Infection, Genetics and Evolution xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Spoligotyping of *Mycobacterium tuberculosis* complex isolates using hydrogel oligonucleotide microarrays

Julia A. Bespyatykh [a,*], Danila V. Zimenkov [b], Egor A. Shitikov [a], Elena V. Kulagina [b], Sergey A. Lapa [b], Dmitry A. Gryadunov [b], Elena N. Ilina [a], Vadim M. Govorun [a]

[a] Research Institute of Physical – Chemical Medicine, Moscow, Russian Federation
[b] Engelhardt Institute of Molecular Biology, Moscow, Russian Federation

ARTICLE INFO

Article history:
Received 20 January 2014
Received in revised form 26 March 2014
Accepted 30 April 2014
Available online xxxx

Keywords:
Tuberculosis
Genotyping
Biochips
Hybridization
Spoligotyping

ABSTRACT

Mycobacterium tuberculosis remains a leading cause of morbidity and mortality worldwide. This circumstance underscores the relevance of population studies of tuberculosis for transmission dynamics control. In this study, we describe a conversion of the spoligotyping of *M. tuberculosis* complex isolates on a platform of custom designed hydrogel microarrays (biochips). An algorithm of automated data processing and interpretation of hybridization results using online database was proposed. In total, the 445 samples were tested. Initially, 97 samples representing multiple species of *M. tuberculosis* complex and nontuberculous mycobacteria were used for protocol optimization and cut-off settings. The developed assay was further evaluated on the out-group of the 348 mycobacterial samples. Results showed high concordance with the conventional membrane-based spoligotyping method. Diagnostic sensitivity and diagnostic specificity of the spoligo–biochip assay were 99.1% and 100%, respectively. The analytical sensitivity was determined to be 500 genomic equivalents of mycobacterial DNA. The high sensitivity and specificity, ease of operation procedures, and the automatic processing of measured data make the developed assay a useful tool for the rapid and accurate genotyping of *M. tuberculosis*.

1. Introduction

Mycobacterium tuberculosis (MTB), the bacterial agent responsible for the disease tuberculosis (TB), is one of the main infectious causes of death worldwide, with more than eight million new cases of active disease and nearly two million deaths every year (WHO, 2012). Population-based epidemiological studies of TB have aimed to trace the putative chains of TB transmission by identifying patients with identical isolates and linking these molecular typing results to individual contact data. Currently, the primary TB genotyping methods are restriction fragment length polymorphism typing based on the IS*6110* insertion sequence, spacer oligonucleotide typing (spoligotyping), and the 24-locus mycobacterial interspersed repetitive unit-variable number tandem repeats typing (MIRU-VNTR) (Barnes and Cave, 2003; Supply et al., 2006).

Spoligotyping is one of a commonly used amplification-based genotyping method that assesses the genetic diversity of the direct repeat (DR) locus (Kamerbeek et al., 1997). The DR locus contains multiple 36-bp DRs that are separated by 30- to 40-bp unique spacer sequences. DR locus is a member of the clustered regularly interspaced palindromic repeats genetic family (Jansen et al., 2002) and spans up to 5 kb, representing 0.1% of the MTB genome.

Traditionally, spoligotyping is performed by the reverse line blot hybridization of biotinylated PCR products to spacers that are applied to a nitrocellulose membrane. The presence of the spacers is detected by the chemiluminescent staining of the membrane. This method was described by Kamerbeek et al., 1997 and served as the basis for the development of publicly available strain databases, such as SITVIT_WEB (Demay et al., 2012) and SpolDB4 (Brudey et al., 2006). Over the last several years, a variety of surface microarrays have been proposed for spoligotyping assays, including PixSysn QUAD 4500 Microarrayer (Song et al., 2007), DNA microarray (Ruettger et al., 2012) and "Spoligoriftyping" (Gomgnimbou et al., 2012). Additionally, the alternative spoligotyping method based on a matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF MS) platform was suggested by two scientific grousing dependently (Honisch et al., 2010; Shitikov et al., 2012).

