and 23 differences, respectively. Among phage types with distinct RLB patterns, PT 208 and PT 197 had 12 and 10 target differences between isolates, respectively.

The probes CA4-P2, CC2-P2 and gtgB-P1 produced strong positive signals for all phage types and therefore could be used as control markers in future work. Amplicons which produced different signal intensities with the same probe were sequenced. The results showed that those producing strong signals (score 2) were identical to corresponding probe sequences; those that gave weak signals (scored as 1) had one (isolates 146, 158 and 187) or more than one (isolates 127 and 128) nucleotide base difference. Isolates that produced amplicons (as shown by single PCR and gel electrophoresis), but gave no signal with the corresponding probe (scored as 0), differed by more than two nucleotides (isolates 100, 123, 146, 158 and 187) (Table 4).

MLVA typing

The loci showed varied degrees of polymorphism from 21 alleles of STTR5 to five of STTR9. The polymorphism indices for each locus were as follows: STTR3, 0.63; STTR5, 0.91; STTR6, 0.92; STTR9, 0.55; and STTR10pl, 0.84. The combination of five loci gave a polymorphism index of 0.9854. Three loci, STTR3, STTR5 and STTR9, were present in all isolates tested, while STTR6 and STTR10pl were absent from 6 (3.6%) and 25 (14.9%) isolates, respectively.

The 168 *S. Typhimurium* isolates were separated into 97 MLVA allele strings which were numbered from 1 to 97 (Fig. 1). Twenty-six of the 97 MLVA types were represented by multiple isolates, and most of these corresponded with a single phage type. For example, MLVA types 1, 6 and 25 corresponded with PT 160, PT 44 and PT U307, respectively, and MLVA 12 corresponded with PT 135 (six of 15 PT 135 isolates belonged to other MLVA types).

Some MLVA types contained two or more phage types, suggesting close relationships between them. For example, MLVA 2 contained five phage types, MLVA 84 contained four and MLVA 35 and 69 each contained two. On the other hand, even small numbers of some phage types were represented by several MLVA types; for example, six PT 1 isolates were represented by four MLVA types, 15 PT 135 isolates were represented by seven MLVA types and five PT 101 isolates were represented by five MLVA types (Fig. 1).

In many cases, individual RLB patterns included multiple MLVA types and vice versa; for example, RLB 41 contained

Fig. 1. Comparison of molecular typing methods using mPCR/RLB and MLVA for 168 *S. Typhimurium* isolates of 46 phage types. The dendrogram is divided into five larger groups, A, B, C, D and E, indicated by horizontal lines. Isolates 17, 19, 26 and 35 (RLB 41) were from the drug and alcohol rehabilitation centre outbreak in New South Wales in 2004; *RLB types unique for specific phage types.

Table 1. Isolates tested in the investigation of three outbreaks using mPCR/RLB and MLVA

