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Comparison of semi-automated commercial rep-PCR fingerprinting, spoligotyping, 12-locus MIRU-VNTR typing and single nucleotide polymorphism analysis of the *embB* gene as molecular typing tools for *Mycobacterium bovis*

Federica Armas,[1] Cristina Camperio,[1] Luana Coltella,[2] Serena Selvaggini,[3] Maria Beatrice Boniotti,[4] Maria Lodovica Pacciarini,[4] Vincenzo Di Marco Lo Presti[5] and Cinzia Marianelli[1,*]

Abstract

Purpose. Highly discriminatory genotyping strategies are essential in molecular epidemiological studies of tuberculosis. In this study we evaluated, for the first time, the efficacy of the repetitive sequence-based PCR (rep-PCR) DiversiLab *Mycobacterium* typing kit over spoligotyping, 12-locus mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing and *embB* single nucleotide polymorphism (SNP) analysis for *Mycobacterium bovis* typing.

Methodology. A total of 49 *M. bovis* animal isolates were used. DNA was extracted and genomic DNA was amplified using the DiversiLab *Mycobacterium* typing kit. The amplified fragments were separated and detected using a microfluidics chip with Agilent 2100. The resulting rep-PCR-based DNA fingerprints were uploaded to and analysed using web-based DiversiLab software through Pearson's correlation coefficient.

Results. Rep-PCR DiversiLab grouped *M. bovis* isolates into ten different clusters. Most isolates sharing identical spoligotype, MIRU-VNTR profile or *embB* gene polymorphism were grouped into different rep-PCR clusters. Rep-PCR DiversiLab displayed greater discriminatory power than spoligotyping and *embB* SNP analysis but a lower resolution power than the 12-locus MIRU-VNTR analysis. MIRU-VNTR confirmed that it is superior to the other PCR-based methods tested here.

Conclusion. In combination with spoligotyping and 12-locus MIRU-VNTR analysis, rep-PCR improved the discriminatory power for *M. bovis* typing.

INTRODUCTION

Mycobacterium bovis, the main aetiological agent of bovine tuberculosis (bTB), is the most successful pathogen among *Mycobacterium tuberculosis* complex (MTC) members because it is able to infect a wide range of mammalian hosts: cattle, goats, swine, water buffaloes, deer, bison, brush-tailed possums, badgers and humans. Wild animals may act as reservoir hosts and thus contribute to the persistence of bTB in livestock [1]. To establish effective bTB control programmes, the sources of *M. bovis* infection, as well as its routes of transmission and dissemination in the environment, are of great value. Highly discriminatory genotyping strategies are essential in molecular epidemiological studies.

Today, a wide range of molecular methods are available for the genotyping of MTC and nontuberculosis mycobacteria (NTM) species [2–4]. Insertion sequence IS*6110*-based restriction fragment length polymorphism (RFLP) analysis is considered the gold standard for the genetic fingerprinting of *M. tuberculosis* [5, 6]. However, other molecular methods have been demonstrated to possess high-resolution power; these include spoligotyping [7], mycobacterial interspersed repetitive unit-variable number tandem repeats (MIRU-VNTRs) [8, 9], single nucleotide polymorphisms (SNPs) [10,

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Author affiliations: [1]Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy; [2]Microbiology Laboratory, Bambino Gesù Paediatric Hospital, Rome, Italy; [3]bioMérieux Italia SpA, Italy; [4]National Reference Centre for Mycobacterium Bovis Tuberculosis, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy; [5]Istituto Zooprofilattico Sperimentale della Sicilia, Barcellona Pozzo di Gotto, Messina, Italy.

***Correspondence:** Cinzia Marianelli, cinzia.marianelli@iss.it

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Abbreviations: bTB, bovine tuberculosis; DI, discriminatory power index; DR, direct repeat; ID, code number; MIRU-VNTRs, mycobacterial interspersed repetitive unit-variable number tandem repeats; MTC, *Mycobacterium tuberculosis* complex; NTM, nontuberculosis mycobacteria; rep-PCR, repetitive sequence-based PCR; RFLP, restriction fragment length polymorphism; SNPs, single nucleotide polymorphisms.

