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A rapid multiplex DNA suspension array method for *Salmonella typhimurium* subtyping using prophage-related markers

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ABSTRACT

In this study we developed a preliminary proof of concept of method for *Salmonella typhimurium* subtyping using multiplex PCR-based phage locus typing and a multiplex Luminex DNA suspension array for product detection. Thirty markers were selected from prophages ST64B, ST64T, ST104, P22, Gifsy-1, sopEΦ and mostly phage-related AFLP fragments, and organised into two multiplex PCRs of 15 markers each. A two-group DNA suspension array was developed using a combination of flow cytometry and Luminex xMAP® technology. To assess its subtyping capability the method was applied to 438 non-epidemiological related *S. typhimurium* isolates of 56 phage types. Eighty-one profiles were generated. Isolates were divided into sixteen main prophage marker profile types. There was a strong tendency for isolates with the same phage type to have the same or closely related profiles and for groups of phage types to share the same profile. The discriminatory power of this method expressed as the Simpson's Index of Diversity (D) was 0.954. A panel of 12 selected markers achieved almost the same D value (0.952) as the 30 markers. This new method provides an alternative typing scheme for *S. typhimurium* epidemiological investigations. The developed array is in a high-throughput format which could easily be semi-automated, making the test fast and economical.

1. Introduction

Salmonella enterica serovar *typhimurium* is a pathogen of reptiles, birds, and mammals and is a frequent food-borne pathogen of humans worldwide. In Australia, *Salmonella typhimurium* is the most common aetiological agent of salmonellosis and is a common cause of foodborne disease outbreaks (OzFoodNet Working Group, 2007, 2008).

Subtyping is an important tool for surveillance and outbreak investigations of human *S. typhimurium* infections. Phage typing is often used as an initial step in subclassifying *S. typhimurium* isolates (Anderson et al., 1977). Phage typing is based on the patterns of lysis produced by isolates when challenged by a set of distinct and agreed phages. Phage typing is a useful first-line subtyping tool but has its limitations. Some strains are not amenable to classification by the current phage-typing scheme. The procedure requires standardisation and a high level of expertise for results to be comparable between laboratories and consequently is only available at one or two reference laboratories for each country. This can lead to long turnaround times for subtyping results, limiting the public health response to outbreaks. Furthermore, some phage types can spontaneously convert from one type to another following infection with a temperate phage or plasmid (Baggesen et al., 1997; Brown et al., 1999; Frost et al., 1989; Mmolawa et al., 2002; Rankin and Platt, 1995; Ridley et al., 1996).

A number of molecular genotyping approaches have been developed to discriminate between *S. typhimurium* isolates. These include pulsed-field gel electrophoresis (PFGE) (Hudson et al., 2001; Meays et al., 2004; Threlfall et al., 1999), fluorescent amplified fragment length polymorphism (fAFLP) (Desai et al., 2001; Lawson et al., 2004; Lindstedt et al., 2000), multi-locus sequence typing (MLST) (Kotetishvili et al., 2002; Urwin and Maiden, 2003) and multi-locus variable number tandem repeat analysis (MLVA) (Lindstedt et al., 2004). These methods have one or more of the following shortcomings: portability, labour-intensive, high set-up cost and, long turnaround time (Baker et al., 2010). Bacteriophages are a key factor driving the microevolution of *Salmonella enterica* (Thomson et al., 2004). The origin of much of the genetic diversity among closely related isolates of *S. enterica* comes from mobile elements (mostly phages). Consequently, a number of subtyping approaches based on detection of prophage and plasmid sequences have been developed (Cooke et al., 2007; Drahovska et al., 2007; Lan et al., 2007; Mikasova et al., 2005; Ross and Heuzenroeder, 2005; Rychlik et al., 2008; Wang et al., 2008). These methods have the advantage that the results most closely mimic those of traditional phage-typing. Thus, Ross and Heuzenroeder applied PCR amplification followed by sequencing to a selection of phage loci derived from the sequenced phages ST64B, ST64T and P22. For 73 isolates belonging to nine phage types a good level of discrimination between isolates was

