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Rapid Spoligotyping of *Mycobacterium tuberculosis* Complex Bacteria by Use of a Microarray System with Automatic Data Processing and Assignment

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Membrane-based spoligotyping has been converted to DNA microarray format to qualify it for high-throughput testing. We have shown the assay's validity and suitability for direct typing from tissue and detecting new spoligotypes. Advantages of the microarray methodology include rapidity, ease of operation, automatic data processing, and affordability.

Spacer oligonucleotide typing or spoligotyping was the first PCR-based genotyping method (6) for the causative agents of tuberculosis and has become widely accepted. The test detects the presence or absence of 43 specific DNA spacer sequences in the direct repeat (DR) genomic region of *Mycobacterium tuberculosis* complex (MTC) organisms, i.e., *M. tuberculosis* and other *Mycobacterium* species, such as *M. bovis*, *M. caprae*, and *M. africanum*. The spoligotyping pattern is characteristic of a particular evolutionary lineage of strains and can be used for epidemiological tracking (7, 8, 10). To digitize hybridization data, conversion of spoligotyping signals into a numerical code was introduced (3), which led to the creation of the international spoligotyping databases SpolDB4.0 (1) and Mbovis.org (9).

Several protocols have been proposed to conduct spoligotyping (4). The classical procedure, also termed reverse line blot hybridization, utilizes a nylon membrane carrying all 43 spacer-specific oligonucleotide probes (6). For higher throughput, Luminex technology (2) involving hybridization on spacer oligonucleotide-conjugated microspheres in liquid phase was used. Honisch et al. (5) suggested automated matrix-assisted laser desorption ionization–time of flight mass spectrometry as an alternative approach.

In the present study, we have converted the spoligotyping assay to the DNA microarray format of the ArrayStrip platform (Alere Technologies GmbH, Jena, Germany) to further improve its performance and make it a genuine routine diagnostic test. For probe design, the oligonucleotide sequences of the original panel of spoligotyping probes (6) were either retained ($n = 15$) or adapted to the ArrayStrip platform by adding one to four 5′- or 3′-located complementary nucleotides ($n = 26$) or removing two nucleotides ($n = 2$) in order to adjust their thermodynamic parameters. The complete list of oligonucleotide probes and parameters is given in Table S1 in the supplemental material. Each probe was spotted 4-fold. A staining control (biotinylated oligonucleotide) and negative control (spotting buffer) were also included. The experimental procedure as schematically depicted in Fig. 1A includes the following steps: (i) standard DNA extraction; (ii) amplification of the DR region using 5′-biotinylated primers DRa/DRb (6); (iii) hybridization on ArrayStrips using the hybridization kit (Alere) with hybridization at 60°C and wash steps at 55°C, otherwise following the instructions of the manufacturer; (iv) recording of stained microarrays using an ArrayMate transmission reader (Alere); and (v) automatic processing using the adapted instrument's software (Alere). The latter includes normalization to the background level, automatic spot recognition, and signal intensity output in a gray value median table. Signal intensities higher than 0.3 (on a scale from 0 to 1.0) were considered positive for the respective probe. The signals at all 43 probes were condensed into a binary code, with "1" for positive and "0" for negative. These binary code data were automatically compared with SpolDB4.0, Mbovis.org, and MIRU-VNTR*plus* (http://www.miru-vntrplus.org/MIRU/index.faces) database entries to identify concordant species and lineages or the absence of them. The final experiment report delivered by the system identifies the species and its respective lineage, providing binary, octal, and HEX codes of the strain. In the case of a new spoligotype, differing signals between sample and best match from database are highlighted.