11], repetitive sequence-based PCR (rep-PCR) [12] and whole genome sequencing [13–15]. Each method has its own benefits and shortfalls, and none of them has proven to be clearly superior to any of the others. It has been suggested that the choice of the optimal typing system depends heavily on the sample under investigation, the setting in which the typing is performed and the expected outcome [3].

Spoligotyping is a hybridization assay that detects variability in the direct repeat (DR) locus, which comprises a series of well-conserved 36 bp DRs interspersed with unique, non-repetitive spacer sequences of 34–41 bp [3]. Spoligotyping is one of the most frequently used PCR-based molecular typing methods because of its simplicity. The main disadvantage of spoligotyping is that all genetic polymorphisms are restricted to a single genomic locus, the DR cluster, which limits the resolution. Therefore, spoligotyping alone does not usually provide sufficient discrimination among strains of *M. bovis* to be used as the sole typing method, and is thus often combined with supplementary techniques [16].

MIRU-VNTR analysis involves PCR amplification of repetitive DNA elements, followed by determination of the amplicon sizes. Polymorphism can occur as a result of variations in the number of repeat units at each analysed locus. The MIRU-VNTR method is a reliable and efficient typing system, whose discriminatory capacity increases with the number of loci evaluated [16]. Initially, *M. tuberculosis* typing involved a 12-locus set, which was considered efficient for epidemiological purposes [17]. A 15- or 24-locus subset has been shown to ensure better discrimination [18]. However, no specific set of loci have been agreed upon as a standard, and the allelic diversity of loci can vary by country and by MTC species, requiring localized selection of suitable loci. In Italy, a panel of 12 loci providing the greatest discriminatory power were selected for *M. bovis* typing [19]. The MIRU-VNTR method offers advantages in simplicity, digitalization of results and reproducibility, since the repeating units are highly stable in the MTC genome [20]. This method is considered to be the new standard for molecular epidemiological studies [18].

Screening for SNPs is an additional molecular typing tool that provides insights into the phylogenetic population structure of mycobacterial strains. For *M. tuberculosis* typing has been used for a combination of SNPs, such as those associated with genes encoding either antibiotic-resistance proteins, such as*katG* and *gyrA* genes [11] or 3R system components (DNA replication, recombination and repair) [10]. Recently, our group has characterized SNPs in the *embB* gene of sensitive *M. bovis* strains, suggesting that these polymorphisms are new potential markers for the epidemiological study of MTC, at least in our setting. Although SNPs represent the most reliable markers for lineage classification of MTC, their use is hampered by the need to test a large set of genes to achieve satisfactory resolution [3].

A rep-PCR system is a genotyping method based on the PCR amplification of repetitive elements interspersed throughout the bacterial genome. It was initially developed for DNA fingerprinting of nosocomial pathogens [21] and enteric Gram-negative rods [22]. A commercial kit has been developed for mycobacteria, showing high discriminatory ability for *M. tuberculosis* [12, 23], *M. avium* subsp. *avium* [12] and NTM [24]. Like spoligotyping and MIRU-VNTR typing, rep-PCR is a fast, time- and labour-saving method that can generate real-time strain typing results. The discriminatory power of this system has not been evaluated yet for *M. bovis* typing, to the best of our knowledge.

In this study we have assessed the discriminatory capacity of the rep-PCR in *M. bovis* typing by comparing results to those previously obtained by our group with spoligotyping, 12-locus MIRU-VNTR typing and SNP analysis of the *embB* gene. We have also evaluated the resolving power of combined genotyping methods.

METHODS

M. bovis strains

A total of 49 *M. bovis* strains from our collection, including *n*=31 isolates from Sicilian black pigs, *n*=17 from cattle and *n*=1 from a sheep, were typed by rep-PCR. *M. bovis* ATCC 19210, used here as a control, was included in the rep-PCR analysis.