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observed (Ross and Heuzenroeder, 2005). Lan et al. applied 22 AFLP-derived mostly prophage and plasmid sequences to the PCR typing of 121 *S. typhimurium* isolates from 33 phage types (Lan et al., 2007). The prophage sequences were located in Gifsy-1 and -2, in P22-like phages ST64T, ST104 and P22, and in ST64B and SopEΦ. Drahovska et al. tested for twelve prophage markers in a panel of 152 *S. typhimurium* isolates from 23 phage types and compared results with those for MLVA typing (Drahovska et al., 2007). Both methods produced similar groupings which correlated well with phage type. Rychlik et al. developed four triplex PCRs specific for twelve target sequences of mostly prophage origin, and tested them with 102 isolates, mainly in DT104 and DT2 strains. The results showed slightly less discriminatory power than PFGE method, but its simplicity and user-friendly advantages produced a potential alternative to PFGE typing of *S. typhimurium* (Rychlik et al., 2008). Cooke et al. designed a multiplex PCR which targeted some of the hot spots of prophage sequences and the antibiotic resistance island SGI1, which is known to be present in the antibiotic-resistant DT104 strains (Cooke et al., 2007). Wang et al. developed a phage locus typing method in which 38 probes were used in a reverse line blot assay to detect amplified product from a multiplex PCR. A good level of discrimination was achieved (Wang et al., 2008).

The aim of this study was to develop a convenient and highly discriminatory prophage marker-based typing method to differentiate *S. typhimurium* isolates. We have proposed to rationalise the choice of markers from the five reports (Drahovska et al., 2007; Hu et al., 2002; Lan et al., 2007; Mikasova et al., 2005; Ross and Heuzenroeder, 2005) in order to maximise discriminatory potential for a wide range of phage types. We have developed a unique approach to *S. typhimurium* subtyping which combines a multiplex PCR amplification of prophage markers with a multiplex Luminex DNA suspension array for marker detection. This test platform is relatively cheap, rapid and easy to operate. The subtyping scheme detects either the presence or absence of these particular prophage markers in individual isolates. Since the results of the individual prophage markers are in a binary form (positive and negative) the results can be easily communicated between laboratories. Furthermore, the subtyping results are presented in a table format, which makes the relationship between phage type and phage marker profile easier to identify.

2. Materials and methods

2.1. Bacterial strains and DNA isolation

A total of 438 *S. typhimurium* isolates from 58 definitive phage types (DT) and including 11 untypable and 5 reactions-do-not-conform (RDNC) isolates were selected from a *Salmonella* collection of the Queensland Health Enteric Pathogens Reference Laboratory. The samples were all isolated locally from sporadic patient stool samples and they did not have proven epidemiological relatedness. They were phage typed (Anderson et al., 1977) by the Microbiological Diagnostic Unit, University of Melbourne. DNA preparations for PCR were made by taking a single colony from an overnight culture into 400 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and boiling for 5 min.

2.2. Primers and probes

For the 30 selected prophage markers (see below) the primers designed for this study and the primers taken from the references are shown in Table 1. For efficient amplification by multiplex PCR (mPCR) and efficient capture by probes new primer pairs were designed to yield PCR products between 100 and 300 bp. The primers were designed with OLIGO 6.71 software (Molecular Biology Insights, Inc) (Rychlik and Rhoads, 1989) and had a Td (= Tm calculated by nearest neighbour method) higher than 65 °C. The specificity of the candidate sequences of the primers were assessed by GenBank BLAST (www.ncbi.nlm.nih.gov). For the Luminex DNA array tests, the reverse primers were biotinylated at the 5′ end (Geneworks, Australia) to enable detection of PCR amplicons after capture by probes.