In the present study, we describe a new approach for MTB spoligotyping. The suggested scheme includes the amplification of the MTB DR locus and the subsequent hybridization of the PCR products to a custom-designed three-dimensional hydrogel microarray

* Corresponding author. Tel.: +7 9099611846.
E-mail address: JuliaBespyatykh@gmail.com (J.A. Bespyatykh).

http://dx.doi.org/10.1016/j.meegid.2014.04.024
1567-1348/

(biochip). The dedicated chip-reader supplies the automatic processing of measured signals. The developed assay displays sensitivity and specificity relative to a conventional membrane-based testing procedure. The three-dimensional hydrogel microarrays were initially worked out at the Engelhardt Institute of Molecular Biology (EIMB) (Yershov et al., 1996), and now are widely used in the molecular diagnostics of TB, viruses, the human genome, and more (Gryadunov et al., 2011). Technically, the presented spoligotyping scheme could be integrated with TB drug resistance analysis within the single chip. This can help to develop a bedside laboratory device suitable for the analysis of individual samples (one chip = one patient) supporting the principals of personal medicine.

2. Materials and methods

2.1. Strains

Two sets of DNA samples were used in the present study. A test set consisted of 67 *M. tuberculosis* complex (MTBC) and 30 nontuberculous mycobacteria (NTM) (*M. abscessus* (1), *M. asiaticum* (1), *M. avium* (5), *M. chelonae* (1), *M. duvalii* (1), *M. flavescens* (1), *M. fortuitum* (1), *M. gastri* (1), *M. gordonae* (1), *M. interjectum* (1), *M. intermedium* (1), *M. intracellulare* (1), *M. kansasii* (1), *M. kumamotonense* (1), *M. malmoense* (1), *M. mantenii* (1), *M. marinum* (1), *M. paratuberculosis* (1), *M. phlei* (1), *M. scrofulaceum* (1), *M. septicum* (1), *M. simiae* (1), *M. smegmatis* (1), *M. szulgae* (1), *M. terrae* (1), *M. xenopi* (1)) DNA samples. These samples were provided by the EIMB and utilized for assay optimization, setting of cut-off values, and reproducibility studies. 65 MTB strains were represented by 20 different spoligotype international types (SITs). For more information see Supplementary Table S1 in Zimenkov et al. (2013). *M. tuberculosis* strain H37Rv was used for estimation of analytical sensitivity.

An additional set of 348 DNA samples was used for the evaluation of the method. These samples were available in prior collection from the Research Institute of Physical – Chemical Medicine and included two species of MTBC (342 of MTB and six of *Mycobacterium bovis* samples) (Shitikov et al., 2012). For all these samples, the spoligotypes obtained by the reverse line blot hybridization method were available.

2.2. Oligonucleotides and biochip design

For spoligotyping, new 43 oligonucleotide probes were constructed based on the 43 spacer sequences in the informative DR locus of MTBC (Table 1). The OligoAnalyzer program (Integrated DNA Technologies, http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) was utilized to design these probes. The lengths of the oligonucleotides were adjusted to keep the melting temperatures within 2–3 °C. The oligonucleotides used for immobilization on the biochip and the primers for amplification were synthesized and purified as described (Rubina et al., 2004). The biochips were manufactured as described previously (Rubina et al., 2004) and consisted of 43 elements containing immobilized oligonucleotides, three marker elements (M), and three reference gel pads without probes as negative controls for processing the hybridization image (Fig. 1B).

2.3. Amplification of the DR region, hybridization to the biochip and registration of the results

Single stranded fragments containing spacers flanked by direct repeats were amplified by asymmetric PCR using the following primers: DR-F: 5′-CCCCGAGAGGGGACGGAAAC-3′ and DR-R: 5′-CGGGGTTTTGGGTCTGACGAC-3′. The nucleotide sequences of DR-F and DR-R are based on the sequence of the DR locus; they are outward-directed primers with a head-to-head orientation.

Table 1
Oligonucleotides utilized for the biochip.

