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Serotype determination of *Salmonella* by xTAG assay

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ABSTRACT

Currently, no protocols or commercial kits are available to determine the serotypes of *Salmonella* by using Luminex MAGPIX®. In this study, an xTAG assay for serotype determination of *Salmonella* suitable for Luminex MAGPIX® is described and 228 *Salmonella* isolates were serotype determined by this xTAG assay. The xTAG assay consists of two steps: 1) Multiplex PCR to amplify simultaneously O, H and Vi antigen genes of *Salmonella*, and 2) Magplex-TAG™ microsphere hybridization to identify accurately the specific PCR products of different antigens. Compared with the serotyping results of traditional serum agglutination test, the sensitivity and specificity of the xTAG assay were 95.1% and 100%, respectively. The agreement rate of these two assays was 95.2%. Compared with Luminex xMAP® *Salmonella* Serotyping Assay (SSA) kit, the advantages of this xTAG assay are: First, the magnetic beads make it applicable to both the Luminex®100/200™ and MAGPIX® systems. Second, only primers rather than both primers and probes are needed in the xTAG assay, and the process of coupling antigen-specific oligonucleotide probes to beads is circumvented, which make the xTAG assay convenient to be utilized by other laboratories. The xTAG assay may serve as a rapid alternative or complementary method for traditional *Salmonella* serotyping tests, especially for laboratories that utilize the MAGPIX® systems.

1. Introduction

Salmonella is a major cause of food-borne disease in many countries (Hendriksen et al., 2011; WHO, 2016). A total of 2659 serotypes have been found so far (Grimont and Weill, 2007; Issenhuth-Jeanjean et al., 2014). Serotype determination of *Salmonella* is important for disease assessment, infection control, and epidemiological surveillance (CDC, 2011). Serum agglutination test, which is based on the surface antigen identification of O, H and Vi antigens according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007; Issenhuth-Jeanjean et al., 2014) has been regarded as the gold standard for *Salmonella* serotyping. However, it shows some limitations due to subjectivity, cross-reaction, and is not efficient enough for serotyping multiple strains of *Salmonella* rapidly.

In recent years, nucleic acid-based assays have been used for the serotype determination of *Salmonella* (Bugarel et al., 2017; Fan et al., 2015; Guo et al., 2013; Muñoz et al., 2010; Ranieri et al., 2013). The *rfb* gene cluster, *fliC*, *fljB* and *viaB* genes, which have been reported to be involved in the expression of O, phase 1 H, phase 2 H and Vi antigens of *Salmonella* (Liu et al., 2014; McQuiston et al., 2004), were often selected as the target genes. Bead-based suspension array, due to its high throughput and great flexibility in array preparation, offers an efficient way for identification of multiplex PCR products, and has been used in the serotype determination of *Salmonella*, such as the xMAP® *Salmonella* Serotyping Assay (SSA) kit manufactured by Luminex corporation based on published research (Fitzgerald et al., 2007; McQuiston et al., 2011). Multiplex PCR were performed to amplify the variable regions of the target genes and the amplicons were hybridized with antigen-specific oligonucleotide probe-coupled microsphere mixture. The evaluations demonstrated that it had a good congruency with traditional serotyping (Dunbar et al., 2015; Liang et al., 2016; Yoshida et al., 2016). However, the microspheres used in the SSA kit are non-magnetic and thus cannot be applied to the Luminex MAGPIX®, which utilizes the technology of charge-coupled device (CCD) rather than flow cytometry (FCM) and magnetic microspheres are required. Moreover, since the process of coupling antigen-specific oligonucleotide probes to microspheres is complicated, with each coupling reaction taking more than 20 steps (Angeloni et al., 2016), the preparation of bead arrays acquires considerable effort and the xMAP assays reported before (Fitzgerald et al., 2007; McQuiston et al., 2011) are not easily shared between laboratories. Thus, for laboratories facilitated with MAGPIX® rather than Luminex®100/200™, such as our laboratory, no protocols or commercial kits are available for high-throughput serotype determination of *Salmonella*.

In this study, a multiplex PCR-based TAG microsphere suspension array suitable for both the MAGPIX® and Luminex®100/200™ systems is

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described and 228 *Salmonella* isolates were serotype determined by this xTAG assay. With the adoption of xTAG® technology and MagPlex-TAG™ microspheres, which use a universal tag system that is convenient for development and optimization of nucleic acid assays (Luminex, 2017a), oligonucleotide probes and the process of coupling oligos to beads are circumvented. MagPlex-TAG™ microspheres are oligonucleotide-coupled polystyrene microparticles, or "beads", that have been dyed into spectrally distinct sets (Luminex, 2017b). Each set of these uniquely color coded beads is covalently coupled with unique 24-base DNA sequences, called "anti-TAGs", allowing the beads to capture complementary TAG sequences generated on target molecules. In this xTAG assay, biotinylated forward primers and TAG-containing reverse primers were designed and synthesized based on the *rfb* gene cluster, *fliC*, *fljB* and *viaB* genes. After amplification and labeling with TAG sequence and biotin, the PCR products of specimens were hybridized with the mixture of MagPlex-TAG™ microspheres, and the hybridization results were analyzed. The serotypes of 228 *Salmonella* isolates were determined by this multiplex PCR-xTAG suspension array and the results indicate that this xTAG assay could serve as a rapid alternative or complementary approach for traditional *Salmonella* serotyping methods.