Phage type	No. of isolates tested	RLB pattern (n)*	MLVA type (n)†	MLVA pattern‡	Source
PT 135	23	RLB 41 (23)	12 (20)	3-11-10-12-8	Outbreak 1: drug and alcohol rehabilitation centre outbreak in NSW (2004)
			98 (1)	3-10-10-10-8	
			99 (1)	3-5-15-1-8	
			28 (1)	3-15-7-12-8	
PT 135	15	RLB 41 (11)	16 (5)	3-12-10-12-8	Sporadic isolates (2001–2003)
			100 (3)	3-11-10-10-8	
			101 (2)	3-12-10-8-8	
			102 (1)	3-12-9-10-8	
		RLB 7 (4)	103 (1)	3-12-11-10-8	
			8 (1)	3-10-10-12-8	
			12 (1)	3-11-10-12-8	
			23 (1)	3-13-9-10-8	
PT 126	2	RLB 3 (2)	33 (1)	3-16-19-12-8	
			37 (1)	3-17-14-12-8	
PT 170	14	RLB 103 (14)	5 (12)	3-9-8-12-8	Outbreak 2: restaurant outbreaks in NSW (2006)
			2 (2)	3-9-7-12-8	
	1	RLB 103 (1)	2 (1)	3-9-7-12-8	Food isolate
PT 170	22	RLB 103 (17)	5 (12)	3-9-8-12-8	Sporadic isolates (2000–2005)
			2 (3)	3-9-7-12-8	
			4 (1)	3-9-7-14-8	
			104 (1)	3-9-9-12-8	
		RLB 104 (1)	55 (1)	3-31-10-18-6	
		RLB 105 (1)	105 (1)	3-10-8-12-8	
		RLB 106 (2)	106 (1)	4-15-9-0-7	
			107 (1)	4-16-8-0-7	
		RLB 107 (1)	5 (1)	3-9-8-12-8	
PT 170	17	RLB 58 (17)	108 (17)	3-11-7-12-8	Outbreak 3: catering college outbreak in NSW (2006)
	2	RLB 58 (2)	108 (2)	3-11-7-12-8	Chocolate mousse
	2	RLB 58 (2)	2 (2)	3-9-7-12-8	PT 170 controls
PT 126	2	RLB 3 (1)	33 (1)	3-16-19-12-8	PT 126 controls
		RLB 10 (1)	37 (1)	3-17-14-12-8	
PT 135	3	RLB 4 (2)	12 (2)	3-11-10-12-8	PT 135 controls
		RLB 8 (1)	18 (1)	3-12-11-12-8	
PT U290	2	RLB 27 (2)	16 (2)	3-12-10-12-8	PT U290 controls
S. Java	2	RLB 108 (2)	S. Java 1 (2)	1-13-0-0-4	Fish
S. Sofia	2	RLB 109 (2)	S. Sofia 1 (1)	0-10-0-0-0	Chicken
			Not typed (1)	0-0-0-0-0	Chicken

*RLB types are numbered consecutively as they are identified, based on a 38-digit string determined by probe reaction strengths. RLB types 103–109 are new types not identified among 168 isolates initially tested.

†MLVA types are numbered consecutively as they are identified, based on a 5-digit string or pattern. MLVA types 98–108 are new types not identified among the 168 isolates initially tested.

‡MLVA pattern consists of a number of repeats (+1) present at loci STTR9, STTR5, STTR6 and STTR10 (in that order) and allele number for STTR3 [see text for further details: 0=no amplicon; 1=amplicon equivalent to flanking region (no repeat sequence present)].

five MLVA types and MLVA 12 contained five RLB types (Fig. 1), indicating that RLB and MLVA types are generally complementary and together provide a higher level of discrimination than either alone. However, there were some exceptions, where the RLB, MLVA and PT types were consistent. For example, four PT 99 isolates shared the

same RLB 41 and MLVA 49 types, three PT 44 isolates shared RLB 22 and MLVA 6 types, and three untypable isolates had the same RLB 61 and MLVA2 types (Fig. 1). Further information is required to determine whether isolates with consistent RLB, MLVA and phage types are epidemiologically related.

Table 2. Primers used in the multiplex PCR for identifying phage types of *Salmonella* Typhimurium