For validation of the assay, DNA extracts from 65 field isolates submitted to the National Veterinary Reference Laboratory from 2003 to 2008 were blindly examined in parallel by reverse line blot hybridization using the spoligotyping kit (Ocimum Biosolutions, Hyderabad, India) and the present DNA microarray. The specimens originated from cattle, wildlife, and zoo animals (see Table S2 in the supplemental material). The results summarized in Table 1 show complete agreement of the spoligotyping results. As an example, test results of both methods are illustrated in Fig. 1B. Furthermore, testing of a dilution series of *M.*

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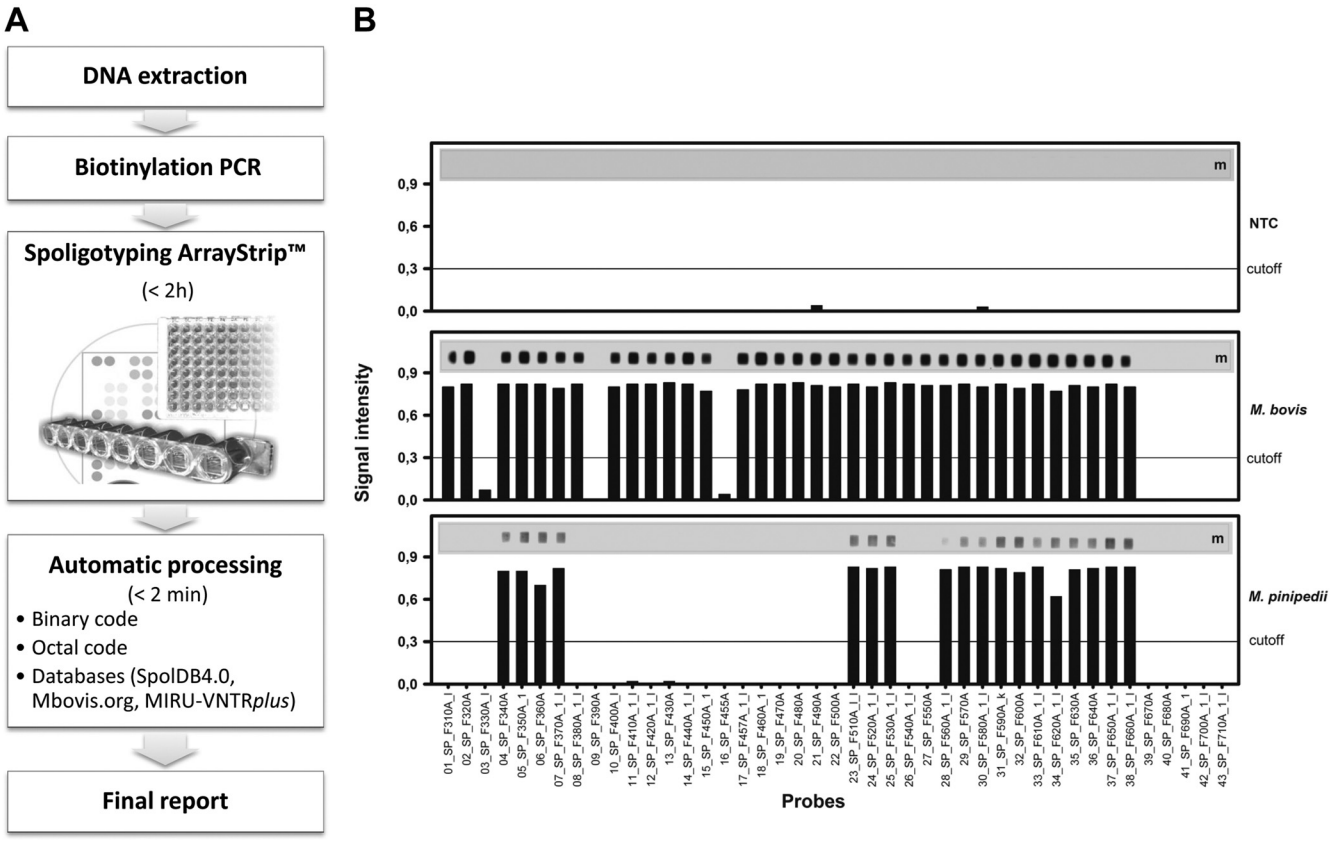

FIG 1 Illustration of ArrayStrip spoligotyping. (A) Workflow diagram. (B) Presentation of experimental output from membrane-based reverse line blot hybridization (m) and ArrayStrip spoligotyping of *Mycobacterium bovis* BCG (SB0120) and *Mycobacterium pinipedii* strains, as well as a nontemplate control (NTC). The ArrayStrip platform utilizes 4- by 4-mm microarrays mounted on the bottom of reaction vessels that are arranged in strips of 8 and fit into the 96-well microtiter plate format.

bovis BCG revealed that 30 genomic copies were sufficient to generate a correct spoligotyping pattern after amplification (data not shown).