2. Materials and methods

2.1. Bacterial strains

All bacterial strains were from the collection of the Hangzhou Center for Disease Control and Prevention, China. Five *Salmonella* serovar reference strains (*Salmonella* Typhimurium ATCC 14028, *Salmonella* Typhimurium CMCC 50013, *Salmonella* Enteritidis CMCC 50335, *Salmonella* Typhi CMCC 50071 and *Salmonella* Braenderup H9812) and 228 *Salmonella* strains isolated from clinical samples in food-borne disease surveillance program during the period of 2014–2016 were analyzed in this study. All the isolated strains were identified as *Salmonella* by using VITEK® 2 Compact (bioMèrieux, France).Serum agglutination tests and phase inversion analyses were performed by using SSI® *Salmonella* antiserum for slide agglutination (SSI Diagnostica, Denmark) according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007; Issenhuth-Jeanjean et al., 2014).The manufacturer's instructions were followed strictly.

2.2. Nucleic acid extraction

After bacterial growth overnight at 37 °C in LB broth (BD Diagnostics, USA), bacterial nucleic acids were extracted by using a QIAamp® DNA mini kit (QIAGEN, Germany). The manufacturer's instructions were followed strictly. One milliliter of bacterial culture was used and the purified nucleic acids were finally eluted with 200 μL of the elution buffer.

2.3. Primer preparation

The *rfb* gene cluster, *fliC*, *fljB*, and *viaB* genes were selected as the target genes. The homology of the sequences was analyzed by Clustal W program (Chenna et al., 2003). Based on the highly conserved regions (see Fig. S1 in the Supplemental material), common primers were designed by using Primer Premier 6.0, and the corresponding primers, which were specific, were designed based on the variable regions. An appropriate TAG sequence, which is complementary with the anti-TAG sequence pre-coupled on one MagPlex-TAG™ microsphere set by Luminex corporation (Luminex, 2017b), was selected for each specific reverse primer. Oligo 7.58 was used to evaluate the duplex formation, hairpin formation, false priming sites, GC composition and melting temperature (Tm) of the primers. A total of 60 primers were used (Table 1), of which FO1 is the common forward primer for R1 and R2, FO2 is the common forward primer for R3 and R4, FO3 is the common forward primer for R5 and R6, FH1 is the common forward primer for R14–R33, FH2 is the common forward primer for R34–R37, and FH3 is the common forward primer for R38–R41. During synthesis (Sangon Biotech, China), the 5′ end of each forward primer was labeled with biotin, and the 5′ end of each reverse primer was modified with its specific TAG sequence. Between the specific reverse primer and its TAG sequence, a 12 carbon atom spacer was inserted. The primers and MagPlex-TAG™ microspheres used in the study are listed in Table 1.Those for H antigens are from Zheng et al. (2016).

2.4. Multiplex PCR and labeling of PCR amplicons

Three multiplex PCRs were performed to amplify the target sequences, one for O serogroups, Vi antigen as well as a *Salmonella*-specific control (*invA*), and two for H antigens. Multiplex PCR amplification was performed using a QIAGEN® Multiplex PCR Plus Kit (QIAGEN, Germany) according to the manufacturer's instructions. Each 50 μL reaction mixture contained 25 μL 2 × Multiplex PCR Master Mix, 2 μL primer mixture (5 μM each except primer FH1, which was 7.5 μM), and 2 μL template DNA. The remainder of the reaction mixture was complemented by RNase-Free Water. PCR was performed on a S1000 PCR Thermal Cycler (Bio-Rad, USA) with the following parameters: initial activation at 95 °C for 5 min; then 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 90 s, and extension at 72 °C for 30 s; final extension at 68 °C for 10 min.

Because of the modified primers, each amplicon was ligated with biotin at one end and a 24-base TAG sequence at the other after PCR amplification. The PCR products were used directly to hybridize with the MagPlex-TAG™ microspheres (Luminex, USA) as described below.

2.5. Hybridization and detection of signals

A total of 3MagPlex-TAG™ microsphere mixtures were prepared, in which the appropriate MagPlex-TAG™ microsphere sets listed in Table 1 were contained. The microsphere mixtures were diluted to 125 microspheres of each set per microliter in 1 × TM hybridization buffer (0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100, pH 8.0). Eight microliters of each microspheres mixture were mixed with 5 μL of PCR products in a single well of a 96-well microtiter plate, and then 75 μL of 5 μg/mL SAPE (Streptavidin-R-phycoerythrin) were added. The reaction mixture was mixed and incubated at 42 °C for 25 min to allow the hybridization of the PCR amplicons to MagPlex-TAG™ microspheres.

The hybridization results were analyzed by a MAGPIX® (Luminex, USA). Mean fluorescence intensity (MFI) and data analysis were performed with xPonent®4.2 software (Luminex, USA). The ratio of sample MFI to background MFI (MFIs/b ratio) was considered as the criterion for detection. The threshold for detection was determined to be a MFIs/b ratio of 5 (Janse et al., 2012).

3. Results

3.1. Serotyping results of Salmonella isolates

A total of 228 *Salmonella* strains isolated from clinical samples were serotype determined by the xTAG assay, and the results were compared with those of the traditional serum agglutination test (gold standard). The sensitivity and specificity of this xTAG assay were 95.1% and 100%, respectively (Table 2).

Among 228 *Salmonella* strains, a total of 38 serovars were detected (Table 3). Of these, 2 *Salmonella* Stanley and 1 *Salmonella* Gallinarum, which were determined by serum agglutination test, were detected as *Salmonella* Saintpaul, *Salmonella* Typhimurium and *Salmonella* Enteritidis by xTAG assay, respectively. All were further confirmed by serum agglutination test after phase inversion. Among 6 *Salmonella* isolates which failed to be serotyped by serum agglutination, 3 could be assigned to serovars by xTAG assay, and the results were also confirmed

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