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A liquid bead array for the identification and characterization of *fljB*-positive and *fljB*-negative monophasic variants of *Salmonella* Typhimurium

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ABSTRACT

Salmonella 1,4,[5],12:i:- accounts currently for one of the most common serotypes observed worldwide. These isolates do not express the FljB flagellin and mostly derive from *Salmonella* Typhimurium. They are therefore termed *Salmonella* Typhimurium monophasic variants (STMV) and are considered of comparable public health risk. Since serological identification of the somatic and flagellar antigens of STMV is not sufficient to demonstrate relatedness with *Salmonella* Typhimurium, additional assays detecting genetic markers unique to *Salmonella* Typhimurium are required. In addition, identification of the mutations affecting expression of the flagellar gene *fljB* can be useful to support the monophasic character observed phenotypically. Finally, genetic subtyping of the various mono- and biphasic *Salmonella* Typhimurium clonal groups can facilitate their epidemiological follow-up. Here, we present a home-made liquid bead array able to fulfill these requirements. This array confirmed the monophasic character of 240 STMV isolates collected in Belgium during 2014–2015 and identified 10 genetic subtypes. Microevolution in and around the *fljB* locus linked to IS*26* insertions is probably one of the driven force accounting for STMV population diversity. Thanks to its open design, other genetic signatures could later be merged to the assay to subtype additional STMV clonal groups and to detect rare mutations.

1. Introduction

Salmonella Typhimurium Monophasic Variants (STMV) strains serotyped as 1,4,[5],12:i:- account currently for one of the most common serotypes observed in many countries worldwide (Switt et al., 2009). Such strains caused several outbreaks during the recent years, including three foodborne outbreaks in France in 2010 and 2011 (Anonymous, 2010; Bone et al., 2010; Gossner et al., 2012; Raguenaud et al., 2012; Switt et al., 2009), an epidemic in the United Kingdom from 2005 to 2010 (Petrovska et al., 2016), an outbreak associated with the consumption of dried pork sausage in Spain in 2011 (Arnedo-Pena et al., 2016) and an outbreak in Central Italy in 2013–2014 (Cito et al., 2016). Isolates serotyped as *Salmonella* 1,4,[5],12:i:- can be monophasic variants of several serovars: Typhimurium, Lagos, Agama, Farsta, Tsevie, Gloucester and Tumodi. The relatedness of *Salmonella* 1,4,[5],12:i:- strains to the Typhimurium serovar is important to determine due to the high risk for public health associated with *Salmonella* Typhimurium strains (Anonymous, 2011) and their monophasic variants (Anonymous, 2010). Since serological identification of the somatic and flagellar antigens is not sufficient to determine this relatedness, a duplex PCR assay is recommended by the European Food Safety Authority (EFSA) to discriminate between biphasic *Salmonella* Typhimurium strains, the monophasic variants of *Salmonella* Typhimurium and the strains belonging to other serovars expressing the O:4 and H:i antigens (Anonymous, 2010; Tennant et al., 2010). This PCR assay targets (i) the *fliB-fliA* intergenic region hosting an IS*200* element

Abbreviations: STMV, *Salmonella* Typhimurium Monophasic Variants; STM, *Salmonella* Typhimurium.

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found only in the Typhimurium or Farsta serovars or their variants (among the serovars expressing the O:4 and H:i antigens) and (ii) *fljB*, the gene coding for the phase 2 flagellin. This PCR identifies STMV isolates lacking the *fljB* coding sequence but cannot differentiate *fljB*-positive STMV strains from phenotypically biphasic *Salmonella* Typhimurium strains. STMV isolates harboring an *fljB* coding sequence were reported in several countries: the United States (Zamperini et al., 2007), Germany (Hauser et al., 2010b), Italy (Barco et al., 2011, 2014), France (Bugarel et al., 2012), Poland (Wasyl and Hoszowski, 2012), England and Wales (Hopkins et al., 2012), Ireland (Prendergast et al., 2013), Greece (Mandilara et al., 2013), Denmark (Arguello et al., 2014) and Japan (Ido et al., 2014). To our knowledge, the highest prevalence of *fljB*-positive STMV isolates was reported in Belgian farmed animals during the period 2008 to 2011 with 43.8% of all *Salmonella* 4,[5]:i:- isolates being *fljB*-positive (Boland et al., 2014). Next to *fljB*-negative isolates, detection and characterization of *fljB*-positive STMV isolates is thus critical for the global monitoring of the various monophasic *Salmonella* Typhimurium lineages circulating worldwide.

Here, we describe a home-made liquid bead array able to (i) identify monophasic isolates as Typhimurium variants, (ii) detect the absence of an *fljB* coding sequence in *fljB*-negative isolates, (iii) identify the genetic mutations causing the monophasic character in *fljB*-positive isolates, and (iv) further subtype the clonal groups of mono- and biphasic *Salmonella* Typhimurium isolates to help their molecular epidemiological follow-up. A collection of 241 isolates serotyped as 4,[5]:i:- and collected in Belgium during 2014 and 2015 was used to assess the ability of the bead array to characterize these isolates at genetic level.

