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Methodologies towards the development of an oligonucleotide microarray for determination of *Salmonella* serotypes

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Abstract

A DNA-based microarray designed to detect somatic (O) and flagellar (H) antigens present in the five most commonly isolated *Salmonella* serovars within Canada was developed as an alternative to the traditional Kauffmann–White serotyping scheme currently used to serotype salmonellae. Short oligonucleotide probes were designed based on publicly available sequence data of selected genes responsible for O and H antigen biosynthesis. These targets included: antigen-specific sequences within the flagella (H) antigen phase 1 (*fli*C) and phase 2 (*flj*B) genes and somatic (O) antigen biosynthesis genes within the *rfb* cluster (Groups B—*rfb*J, C1—*wba*A, C2—*rfb*J, D1—*rfb*S). A prototype microarray with 117 O and H antigen-specific probes and controls was used to assess probe performance against two pools of gene target PCR amplicons. A set of 31 of these antigen-specific probes (8 O and 23 H) with high specific signal and low non-specific signal were selected based on *t*-test (*p*-value <0.01) and $\log_2$ ratio distribution analysis to create a prototype microarray. The microarray was tested against 16 *Salmonella* strains of known serotype. Based on the strains tested in this study, these probes successfully identified and differentiated 11 of the 12 antigens targeted. The prototype DNA-based typing microarray described here has the potential to be an automated alternative to the traditional antigen–antibody serotyping scheme currently used for *Salmonella*.

Keywords: Microarray; *Salmonella*; Serotyping

1. Introduction

Salmonella enterica is one of the leading causes of foodborne gastroenteritis in humans worldwide. There are about 30,000 culture-confirmed cases of salmonellosis reported annually in the US (CDC, 2005), although many cases are not diagnosed or reported, and the actual annual number of infections is estimated at 1.4 million (CDC, 2005). Canadian statistics reported 11,201 culture-confirmed cases of salmonellosis in 2003 (Demczuk, 2005), and for every *Salmonella* infection reported, it is estimated that there are 13 to 37 cases annually (Thomas et al., 2006).

Serotyping is central to the epidemiological classification of *Salmonella* strains, surveillance studies used to identify trends in disease transmission, detection of outbreaks, and monitoring of control efforts (Olsen et al., 2001).

The Kauffman–White serotyping scheme is a widely accepted method used to characterize *Salmonella* based on the antigenic variability of the lipopolysaccharide moieties (O antigen), flagellar proteins (H antigen), and capsular polysaccharides (Vi antigen) (Popoff, 2001; Popoff et al., 2004). There are currently over 2500 recognized *Salmonella* serotypes according to the Kauffman–White serotyping scheme (Popoff, 2001; Popoff et al., 2004). *Salmonella* has two flagellar antigens that are co-ordinately regulated by phase variation between the *fli*C gene, which encodes phase 1 (H1) flagella, and the *flj*B gene, which encodes phase 2 (H2) flagella (Silverman, 1979). Despite the usefulness of traditional serotyping, it has

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many limitations, including the high cost and the time involved in producing antisera, the laborious quality control necessary to maintain the hundreds of antisera required for serotyping, and a lack of standardization of antisera in the current *Salmonella* serotyping method (Seyfarth et al., 2003).

This study describes the development of a prototype oligonucleotide microarray method towards serotyping the five most frequently isolated salmonellae from humans in Canada; *Salmonella* serovars Typhimurium (1,4,[5],12:i:1,2), Enteritidis (1,9,12:g,m:–), Heidelberg (1,4,[5],12:r:1,2), Hadar (6,8:z_{10}:e,n,x), and Thompson (6,7,14:k:1,5), respectively. These five *Salmonella* serovars represent 59.2% of human isolates and 50.6% of non-human isolates collected in Canada in 2002 (Demczuk, 2005). The DNA-based microarray method serves as a feasible alternative to the traditional serotyping scheme, and provides an antigenic formula consistent with the Kauffman–White nomenclature, including the O, H1 and H2 antigen genes. This study represents the first step towards developing a *Salmonella* serotyping microarray to detect the 100 most frequently isolated *Salmonella* serovars in Canada.