Rep-PCR fingerprints of *M. bovis* isolates were then compared with spoligotypes, 12-locus MIRU-VNTR profiles and *embB* gene polymorphisms, previously characterized by our group [25, 26]. The 12-locus MIRU-VNTR profiles of *M. bovis* isolates derived from the selection of 12 genomic loci were, according to Boniotti and colleagues [19]: VNTR loci 2165, 2461, 0577, 580 and 3192 (i.e. ETR-A to –E), VNTR locus 2996 (i.e. MIRU26), VNTR loci 2163a, 2163b, 3155 and 4052, and VNTR loci 1895 and 3232. The *embB* gene polymorphisms of *M. bovis* isolates derived from four different loci of the *embB* gene previously characterized by Marianelli and colleagues [26] were: one synonymous SNP (CTG → CTA) at codon 220 (SNP1), and three non-synonymous SNPs – T610K, Q998R, and F1012S – at codon positions 610 (SN2), 998 (SNP3) and 1012 (SNP4), respectively.

All *M. bovis* strains were isolated from lymph node and tissue samples collected at the abattoir during the postmortem inspection for the purpose of detecting macroscopic lesions suggestive of tuberculosis, in accordance with national laws.

Semi-automated commercial rep-PCR typing

DNA was extracted from bacterial cultures using the NucliSENS miniMAG Kit (bioMérieux, France) and following the manufacturer's instructions. Samples were assigned arbitrary code numbers (ID). Genomic DNA was amplified by the DiversiLab *Mycobacterium* typing kit (bioMérieux, France), according to the manufacturer's recommendations. The amplified fragments of various sizes and fluorescent intensities were separated and detected using a microfluidics chip with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The resulting rep-PCR-based DNA fingerprints, also called electropherograms, were

uploaded to and analysed using web-based DiversiLab software (version 3.4) through Pearson's correlation coefficient. The report generated by the DiversiLab System contained a dendrogram and a scatter plot for sample comparison. The report also included electropherograms, gel-like images and selectable demographic fields. A cut-off value of 95 % similarity was used to establish strain identity, according to Barnes and Cave [27].

The discriminatory power index (DI)

The discriminatory capacity of each typing method and combination of them was estimated by the numerical index of discrimination (DI) described by Hunter and Gaston [28] and expressed by the formula of Simpson's index. The DI expresses the average probability that the typing system will assign a different type to two unrelated strains randomly sampled in the microbial population of a given taxon. The Discriminatory Power Calculator tool available at http://insilico.ehu.es/mini_tools/discriminatory_power/index.php?show=formula) was used to calculate the DI.

RESULTS

Rep-PCR DiversiLab fingerprints

Forty-nine *M. bovis* isolates from domestic animals and the control *M. bovis* ATCC 19210 were genotyped by automated rep-PCR. The dendrogram and virtual gel images, indicating strain level grouping, are shown in Fig. 1. Rep-PCR DiversiLab identified 11 main clusters (I–XI): the majority of isolates (*n*=26) clustered into cluster I, five isolates grouped into cluster X, four isolates grouped into three clusters (III, V and IX), and two isolates into cluster VI. Four isolates were placed alone into clusters (II, IV, VII and VIII). *M. bovis* ATCC 19210 clustered separately from all the *M. bovis* isolates, as expected (XI). The rep-PCR fingerprints were then compared with spoligotypes, MIRU-VNTR profiles (here indicated from MV-A to MV-Q) and *embB* gene polymorphisms previously defined by our group [25, 26]. All genotypes are detailed in Fig. 1. The rep-PCR clusters were somewhat different from those obtained by the other molecular typing methods. Most strains clustered in I, although they displayed different spoligotypes (SB0120, SB0133, SB0833, SB0841 and SB2018), MIRU-VNTR types (MV-A, -B, -E, -F, -G, -H, -I, -K, -L, -M, -N, -O and –P) and *embB* SNPs (1 1 0 0, 1 0 0 0 and 1 1 0 1). Different molecular patterns were also observed in rep-PCR clusters III and V. In clusters VI and X, strains displayed the same spoligotype, the SB0120, but clearly distinct MIRU-VNTR profiles (MV-A, -B, -J and -L) and *embB* SNPs (1 1 0 0 and 1 0 0 0). Strains of cluster IX shared identical spoligotype (SB0120) and *embB* SNPs (profile 1 1 0 0), but had different MIRU-VNTR profiles (MV-A, -B and –C). No isolate sharing identical spoligotype, MIRU-VNTR profile and *embB* gene polymorphism grouped into a unique rep-PCR DiversiLab cluster.