No.	Primer	Sequence (5'–3')	Size (bp)	T _m (°C)*	Accession no.	Source†
1	BIM4-F1b	29284 GCT GGT ACT GCA ACG TGC C 28302	188	66.9	AY055382	Phage ST64B
	BIM4-R1b	29435 TGG TGA TCT GCT CAA TCG CTT C 29414		68.6	AY055382	
2	BIM4-F2b	29739 TAG TCT GGT CGT GCG TAA CCA C 29760	113	66.1	AY055382	Phage ST64B
	BIM4-R2b	29851 CGA ATG ACA TGG ACA TAA AGT CC 29829		64.1	AY055382	
3	CA3-Fb	56 GCC ATC CCG ATT GTT TAT TG 75	112	65.4	AF500155	AFLP fragments
	CA3-Rb	167 CGA AGC GCA GCA GAG ATA C 149		67.6	AF500155	
4	CA4-Fb	31 TGG GCG CTT TGT TAC TCT TC 50	138	64.1	AF500156	AFLP fragments
	CA4-Rb	168 CGT ACA CAG CGG GAC AC 152		61.5	AF500156	
5	CA7-Fb	5 CAT CGG GGT GAT CAG CAT 22	90	66.6	AF500159	AFLP fragments
	CA7-Rb	94 ATC CCC GCA ATA AAT TTT CC 75		64.7	AF500159	
6	CC2-Fb	28 CGC AGA ATC CGG GAT AGT TA 47	121	64.1	AF500161	AFLP fragments
	CC2-Rb	148 GGT CTC GGC TTG ATT TGT GT 129		62.1	AF500161	
7	CC4-Fb	8 CCC TCG GTG AAA AGT TGC T 27	109	64.1	AF500169	AFLP fragments
	CC4-Rb	108 GAT TTG CAC CGT GCT GCT 91		65.2	AF500169	
8	CT1-Fb	25 CGG CAT CTG CTG ATA GCT TA 44	109	62.9	AF500164	AFLP fragments
	CT1-Rb	133 GGG TCT TGT TTT GGT GCT GT 113		63.9	AF500164	
9	eac-F1b	4790 CGT TAT GCA TGA TTG CAT CC 4809	114	64.8	AF217253	Phage P22
	eac-R1b	4903 CAA TCA AAC GCG AGG ATT TT 4884		63.7	AF217253	
10	eac-F2b	5009 TTT CAG CGG CTG TGG TTA G 5027	120	63.9	AF217253	Phage P22
	eac-R2b	5128 CAG GCC AGA GCA AAT ATT ACG 5108		63.3	AF217253	
11	g8-F1b	27455 GAT AAT GCC AAC GAC AAT GC 27474	105	62.8	AF217253	Phage P22
	g8-R1b	27559 GGC GAA TTC TGC GTT CTT T 27541		64.1	AF217253	
12	g8-F2b	27796 CAA TGC CGT AGC AGA ACA GG 27815	113	65.6	AF217253	Phage P22
	g8-R2b	27908 CAG GGA TGT TGA GCT TTT CC 27889		63.5	AF217253	
13	g13-F1b	20044 CAA AGG AAC AGG GCA TCG 20061	108	64.3	AF217253	Phage P22
	g13-R1b	20151 CGC ACA TCG TTG CGT CTA 20134		64.8	AF217253	
14	g13-F2b	20184 GTC TTC GCC GGA CTG AGT AG 201203	100	63.9	AF217253	Phage P22
	g13-R2b	20283 CAG CGA AGC GTT TGA TTA GC 22264		64.7	AF217253	
15	gipA-F1b	779 GCA AGC TGT ACA TGG CAA AG798	102	62.3	AF246666	Phage Gifsy-1
	gipA-R1b	880 CTG CGG CAT CTT TGG AAA T 862		64.9	AF246666	
16	gipA-F2b	886 TAC TTT GTT TCG TGC CTT TGC 906	105	63.8	AF246666	Phage Gifsy-1
	gipA-R2b	990 GGT ATC GGT GAC GAA CAA AT 971		61.2	AF246666	
17	gtgB-F1b	464 TGC ACG GGG AAA ACT ACT TC 483	101	63.8	AF254763	Phage Gifsy-2
	gtgB-R1b	564 CCC CTG GCA ATT TAC GGT AT 544		64.4	AF254763	
18	gtgB-F2b	800 TTA TCT GGG ATA AAC CAA CAG AGG 823	100	63.8	AF254763	Phage Gifsy-2
	gtgB-R2b	899 TTT GAT GTT TCA GCC CAT CA880		64.0	AF254763	
19	SB6-F1b	4394 ACG ACA AGC GCG TTG AGG C 4412	134	71.0	AY055382	Phage ST64B
	SB6-R1b	4527 ACG TTT CAG CTC AAG CAG CTC 4507		65.9	AY055382	
20	SB6-F2b	4776 ACT GGT GAA TCT GGG TGG TAC G 4797	168	67.0	AY055382	Phage ST64B
	SB6-R2b	4943 GCT TCC ACG TTG AAG AAG GC 4924		65.9	AY055382	
21	SB21-F1b	15411 CTG TAT GGT TAT ATC GAT TAT CTG G 15435	127	59.2	AY055382	Phage ST64B
	SB21-R1b	15537 CAA ATC CCT TCG CAG ATA CGG C 15516		70.3	AY055382	
22	SB21-F2b	15751 CAG GTT ACG CTG ACA CTC TGA C 15772	138	63.8	AY055382	Phage ST64B
	SB21-R2b	15888 GAT TTC CTT TGC CCA GAT GAC G 15867		68.5	AY055382	
23	SB26-F1b	19466 GAC ACC ATC AAT GTA TGG ATC GC 16488	249	67.2	AY055382	Phage ST64B
	SB26-R1b	19714 AAA CAT GGC TGC CAT CAC CTG 19694		69.2	AY055382	
24	SB26-F2b	19804 ACG AAA CAA GGG ACT CCT CTG G 19825	186	67.5	AY055382	Phage ST64B
	SB26-R2b	19989 AGG TTA TCT ATA ATT CCG ACC TGG 19966		62.0	AY055382	
25	SB28-F1b	21366 TGC AGT CAA GAG GAC GTC C 21384	136	64.2	AY055382	Phage ST64B
	SB28-R1b	21501 TGT CGT GAA TGG GGA GAG GAA G 21480		69.4	AY055382	
26	SB28-F2b	21841 GAC ACG GCT TTT TTG CCC AC 21860	154	68.6	AY055382	Phage ST64B
	SB28-R2b	21994 TGC CGA TTA TGC TGA TCT GGC 21974		69.2	AY055382	
27	SB37-F1b	26841 TGG TAG TGA ATT GGT TAG CTG C 26862	132	62.8	AY055382	Phage ST64B
	SB37-R1b	26972 CAA TGT GGG TTA ACA AGG GCG G 26952		70.8	AY055382	
28	SB37-F2b	27186 CAT CCA GTT CGC CCA GAA AGT C 27207	137	68.9	AY055382	Phage ST64B
	SB37-R2b	27322 CGG AAA GCT GTT ACA GCA GG 27303		64.7	AY055382	

