Multiplex, bead-based suspension array for the molecular determination of *Salmonella* H serogroups

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Abstract

Salmonella enterica continues to be an important foodborne pathogen worldwide. Serotyping is the fundamental approach in the epidemiology of *Salmonella*. It consists of the immunologic classification of two surface structures, O-polysaccharide (O antigen) and flagellin protein (H antigen). *Salmonella* serotypes are the basis for national surveillance networks and are critical to the identification of outbreaks.The current conventional serotyping procedure is time-consuming, requiring 3 to 5 days or more to fully determine the serotype, thus delaying information and costing laboratories in overhead. Furthermore, production and quality control of the hundreds of antisera required for serotyping are difficult and time consuming. To avoid the difficulties of antisera production and to take advantage of the automability of DNA technology, the National Centre for Infectious Diseases, CDC, Atlanta has developed a system for the determination of serotypes based on DNA markers within genes responsible for O and H antigen expression. This system combines PCR with a bead-based suspension array detection system. The objective of this paper is to describe the application of the Luminex® Multianalyte profling (xMAP) technology to detect the H antigens of *Salmonella*.

Introduction

Serotyping of *Salmonella* remains the most common subtyping technique and is useful for routine surveillance, identification and characterisation of disease outbreaks, and for understanding population biology and evolution of disease. Traditional *Salmonella* detection and species identification methods are based on cultures using selective media and characterisation of suspicious colonies by biochemical and serological tests. The Kauffmann White serotyping scheme recognizes 46 O serogroups and 115 H antigens in various combinations to define the 2,587 serotypes (Popoff, 2006). A minimum of 3 days is required to determine the serotype of an isolate. Furthermore, traditional serotyping requires the maintenance of hundreds of antisera, which can be expensive and time-consuming. It also requires growth of isolate and is dependent on antigen expression.

To simplify the identification of *Salmonella* serotypes, an alternate approach that utilise modern molecular technology is developed. The genes involved in the biosynthesis of the O group antigens are organised in a large regulon, termed the *rfb* region. Genes typically found in *rfb* region include genes involved in the sugar biosynthesis, genes encoding sugar

transferases that assemble component sugar into an O subunit monomer, an export enzyme (encoded by *wzx*) that exports O subunits to the periplasmic space, and an O antigen polymerase (encoded by *wzy*) that assembles the O subunit into the O antigen. While the *rfb* region can be found in many enteric organisms, the composition of individual *rfb* region is flanked by the same two genes, *gal*F and *gnd*. PCR primers to the flanking sequences can be used to amplify the *rfb* region from unrelated O serogroups. Most flagellar (H) antigens in *Salmonella* sp are encoded by one of two genes, *fli*C or *flj*B, which encode the Phase 1 and Phase 2 antigens, respectively. These loci are co-ordinately regulated so that only one antigen is expressed at a time in a single cell. The approach has been to sequence the genes responsible for serotype in *Salmonella* (*rfb*, *fli*C and *flj*B) and to design antigen-specific probes. This will provide a correlation between serotype determined by traditional and new method.

The Luminex multianalyte profiling (xMAP) technology is a bead-based suspension array that allows the detection of different molecular targets in a single tube/reaction. This technology is a suspension array system that utilizes a flow cytometry-like instrument with two lasers: a classification laser and a reporter laser. In this study, specific probes targetting the H antigen genes are covalently linked to individual fluorescently-encoded microsphere (beads) sets. Each set of beads is internally labelled with a different ratio of red to infrared fluorophores, creating an array consisting of up to 1000 individually addressable bead sets. Each bead is excited by the classification laser to reveal a unique spectral address (Dunbar et al., 2003).

Methods and Materials

PCR amplification. PCR primers corresponding to specific *fli*C and *flj*B genes were used. The forward primer was biotinylated at the 5' end to allow binding of the reporter dye, streptavidin-R-phycoerythrin, and subsequent detection on the Bio-Plex platform. The target sequences for the H groups were amplified in a single, multiplex PCR reaction. PCR amplification was performed in 25ul volumes using a Hot-start PCR kit (Promega, Madison, WI) according to manufacturer's instructions. PCR amplification was carried out in a thermal cycler (MJ Research, MA, USA) with the following cycle parameters: initial denaturation, 95°C, 15mins; then 30 cycles of 94°C, 30 seconds (denaturation); 48°C, 60 seconds (annealing); 72°C, 90 seconds (extension); and 72° C for 10 mins (final extension). PCR amplicons were then used directly with the coupled beads in the hybridization reaction described below and the H group targets detected in a single Bio-Plex assay.