In addition to eliminating the need for production and maintenance of quality control of hundreds of antisera, a *Salmonella* serotyping microarray would facilitate the analysis of many antigens simultaneously, thus producing results more rapidly. The development of a molecular technique also enables the progress towards a standardized global surveillance system for use across research, reference and diagnostic laboratories.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Salmonella strains used to test candidate oligonucleotide probes on the prototype microarray were obtained from the *Salmonella* Typing Lab, Laboratory for Foodborne Zoonoses, Guelph, ON, and the National Laboratory for Enteric Pathogens, Winnipeg, MB. For routine culture, *Salmonella* strains were grown overnight at 37 °C on Luria-Bertani (LB) agar (Fisher Scientific, Nepean, ON).

2.2. Microarray design

Flagellar antigen oligonucleotide probes were designed based on the antigen-specific DNA sequences within the flagella (H) antigen phase 1 (*fli*C) and phase 2 (*flj*B) genes. Phase 1 H antigens included i; g,m; r; z_{10}; and k, and phase 2 H antigens included 1,2; e,n,x; and 1,5 (McQuiston et al., 2004). Somatic antigen oligonucleotide probes were designed using publicly available sequences of gene targets involved in O antigen biosynthesis within the *Salmonella rfb* cluster [Groups B *rfb*J (X56793), C1 *wba*A (M84642), C2 *rfb*J (X61917), D1 *rfb*S (AF442575) and D1 *rfb*S *Salmonella* Typhi-specific (M29682)]. Alignments of homologous sequences were created using MegAlign (DNASTAR Inc., Madison, WI, USA), or Seqman (DNASTAR Inc., Madison, WI, USA) to aid in identification of unique antigenic regions. Eighteen to 35-mer oligonucleotide probes were designed using PrimerSelect (DNASTAR). The predicted specificity of probe sequences was analyzed using GenBank's Basic Local Alignment Search Tool (BLASTN) (Altschul et al., 1990). A prototype microarray was generated, consisting of 117 probes printed in quadruplicate, representing 8 H and 4 O antigens. This prototype microarray contained at least 2 oligonucleotide probes representing each of these O and H antigens.

The probes were spotted in quadruplicate and were randomized across the array to reduce spatial effects. Positive control gene targets on the prototype included *Salmonella* (*inv*A), enteric-specific (*gap*A) probes and *Arabadopsis thaliana* chlorophyll synthetase printed in a dilution series with the corresponding spike-in PCR amplicon. Negative controls included an *A. thaliana*-specific probe targeting the ATS3 gene and printing buffer.

2.3. Microarray printing

For the printing experiment, amino-linked (AL) probes were printed on both Hydrogel Aldehyde slides (NoAb BioDiscoveries, Mississauga, ON) and Epoxide slides (Corning, Acton, MA, USA) at a concentration of 40 μM using the Virtek ChipMaker Pro Version 1.2.1 (Bio-Rad, Mississauga, ON) with an SMP3 pin (TeleChem International, Inc., London, United Kingdom). Unmodified probes were also printed on the same Epoxide slides, which accommodate both probe types, thus allowing a direct comparison of probe performance. The printing buffer from NoAb BioDiscoveries was used to print on both slide surfaces and was also included on the arrays as a negative control.

For the probe and array validation experiments, AL probes were printed on Epoxide slides at a concentration of 40 μM using the Virtek ChipMaker Pro version 1.2.3 (Bio-Rad, Mississauga, ON) with four Stealth SMP3 pins (TeleChem International, Inc., London, United Kingdom). Pronto Epoxide Slide Spotting solution (Corning) was printed as a negative control throughout the array. The printed oligonucleotide probes were immobilized on the arrays by UV crosslinking at 600 mJ and stored in a desiccator (Secador Desiccator™, Structure Probe Inc., West Chester, PA, USA) until use. Quality control of the microarray slides was assessed using Paragon DNA Microarray Stain QC Kit (Molecular Probes, OR, USA) and Spot QC (IDT, Coralville, IA, USA).

2.4. Target preparation

Genomic DNA was isolated from the target *Salmonella* strains grown on LB agar overnight at 37 °C using DNeasy™ (QIAGEN Ltd., Mississauga, ON) or EZ1 DNA Tissue Kit and a BioRobot EZ1 (QIAGEN) according to the manufacturer's instructions. DNA was quantified spectrophotometrically (Nanodrop® ND-1000, Nanodrop Technologies, Inc., Wilmington, DE, USA). For each *Salmonella* strain tested, the microarray targets were prepared by individual PCR amplification reactions. Targeted genes included the *fli*C and *flj*B H antigen genes, selected somatic genes within the *rfb* cluster (Table 1), a conserved enteric gene (*gapA*), a *Salmonella*-specific gene