Spacer	Sequence 5′ to 3′	Length (bp)
1	agggtcgccggttctggatc	20
2	ctcatgcttgggcgacag	18
3	ccgtgcttccagtgatcgc	19
4	catacgccgaccaatcatca	20
5	ccacttgtgcgggattagc	19
6	tttccggcttcaatttcagc	23
7	tactcggggctgccgtctg	22
8	cagcctcgccggggcc	16
9	catgtgctgacagcggattc	20
10	ccgggcagcgttcgaca	17
11	caacggcggcggcaac	16
12	gggagatgctgtccgagg	18
13	tcgaccatcattgccattccc	21
14	cctttcggtgtgatgcggatg	21
15	cttgaataacgcgcagtgaatt	22
16	cgagttcccgtcagcgt	17
17	gcccgcgcggatgact	16
18	cgggcgagctgcagatg	17
19	actggcttggcgctgatcct	20
20	gacctcgccaggagagaaga	21
21	cgcgtcgatgtcgatgtccc	20
22	tgtcaccgcagacggcacgattg	23
23	cagcatcgctgatgcggtccag	22
24	ctgggtgagacgtgctcg	18
25	cagcgaccaccgcaccct	18
26	ccaccatcatccggcgc	17
27	ggattcgtgatctcttcccgc	21
28	cggcgtttagcgatcacaaca	21
29	tacaggctccacgacacgacc	21
30	cgcgccctttccagccg	18
31	cagacaggttcgcgtcgatca	21
32	gaccaaataggtatcggcgtg	21
33	catgacggcggtgtcgcac	19
34	cacctcgcccacaccgtc	18
35	acgctcgaaacgcttccaacg	21
36	cgaaatccagcaccacatcc	20
37	cgcgaactcgtccacagtcc	20
38	cgtggatggcggatgcgttg	20
39	tcggcgtgggtaaccgatc	19
40	gtgcctcatacaggtccagtgc	22
41	ggcacggagctttccggct	19
42	gggacatggacgagcgcgac	20
43	gggtgcgggaggtgcagca	17

PCR amplification was carried out in a 30 μL reaction mixture containing a 3 μl dilution of HS Taq buffer; a 3μl dilution of HS Taq DNA polymerase (Evrogen JSC, Moscow); 0.2 μM each deoxynucleoside triphosphate (dNTP); 0.3 μM and 3 μM DR-F and DR-R, respectively; 8 μM fluorescently labeled dUTP-ImD#49 (Biochip-IMB, LLC, Moscow); and a 3 μl dilution of the DNA sample. The cycling conditions were as follows: an initial denaturation at 95 °C for 5 min; 52 cycles of denaturation at 95 °C for 40 s, annealing at 63 °C for 40 s and extension at 72 °C for 40 s; and a final extension step at 72 °C for 7 min. A negative control was included in each series of experiments to identify any possible amplicon contamination.

The hybridization mixture was prepared by adding 10 μL of the PCR mixture to 20 μL 1.5 M guanidine thiocyanate (GuSCN), 0.075 M HEPES (pH 7.5), and 7.5 mM EDTA. The biochip hybridization chamber was filled with the resultant mixture and incubated for 3–5 h at 37 °C. The chamber was then removed, and the microarray surface was washed twice (approximately 30 s each) with water at 37 °C and air-dried. The fluorescent pattern of the biochips was recorded using a fluorescence analyzer (Biochip-IMB, Russia, http://www.biochip-imb.ru/index.php/biochip-reader) and ImaGeWare software (Biochip-IMB, LLC, Russia).

2.4. Interpretation of hybridization results

The individual cut-off value was established for each element with a spacer-specific immobilized oligonucleotide. These values

Please cite this article in press as: Bespyatykh, J.A., et al. Spoligotyping of *Mycobacterium tuberculosis* complex isolates using hydrogel oligonucleotide microarrays. Infect. Genet. Evol. (2014), http://dx.doi.org/10.1016/j.meegid.2014.04.024