Coupling of probes to microspheres (beads). Oligonucleotide probes based on *fli*C *and flj*B genes were synthesized with an amino-modified 6-carbon spacer at the 5' end of each probe. Carbodiimide coupling was then used to covalently attach the probes to carboxylated beads. In brief, 200 pmol of each oligonucleotide probe, 2.5 x 10^6 microspheres (Luminex Corp., Austin, TX) and 25μg of freshly prepared N-(3-dimethylaminodipropyl)-N'-ethylcarbodiimide (EDC; Pierce Chemicals, IL) were combined in 25ul of 100mM 2-(N-morpholino)-ethanesulfonic acid (MES), pH 4.5. The reaction mixtures were incubated in the dark at room temperature for 30 mins with

constant shaking, and the EDC treatment and subsequent incubation step repeated once. After coupling, the microspheres were washed with 1.0ml of 0.02% Tween 20 (Sigma, St Louis, MO) followed by a second wash with 0.5ml of 0.1% SDS (Sigma). Coupled microspheres were then suspended in 50ul of TE buffer (0.01M Tris-EDTA, pH 8.0) and stored at 4°C in the dark. All H-group specific bead sets were mixed by combining equal volumes (3 ul) of each and the mixture stored in the dark at 4°C until used.

Hybridization of bead-coupled probes to PCR amplicons. Immediately before use in the hybridization reaction, the H-group beads mixture was brought to a final volume of 1ml in 1.5X TMAC buffer (1.5M tetramethylammonium chloride, 75mM Tris, 6mM EDTA, and 0.15% sarkosyl, pH 8.0), 5 ul PCR product and 12ul of TE buffer in a single well of a 96-well microtiter plate. The reaction mixture was incubated for 5 min at 94°C, then 30 min at 52°C to denature the DNA and to allow hybridization of the probes to the PCR amplicons.

Detection of hybridized amplicons. The microspheres were then suspended in 75ul of detection buffer [R-phycoerythrin-conjugated streptavidin, (Molecular Probes, Eugene, OR) diluted to 4 ug/ml in 1X TMAC hybridization buffer]. Samples were incubated an additional 10 min at 52°C and then analyzed on the Bio-Plex (Bio-Rad, Hercules, CA). The median fluorescence intensity (MFI) for each bead set was calculated automatically by using the Bio-Plex software. A positive signal was defined as an MFI giving 6x the background fluorescence intensity for each bead-probe set.

Results and Discussion

Amplification of *fli*C(H1) and *flj*B (H2) were successfully carried out. To assess the utility of the Luminex suspension array system for molecular serotyping of *Salmonella enterica,* probes were designed based on the nucleotide sequences of the *fli*C and *flj*B genes. In this study, probes were designed to target the EN, 1, L, and G complexes. Probes specificity was verified by following through the entire process as briefly outlined in the methodology. At this point, we have successfully evaluated and validated the specificity of specific probes against the intended targets. The specificity of probes was evaluated against 499 Salmonella strains of known serotypes for EN, 1, L, G and z4 complexes (Table 1). Of the 499 *Salmonella* strains tested, 98% and 93% of the Phase 1 and Phase 2 antigens respectively, as determined by luminex technique matched with the data obtained by conventional method. To date, probes for almost 90% of the top 100 serotypes in United States have been tested and evaluated (Table 2).

Table 1: Summary of the specificity of the probes to detect the H antigens of Salmonella spp.

Phase 1 (H1 antigen)	Number of strains tested	% of positives
Isolates tested Match Ref Lab	499 489	 98%
Phase 2 (H2 antigen)		
Isolates tested Match Ref lab	499 462	 93%
NB : The primary reason for mismatch is subspp III in allele diversity		

Table 2 : Progress of work to serotype the top 100 serogroups of *Salmonella* by the luminex technology. Because of the numerous H antigens, the assay is divided into two tiers, the first tier comprises of the most common H antigens.

Tier 1 Antigens tested	Status of testing	Tier 1 Antigens tested	Status of testing	Tier 2 Antigens tested	Status of testing
b	completed	Z15	completed	a	completed
d	completed	G complex	completed	c	completed
e,h	completed	1 complex	completed	g,z23	completed
f	completed	EN complex	completed	l,w	completed
g,m	completed	L complex	completed	l,z13	completed
s	completed			z24	completed
i	completed			z29	completed
r	completed			z6	completed
z	completed			p	completed
zw	completed			y	completed
1,2	completed			z23	completed
1,5	completed			z4 complex	completed
1,6	completed				
1,7	completed				
x	completed				
k	completed				
l,v	completed				
l,z28	completed				
m,t	completed				

For a high-throughput assay to be useful to both research and public health laboratories, the cost of any assay and necessary instrument must be reasonable. In addition, an assay format that can accommodate analysis of a number of different organisms is preferable. The experimental data illustrate the relative ease and efficacy of designing and implementing a suspension array for serotyping *Salmonella.* The estimated cost per isolate is about $4.95 and the amount of time required is less than 5 hours.

The development of a rapid, high-resolution, and genetically informative pathogen-subtyping technology is a necessary component of an effective infectious-disease surveillance system. Although glass microarray slide (planar microarray) subtyping

analysis has been shown to be high resolution and genetically informative, it is technically challenging. Suspension microarray eliminates many of the technical issues associated with planar-microarray such as printing and washing artefacts, post hybridisation images analysis, and data normalisations (Borucki et al, 2005).

Reference

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