The newly developed assay was used to examine 37 positive patient samples. The clinical isolates ($n = 30$) and tissue samples ($n = 7$) (PCR positives) represent a miscellaneous collection of cases treated at Dresden University Hospital between 2005 and 2011. Details of the samples, diagnoses, and results are given in Table 2 (see also Table S2 in the supplemental material). The observed range of *M. tuberculosis* types and lineages is reflective of the epidemiological situation in Central Europe, i.e., a low-prevalence area, where typical cases of tuberculosis are due to reactivation of past infections in elderly patients. The cases of *M. bovis* and *M. caprae* indicate a history of zoonotic transmission. Identification of two lineages from the Indian subcontinent is in line with the country of origin of those two patients.

Furthermore, we examined eight field isolates from Ukrainian cattle selected for diagnostic slaughtering following a positive reaction in mandatory tuberculinization, as well as 13 isolates from swine (details and results in Table 3). Interestingly, one of the bovine strains showed a unique spoligopattern designated SIT3423/SB2097 (lineage BOVIS1). Isolation of *M. tuberculosis* type Beijing from cattle reaffirms the anthropozoo-

TABLE 1 Comparison of test results on 65 MTC strains using ArrayStrip spoligotyping and reverse line blot hybridization

No. of samples	Membrane hybridization result (octal code)	ArrayStrip hybridization result (octal code)	SpolDB4.0 database result (shared-type no., species, lineage)	Mbovis.org database result (SB pattern)
13	676773677777600	676773677777600	481, *M. bovis*, BOVIS1	SB0121
1	676773777777600	676773777777600	482, *M. bovis*, BOVIS1_BCG	SB0120
1	000000000000600	000000000000600	539, *M. microti*, MICROTI	SB0118
5	074000037777600	074000037777600	593, *M. pinipedii*, PIN	SB0155
22	200003777377600	200003777377600	647, *M. caprae*, CAP	SB0418
12	676673757777600	676673757777600	1118, *M. bovis*, BOVIS1	SB0989
11	676673777777600	676673777777600	1601, *M. bovis*, BOV	SB1021

TABLE 2 Examination of 37 human MTC samples using the ArrayStrip spoligotyping assay

Sample	Sample material/diagnosis[a]	SpolDB4.0 database result		
		ST no.[b]	Species[c]	Lineage
Patient sample	Urine[d]	482	*M. bovis*	BOVIS1_BCG
Culture	Cervical lymph node	820	*M. bovis*	BOV
Culture	BAL/pulmonary TB	481	*M. bovis*	BOVIS1
Culture	Skin biopsy/cutaneous TB	647	*M. caprae*	CAP
Culture	Retroperitoneal lymph node	1151	*M. tuberculosis*	CAS
Culture	BAL/pulmonary TB	1264	*M. tuberculosis*	CAS
Culture	CSF/meningitis[e]	26	*M. tuberculosis*	CAS1_DELHI
Culture	Sputum/pulmonary TB[e]	11	*M. tuberculosis*	EAI3_IND
Culture	Biopsy (carina)/pulmonary TB	151	*M. tuberculosis*	H1
Patient sample	Swab of lymph node biopsy/cutaneous TB	47	*M. tuberculosis*	H1
Culture	Abscess caused by *Trochanter maior*	47	*M. tuberculosis*	H1
Culture	Tissue sample (lymph node)	47	*M. tuberculosis*	H1
Culture	Cervical lymph node	50	*M. tuberculosis*	H3
Patient sample	Aspirate (pulmonary focus)	316	*M. tuberculosis*	H3
Culture	Pleural effusion/pulmonary TB	50	*M. tuberculosis*	H3
Culture	Feces	748	*M. tuberculosis*	H3
Culture	Sputum/pulmonary TB[f]	35	*M. tuberculosis*	H4
Culture	BAL/pulmonary TB	60	*M. tuberculosis*	LAM4
Culture	Sputum/pulmonary TB	1697	*M. tuberculosis*	LAM9
Culture	Lymph node (axilla)	54	*M. tuberculosis*	MANU2
Culture	Lymph node	54	*M.tuberculosis*	MANU2
Patient sample	Sputum/pulmonary TB	53	*M. tuberculosis*	T1
Patient sample	Sputum/pulmonary TB	522	*M. tuberculosis*	T1
Culture	Tissue sample (lymph node)	53	*M. tuberculosis*	T1
Culture	Sputum/pulmonary TB	53	*M. tuberculosis*	T1
Culture	Pleural effusion/pulmonary TB	53	*M. tuberculosis*	T1
Culture	Biopsy/pulmonary TB	53	*M. tuberculosis*	T1
Culture	BAL/pulmonary TB	53	*M. tuberculosis*	T1
Patient sample	BAL/pulmonary TB	535	*M. tuberculosis*	T1
Culture	Urine	875	*M. tuberculosis*	T2
Culture	BAL/pulmonary TB	875	*M. tuberculosis*	T2
Culture	Bronchial secretion/pulmonary TB	39	*M. tuberculosis*	T4_CEU1
Culture	BAL/pulmonary TB	1756	*M. tuberculosis*	X3
Culture	BAL/pulmonary TB[g]	1279	NA	T5
Culture	BAL/pulmonary TB	1177	NA	U
Culture	BAL/pulmonary TB	1793	NA	U
Patient sample	BAL/pulmonary TB	1177	*M. tuberculosis*	U