Table 2. cont.

No.	Primer	Sequence (5'–3')	Size (bp)	T _m (°C)*	Accession no.	Source†
29	SB46-F1b	33640 CAT TGA TGG TAT CGA AGT TCG CC 33662	135	68.2	AY055382	Phage ST64B
	SB46-R1b	33774 TCA ATC AGC TCA CGG GTT TGC 33754		69.4	AY055382	
30	SB46-F2b	34001 AGT GCG CCA CAA ACA TCT TCT G 34022	130	68.4	AY055382	Phage ST64B
	SB46-R2b	34130 CCT GGA GTT TCT GGC ACG C 34112		68.2	AY055382	
31	SieB-F1b	11520 ATG GTG GCA GGA GTT AAT GC 11539	107	63.7	AF217253	Phage P22
	SieB-R1b	11626 CGC TTA CCG GCA TAA CAA TAA 11606		63.4	AF217253	
32	SieB-F2b	11718 CGG TGT TGT TAA TTC CGT TT 11737	104	61.2	AF217253	Phage P22
	SieB-R2b	11821 CAA ACA AAT CCC GAA CGA CT 11802		63.7	AF217253	

*The melting temperature (T_m) was provided by the primer synthesizer (Sigma-Aldrich).

†All primers designed by modifications based on AFLP fragments refer to Hu *et al.* (2002), bacteriophage ST64B refers to Ross & Heuizenroeder (2005) and bacteriophage P22, Gifsy-1 and -2 refer to Mikasova *et al.* (2005).

Outbreak investigations

Outbreak 1. The drug and alcohol rehabilitation centre outbreak in 2004 involved 23 residents from whom isolates were obtained. Nineteen residents had a diarrhoeal illness during one week in July; three other cases occurred in September and one isolate was collected from a chef at the centre in October. The outbreak was believed to be either food-borne or person-to-person spread. All 23 isolates belonged to PT 135. The mPCR/RLB clearly distinguished PT 126 from PT 135 isolates. All of the latter had the same RLB type, which was the same as one of two RLB types observed among the sporadic isolates (Table 1). Twenty PT 135 outbreak isolates (16 from July, 3 from September and one from the chef in October) belonged to MLVA 12 and each of the other three isolates was a different type, as were the two PT 126 isolates. By contrast, there were eight MLVA types (including MLVA 12) among 15 sporadic PT 135 isolates (Table 1). Epidemiological investigation, before the molecular typing results were available, failed to identify a common source. The MLVA results suggest that there may have been more than one source but this could not be confirmed.