The probes used in this study are listed in Table 2. The probe sequence is complementary to the biotinylated strand of the target amplicon (Table 1). The designed probes were synthesised with a 5′- end amino C12 modification with standard desalting purification (Geneworks, Australia). The amino modification and C12 spacer facilitated the coupling of the oligonucleotide probe sequence to the carboxylated surface of the microspheres. Each microsphere set contained unique spectral address by combining different ratios of red and infrared fluorochromes. The probes were covalently linked with the unique sets of polystyrene carboxylated microspheres by a carbodiimide method (Dunbar et al., 2003; Fulton et al., 1997; Lee et al., 2004). Briefly, 2.5×10^6 of microspheres (xMAP®, Luminex) were suspended in 50 μl of 0.1 M MES (4-morpholineethanesulfonic acid, Sigma), pH 4.5, with 0.2 nmol of probe. After vortexing, the beads were incubated twice with a final concentration of 0.5 μg/μl of EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, Pierce), and agitated in the dark for 30 min each time at room temperature. The microspheres were washed with 0.5 ml of 0.02% Tween20 (Sigma), followed by 0.5 ml of 0.1% sodium dodecyl sulfate (Sigma). The beads were resuspended in 100 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and kept in the dark at 4 °C. The probe-bead concentrations were determined by counting the particles with a microscope (ZEISS, West Germany).

2.3. Prophage marker selection and validation

Fifty-one marker candidates were tested in the initial screening. Nine of these (*immC cl-5′*, *immC cl-3′*, *immC cro*, *immC* put *c2*, SB6, SB26, SB28, SB37 and SB46) were based on bacteriophage ST64B (Ross and Heuzenroeder, 2005), seven (*mnt*^{64T}, *gtrA, gtrB, gtrC*, *g9-5′*, *g9-3′*and *g17*) on ST64T and/or P22 bacteriophages (Ross and Heuzenroeder, 2005), eight (*eac, eae1*, *g8*, *g13*, *int, mnt*^{P22}, *sieA* and *sieB*) on bacteriophage P22 (Drahovska et al., 2007; Mikasova et al., 2005; Ross and Heuzenroeder, 2005), three (*eae2*, *g45* and *g62*) on bacteriophage ST104 (Drahovska et al., 2007), two (STTR6 and *gipA*) on Gifsy-1, one (*gtgB*) on Gifsy-2, one (*nanH*) on Fels-1 bacteriophage (Drahovska et al., 2007; Mikasova et al., 2005), *sopE* from bacteriophage sopEΦ (Drahovska et al., 2007) and 19 markers (CA-1, CA-2, CA-3, CA-4, CA-7, CA26.1, CA28.4, CC-1, CG-1, CG-2, CC-5, GC-2, CT-1, CT-2, CT29, GA-1, GA12.2, GA27.1 and GC-1) from the AFLP fragments (Hu et al., 2002; Lan et al., 2007). Agarose gel-based PCR was performed using the original published primer pair for each of the marker candidates on a panel of DNA samples, consisting of 16 isolates each of phage types (DTs 9, 12, 12a, 64, 126, 135, 170, 197 and U307) frequently encountered in Queensland, Australia. Only the markers less than 99% positive or negative in the tested samples were selected for further analysis. A total of 30 markers met the criteria and new primers were designed to obtain the amplicons between 100 and 300 bp. The PCR results with the new primer pairs were compared with those using primer pairs from the references. If the new primers produced results consistent with previous results on DNA gel, then the marker was selected for further multiplex PCR and DNA array analysis.

2.4. Multiplex PCRs

The amplification of the 30 markers was organised in two multiplex PCRs (mPCR). The first mPCR consisted of numbers one to fifteen of the primer sets and the second included numbers sixteen to thirty of the primer sets (Table 1). A 25-μl mPCR mixture was set up using the AmpliTaq® Gold PCR reagent kit (Applied Biosystems, USA). The reaction contained 2.5 μl of 10 × PCR buffer, 2.5 mM of $MgCl_2$, 1 U of AmpliTaq Gold, 200 μM of each dNTPs (Amersham, Australia), 2 μl of DNA template, 0.04 μM of each forward primer and 0.2 μM of each reverse primer