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Note

A strip array for spoligotyping of *Mycobacterium tuberculosis* complex isolates

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ABSTRACT

A novel strip array was developed for a nine-spacer spoligotyping scheme of *Mycobacterium tuberculosis* complex (MTBC). The new method was evaluated using 211 MTBC isolates and the results were fully concordant with the traditional spoligotyping approach. The strip array proved to be rapid and convenient for spoligotyping of MTBC.

Tuberculosis (TB) remains one of the world's deadliest communicable diseases. In 2014, an estimated 9.6 million people developed TB and 1.5 million died of it (World Health Organization, 2015). In the TB endemic regions, recognizing outbreaks, identifying infection sources and tracing the transmission of particular strains of *Mycobacterium tuberculosis* complex (MTBC) are vital steps to organize an appropriate public health response.

Spoligotyping is the first widely adopted genotyping method for tracing transmission of MTBC (Driscoll, 2009). It is based on the highly polymorphic direct repeat (DR) loci, which are separated by variable 31- to 41-bp long spacer sequences (Hermans et al., 1991). Currently, MTBC spoligotyping is performed by reverse line blot hybridization (Kamerbeek et al., 1997; van Soolingen et al., 1995) through detection of a 43-spacer set (Groenen et al., 1993). However, this scheme suffers from technical complexity and lengthy operation, and its wide use has been hindered in those high burden and resource-limited countries. Several alternative approaches were reported, including Luminex system (Cowan et al., 2004), DNA chip (Song et al., 2007), mass spectrometry (Honisch et al., 2010; Shitikov et al., 2012), and microarray (Ruettger et al., 2012; Bespyatykh et al., 2014).

We previously reported a universal strip array approach, which combines PCR with a lateral flow detection using multiple oligonucleotide probes immobilized on the strip, and is able to detect multiple targets in a single step within half an hour after PCR (Xu et al., 2014). In the present study, we aimed to develop a rapid spoligotyping scheme based on the strip array approach that could be easily used in clinical settings. Meanwhile, to simplify the assay and reduce the cost, the fluorophore was labeled on one primer instead of a separate detection probe. In this way, detectable product could be generated directly after PCR amplification. Sebban et al. developed a simplified set of classification rules by testing nine spacers (spacer 8, 9, 18, 19, 22, 23, 31, 36 and 43), which were found sufficient to discriminate 4014 MTBC strains in the database (Sebban et al., 2002). We thus chose this nine-spacer detection system for our study.

Fig. 1 displays a schematic representation of the principle of the strip array-based spoligotyping. The entire assay procedure includes three steps: Step 1, an asymmetric PCR to generate single-stranded fluorescent amplicons; Step 2, a lateral flow hybridization between the nine spacer probes and the amplicons; and Step 3, an array scanning step to indicate the presence or absence of the nine spacers. In step 1, the fluorophore-labeled primer (DRa) was in excess relative to the unlabeled primer (DRb). PCR amplification would thus generate a large amount of single-stranded amplicons carrying the fluorophores. To indicate false negative results, *gyrA*, a conserved gene of MTBC, was co-

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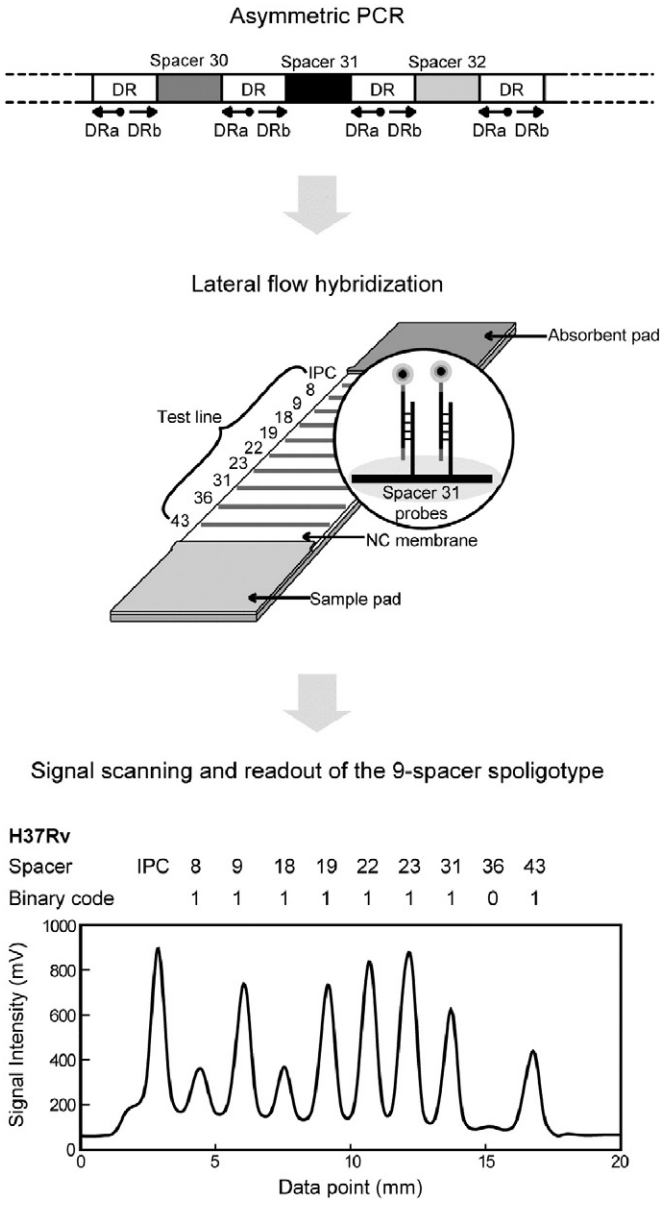