Outbreak 2. The epidemiological investigation of the restaurant outbreaks in 2006 identified two clusters and a possible, but unconfirmed, third. The first cluster involved three cases linked to a restaurant, which also provided a food isolate from prawns. The second cluster of three cases was related to a nearby café, which had the same chicken supplier as the restaurant. Two other affected individuals had eaten food from a pizza shop and another had eaten at the same pizza shop and the café implicated in the second cluster. No epidemiological links were identified for five other cases.

All 15 'outbreak' isolates produced the same RLB pattern (RLB 103), which was also the most common (represented by 17 of 22 isolates) of five patterns observed among the sporadic isolates. The 14 human isolates were divided into two MLVA types (MLVA 2 and 5), which differed by a

single allele. The prawn isolate and two isolates from cluster 2 belonged to MLVA 2 and the remaining 12 belonged to MLVA 5, the most common of the seven MLVA types found among the sporadic isolates (Table 1).

Outbreak 3. This outbreak occurred over a short period among students attending a residential catering college, who cooked and ate together, which suggested a single common food source. All 17 human isolates from the students had the same RLB 58 type, which was the same as that of the PT 170 controls. DNA from four food isolates hybridized with only one to three of 38 RLB targets, suggesting that they were not *S. Typhimurium*. The other two, from chocolate mousse, belonged to the same RLB 58 type as the human isolates.

All human isolates and the two from chocolate mousse also had the same MLVA type (MLVA 107), which differed at only one locus from MLVA 2, which had previously been shown to be associated with PT 170. The other four food isolates did not produce amplicons from two to five of the loci.

Outbreak isolates from the affected students and the chocolate mousse were later identified as *S. Typhimurium* PT 170. The other food isolates were identified as two *Salmonella* Sofia isolates, from chicken, and two *Salmonella* Java isolates, from fish. Epidemiological investigation implicated chocolate mousse, which contained raw egg, as the most likely common food source (Table 1).

DISCUSSION

Our aim was to develop a molecular typing system for *S. Typhimurium* that could, potentially, replace or supplement phage typing, which has significant limitations and limited discriminatory ability. Improvements in commercial 'hot start' PCR *Taq* polymerase technology have made it easier to obtain all expected amplification products from increasing numbers of primers in a single multiplex reaction. If mPCR is used in combination with RLB hybridization, each