2. Materials and methods

2.1. Bacterial isolates

Two hundred forty-one *Salmonella* isolates collected in Belgium during the years 2014 and 2015 at the National Reference Laboratory for Animal Health and serotyped as 4,[5]:i:- after at least one phase-inversion assay were analyzed with the home-made liquid bead array described in point 2.3. Among these, 154 were isolated from pig, 31 from poultry, 10 from cattle, 7 from food, 6 from feed and 1 from horse. The remaining 32 isolates were from unreported origin.

2.2. DNA preparation

DNA was prepared from bacteria grown on Brilliant Green Agar plates (Oxoid, Aalst, Belgium). A few colonies were suspended in 400 μl of sterile Milli-Q water and vortexed. Bacterial concentration was adjusted to reach an absorbance at 600 nm between 1 and 2 and a final volume of 400 μl was kept. Samples were incubated at 100 °C for 15min and centrifuged at room temperature for 5 min at 10,000 g. The supernatant was collected and stored at −20 °C (Anonymous, 2010).

2.3. Liquid bead array

2.3.1. Genetic markers targeted by the 15-plex liquid bead array

Fifteen genetic markers were selected from previous works or from the comparison of published genomic sequences (Table 1). This selection was confirmed with a set of 20 *Salmonella* 4,[5]:i:- and 11 *Salmonella* Typhimurium Belgian isolates (Fig. S1). Among the 15 probes of the array, 2 probes target key genetic elements required for phase 2 flagellin expression: the *fljB* gene and its promoter. The lack of detection of one of these 2 markers is sufficient to genetically confirm the monophasic character of an isolate. Two other probes, "LT2_2915584&IS*26*" and "IS*26*&LT2_2916036", are of particular interest to follow the presence of an IS*26* composite transposon inserted between *hin* and *iroB* which was the hallmark of most *fljB*-positive isolates observed in a previous study in Belgium from 2008 to 2011 (Boland et al., 2015). These probes target the IS*26* elements found at the left and right junctions of this transposon, respectively (Boland et al., 2015).

2.3.2. Ligase Chain Reaction (LCR) assay

The molecular method developed for the identification and characterization of STMV isolates is a multiplex assay based on a Ligase Chain Reaction (LCR) of Padlock-shaped Probes (PLPs) hybridized to MagPlex-TAG™ microspheres coated with unique 24-nt long capture tags and analyzed on a Luminex® 200™ bead-array hybridization platform (Luminex, Austin, Texas). Sequences of primers and probes used in the LCR assay are listed in Table 1. The 15 PLPs were designed as described by Wattiau et al. (2011). Only probes displaying no significant similarity between the target sequence and the rest of the genome were kept. The LCR assay was conducted in three successive steps according to the procedure of Wattiau et al. (2011) with minor modifications. The first step (ligation) was conducted in a 10 μl mixture containing 1 μl of the DNA extracted as described above, 2 U of *Pfu* DNA ligase (Agilent, Santa Clara, CA), 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% Igepal, 0.01 mM rATP, 1 mM dithiothreitol (DTT), and 200 pM of each PLP (or 800 pM for the PLP "LT2_1659055C"). Ligation was conducted with a thermal cycler. After 3 min at 95 °C, 25 cycles of 30 s at 95 °C and 5 min at 65 °C were performed, followed by a 2-min final denaturation at 98 °C. The second step (exonuclease treatment) started with the addition of 15 μl of exonuclease mixture consisting of 67 mM glycine-KOH, pH 9.4, 2.5 mM $MgCl_2$, 50 μg/ml bovine serum albumin (BSA), and 0.0015 U exonuclease λ (New England BioLabs, Ipswich, MA). The resulting samples (25 μl) were incubated at 37 °C for 45 min, followed by inactivation at 95 °C for 10 min. Upon completion of the second step, PCR amplification was conducted by adding 25 μl of 2-fold real-time PCR mix (ABGene, Epsom, United Kingdom) supplemented with UR (0.05 μM) and 5′Cy3-labeled UF (0.4 μM) primers. After 10 min at 95 °C, 30 cycles of 45 s at 95 °C, 45 s at 55 °C, and 1 min at 72 °C were performed, followed by a 15-min final elongation at 72 °C and denaturation at 98 °C for 2 min. The samples were then stored at −20 °C until hybridization and reading on a bead-array platform.

2.3.3. Detection of the LCR products on a bead-array platform

LCR products were hybridized with a mix of Luminex® MagPlex-TAG™ microspheres including the 15 different beads presented in Table 1. Prior to hybridization, the bead mix was pelleted on a magnet and homogenized in 2X Hybridization Buffer (0.4 M NaCl, 0.2 M Tris, 0.16% Triton X-100, pH 8.0) at a concentration of 100 beads of each type per μl. The hybridization mixture consisted of 25 μl of this bead mix and 25 μl of the final LCR product. After denaturation at 96 °C for 90 s, hybridization was conducted at 37 °C for 30 min, immediately followed by three washes performed by pelleting the beads on a magnetic bead separation system (V&P Scientific, San Diego, CA) for 1 min, removing the supernatant by forceful inversion, suspending the beads in 75 μl hybridization buffer (0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100, pH 8.0) and pipetting up and down. The plate was then incubated at 37 °C for 15 min in the Luminex® 200™ instrument and 50 μl of the final solution were analyzed at this temperature. Fluorescence signals were measured on at least 100 beads of each bead type and Median Fluorescence Intensities (MFI) were automatically generated in CSV data.

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