New-Data Letter: **Bacteriology**

Rapid genotyping of *Shigella sonnei* using multiplex high resolution melting

Running title: Multiplex-HRM typing of *Shigella sonnei*

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Shigella sonnei, an emerging global cause of shigellosis, consists of four distinct lineages and the current pandemic involves several geographically associated, multidrug- resistant clones that belong to lineage III (1-3). A typing scheme based on high resolution melting (HRM) of six chromosomal single nucleotide polymorphisms (SNPs) has been described to identify all lineages/sub-lineages (4). HRM detects SNPs in small DNA fragments based on the melting patterns (5, 6). HRM involves minimal labor with rapid turnaround time, and a decreased risk of PCR carryover contamination (7). A sensitive intercalating fluorescent dye, EvaGreen, may yield robust fluorescence intensities and strong sharp melting peaks on melting of the PCR amplicons (8-10). Based on the SNPs summarized in Table 1, we have developed a multiplex-HRM, for identification of lineages I, II, and III using the EvaGreen and a Rotor-Gene 6000 instrument (Qiagen).

Ten strains (2 for each lineage/sublineage) were from a collection held at Institut Pasteur. Only one lineage IV strain is reported so far (1), which is not included in the study. Primers were designed to amplify fragments that contain three SNPs within *kduD*, *deoA* and *emrA* for typing 3 main lineages in a first set run, and to amplify fragments that contain SNPs within *fdX* and *menF* for typing sub-lineages IIIa, IIIb, and IIIc in a second run. Primer sequences are summarized in Table 1.

In the first set run, a 20 µl multiplex real-time PCR reaction was set up, containing 10 µl SensiMix™ with EvaGreen Dye (Bioline), 20 ng of template DNA, and 2.5 µl of each primer of *kduD* (100µM), 1 µl of each primer of *deoA* (100µM) and 1 µl of each primer of *emrA* (50µM). In the second set run, the 20 µl reaction mix was identical, except that the primers used were *fdX* (10 µM) and *menF* (10µM), with a quantity of 2 µl and 1µl, respectively. The PCR reaction was programmed as following: hold at 95°C for 10 min followed by 35 cycles of 95°C for 20s, 60°C for 20s and 72°C for 22s. HRM was performed between 80 and 90°C with a stepwise increase of 0.05°C. The melting temperatures (*Tm*) were determined by the negative derivative of decrease

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fluorescence over increased temperature (*df/dt*) for each SNP using the proprietary software (version 1.7.34).

The first set run clearly separated lineages I, II, and III with distinctive melting curves, and the *Tm* of each allele was at least half degree apart from that of the other allele (Fig. 1A). The second set run distinguished the sub-lineages IIIa, IIIb, and IIIc with distinctive melting curves (Fig. 1B). Table 2 summarizes *Tm* for all alleles. To assess the reproducibility, we blindly run all 10 samples of various lineages randomly in same condition and revealed same results with +/- 0.05 different in *Tm*. Sequencing of five SNPs independently verified reliability of *Tm* data.

A simple and robust genotyping report is important for rapid identification of the emerging multidrug-resistant clones of lineage III (1). The multiplex-HRM approach we present here can be very useful in this regard.

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Figure legend

Fig.1. Multiplex-HRM curve of two set runs. A: multiplex-HRM curve of three SNPs in *kduD*, *deoA* and *emrA* respectively (from left to right), which separates lineages I, II and III (strain ID 1263, 55623 and 54184 respectively). B: multiplex-HRM curve of two SNPs in *fdX* (left) and *menF* (right), respectively, which separates sub-lineages IIIa, IIIb, and IIIc (strain ID 54184, 2073, and 6224 respectively).

Table 1: SNPs and primers used for multiplex-MRM-typing *S. sonnei* in this study:

Gene	SNP position	Ancestor	Derived	Lineage I	Lineage II	Sublineage IIIa	Sublineage IIIb	Sublineage IIIc
kduD	3155111	T	C	C	T	T	T	T
deoA	4803842	G	A	G	A	G	G	G
emrA	2973337	T	C	C	C	T	T	T
fdx	2760031	G	A	G	G	G	A	A
menF	2455693	A	G	A	A	A	A	G
Primers for *kduD*	forward 5'-CGACGGCGAAACACTTTATC-3' reverse 5'-CGCGTATAAGAAGGCACACG-3'							
Primers for *deoAD*	forward 5'-GGAGATGCTTATCTCCGGCAAA-3' reverse 5'-AGTCGGTTGGGCCTTTT-3'							
Primers for kduD	forward 5'-TGCCACCGAAGTACGTAACG-3' reverse 5'-CATCCACCCACATATTGGTG-3'							
Primers for kduD	forward 5'-CAAAGCCTGGGACTGGA-3' reverse 5'-CATGGTTGATAGTGTAACGC-3'							
Primers for kduD	forward 5'-TATTCTCGCGCTGGTTTTTA-3' reverse 5'-GCTTTTCTTGGCTCTTCACC-3'							

Note: These SNPs were extracted using *S. sonnei* strain Ss046 as the reference as described by Holt et al., 2012 (1) and Sangal et al., 2013 (4).

Table 2: Summary of *Tm* for each of the five SNPs used for multiplex-HMR

Main lineages	Tm (+/- 0.05)		
	kduD	*deoA*	*emrA*
I	83.0	85.1	87.7
II	82.5	84.8	87.7
III	82.5	85.2	87.3
Sub-lineages	**Tm (+/- 0.05)**		
	fdX	*menF*	
IIIa	82.5	85.7	
IIIb	82.5	86.0	
IIIc	82.9	85.7