Table 3. Probes designed for the RLB hybridization

No.	Probe*	Sequence (5'–3')	T_m (°C)†	Accession no.
1	BIM4-P1	28305 AGG AAC CTA TCA CCT GGC AC 28324	62.9	AY055382
2	BIM4-P2	29809 CGG TTA AGT TCC TGC ACG 29792	64.5	AY055382
3	CA3-P1	89 AGG TTT CAT AGG AAA TCC CTC C 110	63.0	AF500155
4	CA3-P2	148 CGA TCA GGA AAG TAT CCA CTG G 127	64.5	AF500155
5	CA4-P1	52 GAC CAT CTT ACC TAC CTG TTC TGG 75	63.6	AF500156
6	CA4-P2	140 AAA CAA CGC AAA TTA CGC AC 121	62.4	AF500156
7	CA7-P1	24 ATT ACC CTG TTT GCC CGC 41	64.7	AF500159
8	CA7-P2	71 AGA CAT TTT TAC CGG ACC GG 52	60.8	AF500159
9	CC2-P1	49 CAA TCC AAA ACA GAC CGA CAG 69	65.9	AF500161
10	CC2-P2	125 CAG TTA TTT GGT TGT TCC CTG 105	60.8	AF500161
11	CC4-P1	31 CAG CTC ACG GGT TTG CTT AT 50	64.0	AF500169
12	CC4-P2	72 CGT CCG AAA TAC TGG CTT GAT A 51	64.8	AF500169
13	CT1-P1	49 TCG ATA ATG CCA ACG ACA AT 68	62.8	AF500164
14	CT1-P2	105 CCT TCA GGA CAA TCTCAA AGC 85	62.6	AF500164
15	eacP1	4812 CTC TTT GTT GTA TGC GCG G 4830	64.3	AF217253
16	eacP2	5090 GGT GAG GAT GTT AAG GAG CTT ATC 5067	62.8	AF217253
17	g8-P1	27481 GGA AGA GGG CTT TGA GAT TGT C 27502	64.9	AF217253
18	g8-P2	27863 CTT CGA CGT ATT GCG CTG A 27845	65.1	AF217253
19	g13-P1	20068 TCC TTG CGT TTG CAA TGG 20085	65.8	AF217253
20	g13-P2	20249 GTG CCG ATG TAG CCG ATA 20231	65.2	AF217253
21	gipA-P1	804 AAT CCC CTT AGA CGT GCG 821	62.6	AF246666
22	gipA-P2	963 ACC AAC ATC AAT GCC GAC C 945	65.8	AF246666
23	gtgB-P1	492 GGA TGA AGG CAG TCA GGA GAT ATT 515	65.3	AF254763
24	gtgB-P2	879 CAC ATA GTC TTT TCC TTC TTT ATT TCC 853	62.0	AF254763
25	SB6-P1	4421 AGG AAA AGG GCA AGC TTG 4440	61.9	AY055382
26	SB6-P2	4890 CCG TAG ATT TCC CCC ATG A 4871	64.5	AY055382
27	SB21-P1	15439 GGA ATA TGC TGC CTG ATA TGT G 15460	63.0	AY055382
28	SB21-P2	15842 GGT ATC CAG TAA TAG CGT TCC A 15821	61.6	AY055382
29	SB26-P1	19535 ATG CTC GCT GCG GTT AAC 19552	63.9	AY055382
30	SB26-P2	19904 AGC TCA ACC AGC AAA TGT CAG 19884	64.2	AY055382
31	SB28-P1	21419 CAC GCC TCG TTA AAT CCT TTA T 21437	62.8	AY055382
32	SB28-P2	21938 TGC CGT ATT TGG TGA TGC 21911	63.2	AY055382
33	SB37-P1	26873 GCC GCT TTA AGC TTT CCG 26890	64.6	AY055382
34	SB37-P2	27250 TG AGG GGT TAA CGA TGA ATG 27231	62.3	AY055382
35	SB46-P1	33687 TTA ACG ATT TGC ACC GTG C 33705	64.5	AY055382
36	SB46-P2	34110 CC CAG TAC CGA TGA ATT GGA 34091	64.5	AY055382
37	SieB-P1	11547 CCT GCA AGG AAT GAC ACT TAA AC 11569	63.4	AF217253
38	SieB-P2	11800 TTT CTT CTC TGT CCT TAC GCC 11780	62.2	AF217253

*Probes were designed on both strands inside primer sites listed in Table 3.

†The melting temperature (T_m) was provided by the primer synthesizer (Sigma-Aldrich).

individual amplification product can be identified by its reaction with a specific probe on a nylon membrane array.

Based on published sequence data, we designed or modified 32 primer sets targeting sequences from phages ST64B (Ross & Heuzenroeder, 2005), P22, Gifsy-1 and Gifsy-2 (Mikasova *et al.*, 2005) and *S. Typhimurium* phage type-related AFLP fragments (Hu *et al.*, 2002). They were designed to produce short PCR products with similar physical and biochemical characteristics to allow simultaneous amplification in a multiplex reaction. Similarly, probes were designed so that their optimal hybridization conditions were similar. We designed two primer sets and probes for phage-related targets in order to increase the

number of nucleotide polymorphisms identified and therefore the discriminatory ability of the assay. Each member of the pair serves as an internal control for the other. Because of their short length (100–150 bp), only single primer pairs and probes were used for targets based on phage type-related AFLP fragments.