Fig. 1. Schematic illustration of strip array for simplified spoligotyping of MTBC. Asymmetric PCR with primers DRa and DRb produces single-stranded fluorophore-labeled amplicons. During the lateral flow hybridization, the single-stranded fluorophore-labeled amplicons specifically hybridize with the spacer probes immobilized in order on the nitrocellulose (NC) membrane. By scanning the strip with a portable fluorescence reader, the nine-spacer spoligotype of a certain sample is generated.

amplified as an internal positive control (IPC) in the reaction. In step 2, after the PCR product solution was added to the strip, capillary force would drive the movement of the PCR products from the sample pad to the nitrocellulose (NC) membrane, where the fluorescent single-stranded amplicons would hybridize to the immobilized probes and the probe region would become fluorescent. The lateral flow hybridization procedure lasted for 20 min at 52 °C. In step 3, the strip was inserted into a portable strip scanner, which detected fluorescence intensity on the probe region. For one strip, the scanning procedure could be finished within 15 s. Therefore, the entire assay took less than 0.5 h for one strip after PCR.

The establishment of the strip assay started with evaluation of the primers and probes used in the traditional spoligotyping protocol (Kamerbeek et al., 1997). The two spacer primers, DRa and DRb, were identical to those previously used except that two mismatch bases were introduced in DRa to avoid primer dimer formation. In addition, four spacer probes were redesigned to enhance the specificity of probe binding. The sequences of oligonucleotides used in this assay are provided in Table S1. All probes were synthesized with a 5′ terminal poly(dT) linker and had a biotin group for efficient binding to NC membrane through NeutrAvidin. The strip was fabricated as previously described (Xu et al., 2014).

The array strip-based MTBC spoligotyping was carried out as follows: Briefly, 5 μL of extracted DNA was added to 20-μL PCR reaction (detailed PCR conditions are given in the supplemental material). After amplification, the PCR product was first mixed with 30 μL of flow buffer A, applied to the sample pad of the strip, and followed by adding 30 μL of flow buffer B. The strip was placed in an incubator for 20 min at 52 °C, and then was scanned by a portable strip reader.

The cut-off value of each probe-binding line was determined in order to know whether a spacer or IPC was positive or negative. For this purpose, 33 MTBC strains with different spoligotypes as defined in SITVITWEB were chosen as positive controls, and three nontuberculous mycobacteria (NTM) strains, i.e., *Mycobacterium avium*, *Mycobacterium abscessus* and *Mycobacterium fortuitum*, were chosen as negative controls. The spacer signals of the 33 MTBC strains are given in Table S2. By detecting these strains, each probe site would have a number of positive signal values and negative signal values. These values were normalized by subtracting the background signal values. The cut-off value for each probe site was defined as normalized negative signal plus three times standard deviation (SD). As shown in Fig. 2, such a definition could distinctively differentiate the positive signals from the negative signals on all the probe sites. We also defined that an assay was valid only when the IPC was positive. Following the above definitions, the readout was interpreted directly into a binary code for one strain (where "1" represents positive and "0" represents negative).

We then tested the specificity of the strip array by using 43 plasmids each containing a different spacer (from spacer 1 to spacer 43) flanked by DR and another plasmid containing *gyrA* sequence. The concentration of each plasmid was prepared to be 10^5 copies/μL. The results showed that only those plasmids harboring the corresponding spacers gave the positive signals on their probe sites (Fig. 3A). To confirm if the array might have cross-reactivity with NTM, 40 different NTM isolates (listed in Supplemental material) strains were each tested. The results showed no signals on any probe sites (data not shown). We thus concluded that the strip array was specific for MTBC.

We further studied the analytical sensitivity of the strip array. Two standard strains of MTBC, i.e., H37Rv and BCG, were used for this study. By detection of a serial dilution of their genomic DNA, the limit

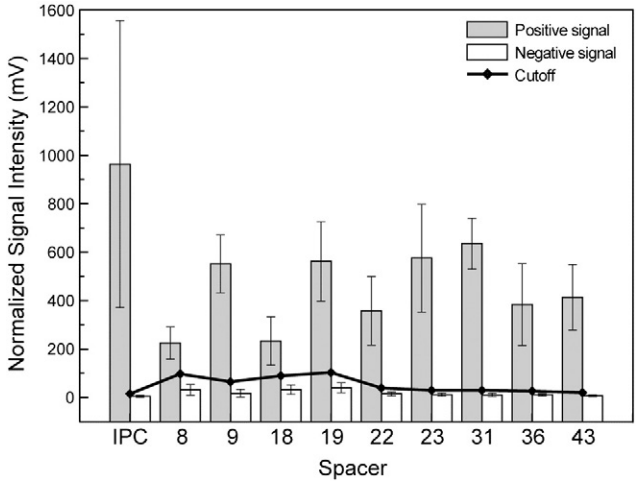

Fig. 2. Normalized signal intensity and resulting cut-off values for each spacer probe by the detection of 33 MTBC and 3 NTM isolates (i.e., *M. avium*, *M. abscessus* and *M. fortuitum*).