[a] BAL, bronchoalveolar lavage; TB, tuberculosis; CSF, cerebrospinal fluid.
[b] ST no., shared-type no.
[c] NA, species identity not available from database.
[d] Intravesical BCG installation due to bladder cancer.
[e] Migrant from India.
[f] Migrant from Russia.
[g] HIV-positive patient.

notic potential of the infection. All porcine *M. caprae* strains showed the same spoligopattern, despite having been isolated from three different farms located hundreds of kilometers apart from each other, which is indicative of its wide dissemination in Ukraine.

In view of the worldwide importance of tuberculosis (11), the availability of efficient diagnostic tools and the steady improvement of these tools are crucial. Microarray-based spoligotyping represents a powerful high-throughput molecular typing method that is suitable for studying strain diversity in relevant populations and geographical areas to uncover epidemiological chains.

Summarizing the findings of this study, we have shown the validity of test results obtained by ArrayStrip spoligotyping and the assay's capability of identifying new spoligotypes and lineages. Compared to the conventional membrane-based spoligotyping, the most striking assets of the microarray methodology are (i) its quick turnaround time (results available within one working day), (ii) ease of operation and use (pipetting microliter volumes into ArrayStrip vessels in 96-well microtiter format instead of handling a membrane in a dot blot manifold and developing a chemiluminescence film in a darkroom), (iii) automatic processing of measured data using online databases (instead of visually inspecting a chemiluminescent image), (iv) relatively low cost, and (v) the possibility of performing the test on cultured material, as well as on the original tissue sample.

TABLE 3 Examination of 21 animal MTC strains from Ukraine using the ArrayStrip spoligotyping assay

No. of samples	Region	Animal	Octal code	SpolDB4 database result			Mbovis.org database result	
				ST no.	Species	Lineage	SB pattern	Species
1	Kyiv	Cattle[a]	000000000003771	1	*M. tuberculosis*	BEIJING		
1	Cherkasy	Cattle[b]	676373777776600	3423 (new)	*M. bovis*	BOVIS1	SB2097 (new)	*M. bovis*
1	Cherkasy	Cattle[b]	676773777777600	482	*M. bovis*	BOVIS1_BCG	SB0120	*M. bovis*
3	Cherkasy	Cattle[b]	200003777377600	647	*M. caprae*	CAP	SB0418	*M. caprae*
2	Kherson	Cattle[a]	200003777377600	647	*M. caprae*	CAP	SB0418	*M. caprae*
13	Lugansk	Swine[b]	200003777377600	647	*M. caprae*	CAP	SB0418	*M. caprae*

[a] Herd with history of tuberculosis.
[b] Herd without history of tuberculosis.

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