This system identified and discriminated subtypes among many of the phage types tested. The RLB patterns were defined by the presence or absence and intensities of hybridization signals; differences in intensity between paired probes were confirmed by sequencing results. The mPCR results were validated by performing single PCRs for each primer set on one representative of each of the 46

Fig. 2. RLB patterns of isolates of *S. Typhimurium* from selected phage types. Lanes: 1–4 (PT 64), isolates 75, 77 (RLB 70), 95 (RLB 71), 131 (RLB 70); 5–8 (PT 6 var1), isolates 118, 111 (RLB 83), 112 (RLB 82), 122 (RLB 81); 9–13 (PT 99), isolates 103, 105, 106, 138, 139 (RLB 41); 14–16 (RDNC), isolates 186 (RLB 41), 187 (RLB 40), 143 (RLB 50); 17–20 (PT U302), isolates 150 (RLB 68), 154 (RLB 69), 155 (RLB 64), 157 (RLB 62); 21–24 (PT U307), isolates 196 (RLB 48), 197 (RLB 49), 198, 199 (RLB 50); 25–28 (untypable), isolates 204 (RLB 73), 205, 207, 208 (RLB 61); 29–30 (PT 186), isolates 137 (RLB 66), 159 (RLB 69); 31–32 (PT 9), isolates 177, 178 (RLB 38); 33 (PT 20 var1), isolate 89 (RLB 102); 34 (PT 30), isolate 148 (RLB 39); 35 (PT 140 var1), isolate 101 (RLB 78); 36 (PT 177), isolate 142 (RLB 97); 37 (PT 179), isolate 146 (RLB 87); 38 (PT 197), isolate 158 (RLB 79); 39 (PT 12), isolate 134 (RLB 66); 40–43 (PT 135), isolates 17, 19, 26, 35 (RLB 41). Left column: probe list.

phage types studied. The method reliably differentiated 21 of 46 individual phage types including some that are closely related, such as PT12 and PT12a. Some common phage types shared RLB types with one other, e.g. PT 8 with 9, PT 12 and 102 with 170, and PT 99 and RDNC with 135, suggesting a close relationship between members of the pair or group. Isolates of most of the common phage types exhibited two or more RLB patterns, indicating the potential for more discriminatory strain typing of common phage types. Multiple RLB patterns were found among the untypable and RDNC isolates, indicating that they represented diverse groups of largely unrelated strains.

The application of this novel mPCR/RLB system to the investigation of outbreaks confirmed its potential value. The first outbreak was suspected when phage-typing results demonstrated an increased number of cases due to the common phage type 135 within a short period of time. The

combination of mPCR/RLB and MLVA helped to define the clusters and identify related cases some time after they had occurred. In the second outbreak, all 15 isolates from three apparently related restaurant outbreaks had the same RLB pattern, which was also one of five patterns exhibited by sporadic isolates of *S. Typhimurium* PT 170. These results support epidemiological data and are compatible with the supposition that the three outbreaks were related to each other and to the food sample. In the third outbreak, investigation began immediately after salmonellae were isolated from cases and three types of food. The outbreak strain and the food source were rapidly identified by mPCR/RLB and predicted the phage type involved more than a week before the phage typing results were available.

Had it been performed routinely on all *S. Typhimurium* isolates, the mPCR/RLB typing alone would have provided

Table 4. Sequencing results based on scoring of RLB hybridization signals

Isolate	Signal*	Sequence† (probe CA4-P1: CCAGAACAGGTAGGTAAGATGGTC)	Signal*	Sequence† (probe CA4-P2: GTGCGTAATTTGCGTTGTTT)
174	2	CCAGAACAGGTAGGTAAGATGGTC	2	GTGCGTAATTTGCGTTGTTT
176	2	CCAGAACAGGTAGGTAAGATGGTC	2	GTGCGTAATTTGCGTTGTTT
127	1	CCAGAACA - - - GGTAAGATGGTC	2	GTGCGTAATTTGCGTTGTTT
128	1	CCAGAACA - - - GGTAAGATGGTC	2	GTGCGTAATTTGCGTTGTTT
100	0	CCAGAACA - - - GGTAAGATGGTC	2	GTGCGTAATTTGCGTTGTTT
123	0	CCAGAACA - - - GGTAAGATGGTC	2	GTGCGTAATTTGCGTTGTTT
		Sequence (probe CC4-P1: ATAAGCAAACCCGTGAGCTG)		Sequence (probe CC4-P2: TATCAAGCCAGTATTTTCGGAC)
17	2	ATAAGCAAACCCGTGAGCTG	2	TATCAAGCCAGTATTTTCGGAC
134	2	ATAAGCAAACCCGTGAGCTG	2	TATCAAGCCAGTATTTTCGGAC
143	2	ATAAGCAAACCCGTGAGCTG	2	TATCAAGCCAGTATTTTCGGAC
146	1	ATAAGCAAACCCGTGA <u>ACTG</u>	0	TATCAAGCCAGTA <u>CTT</u> GGGAC
158	1	ATAAGCAAACCCGTGA <u>ACTG</u>	0	TATCAAGCCAGTA <u>CTT</u> GGGAC
187	1	ATAAGCAAACCCGTGA <u>ACTG</u>	0	TATCAAGCCAGTA <u>CTT</u> GGGAC

*The signal scoring was as described in Methods.

†The deletion of a nucleotide is indicated by '—' and the nucleotide sequence different from the probe is underlined.

early warning of the first two outbreaks, before the phage typing or epidemiological data were available. For the third outbreak, which was immediately apparent because of the large number of related cases presenting within a short period, it rapidly defined the outbreak strain and confirmed the food source. It is more sensitive and specific than phage typing and results are available much sooner.

MLVA is also highly sensitive for strain typing, especially in outbreaks. Three of the loci tested, STTR5, STTR6 and STTR10pl, displayed a high degree of polymorphism indicated by indices of 0.91, 0.92 and 0.84, respectively, which were comparable with published work (Lindstedt *et al.*, 2004). One of the major drawbacks of this typing strategy is that the generation of variation in some MLVA loci is under selection pressure such that two identical clones may appear to differ if isolates have originated from different niches or from hosts with altered immune responses (Lindstedt, 2005). This may be manifested as too high a level of discrimination to correctly cluster isolates from a common source in an outbreak investigation. The PT 135 isolates from the first outbreak were divided into four MLVA types, of which one was predominant and the others differed by two to three alleles. It is not clear whether the three isolates belonging to minor MLVA types were sporadic or variants of the outbreak strain. Further experience with both RLB and MLVA is required to assist interpretation of these results.

Both mPCR/RLB assay and MLVA demonstrated similar discriminatory power in this study. For some phage types, the strains identified were the same between the two methods. For example, both methods separated PT 30, PT 141 and RDNC into different molecular types for each isolate tested (Fig. 1). Some phage types contained the same number of molecular types identified by both

methods; for example, four isolates of PT 102 contained three molecular types and seven isolates of PT 9 contained four. Both methods demonstrated a high level of similarity between isolates of some groups of phage types such as PT 135, PT 102 and PT 170 (Fig. 1).

Based on these results, mPCR/RLB is as sensitive and specific as MLVA. Both methods can provide informative strain typing data and can presumptively identify many common phage types within 24 h, compared with at least 3 weeks for phage typing at a reference laboratory. This difference represents a highly significant advantage for prompt identification of an outbreak source. An advantage of mPCR/RLB over MLVA is that the PCR amplicons are detected by hybridization rather than fragment size, which overcomes the problem of reproducibility between different platforms. Based on our results, mPCR/RLB showed less variation than MLVA among outbreak isolates, which made interpretation easier. MLVA is less labour intensive than mPCR/RLB and apparently more sensitive for outbreak investigations, but further experience with both methods is required to determine which more accurately reflects the epidemiology.

mPCR/RLB is a promising tool that can be developed further by the introduction of additional gene markers, including serotype-specific markers, to increase its sensitivity and discriminatory ability for *Salmonella* species other than *S. Typhimurium*. It has the potential to replace phage typing in the future.

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