Discriminatory capacity

The discriminatory ability of the four molecular typing methods used here was assessed by calculating the DI. The rep-PCR DiversiLab typing system, as detailed above, clustered 49 isolates into 10 main groups (I–X) and the control *M. bovis* ATCC into a different group (XI). This method showed a DI=0.699. The main group was I with 26 isolates. Spoligotyping divided the isolates into five clusters (SB0120, SB0841, SB0833, SB0133 and SB2018) and showed a DI=0.433. SB0120 was the most common spoligotype with 36 clustered isolates. The SNP analysis of the *embB* gene reduced the number of genotypes to four with two main clusters – SNP1 with 19 isolates and SNP1/SNP2 with 27 isolates – but showed a greater discriminatory power than spoligotyping (DI=0.555). The SNP1/SNP3 cluster grouped two isolates that showed identical and unique genotype (SB0120/MV-Q). One isolate with unique SB0120/MV-K genotype clustered into the SNP1/SNP2/SNP4 group. The 12-locus MIRU-VNTR analysis, on the other hand, differentiated 17 profiles (MV-A to MV-Q) and showed a DI=0.873. The largest clusters were MV-A and MV-B (12 isolates each), followed by MV-F (five isolates) and MV-G (three isolates); four MV types clustered into two isolates, and nine MV types were unique. Results are shown in Fig. 2. Comparing all of the above DIs, the 12-locus MIRU-VNTR analysis proved to be more efficient at differentiating *M. bovis* strains.

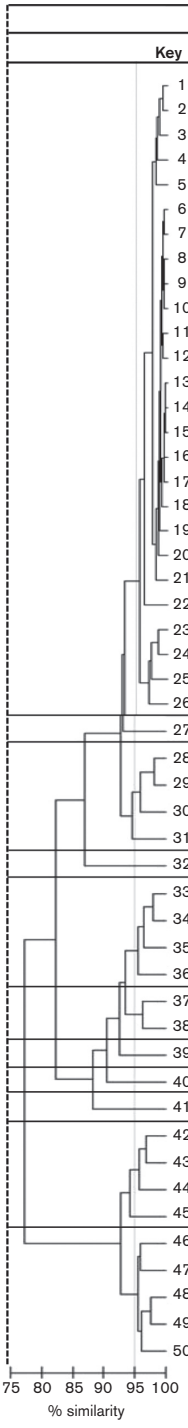
We also analysed combinations of the most efficient methods. When combining the 12-locus MIRU-VNTR types with *embB* SNPs, spoligotypes or rep-PCR profiles, the number of genetic profiles increased, respectively, to 18 (DI=0.875), 20 (DI=0.896) and 34 (DI=0.967). When combining the 12-locus MIRU-VNTR types with spoligotypes and rep-PCR profiles, the number of genetic profiles further increased to 37 and the DI rose to 0.976.

DISCUSSION

Differentiation of *M. bovis* strains at the molecular level provides important insights into the source of infection and routes of transmission and maintenance of bTB. Currently, many PCR-based techniques are available to type *M. bovis*. Spoligotyping and MIRU-VNTR typing are the most extensively used of these.

A semi-automated, highly standardized rep-PCR has proven to discriminate strains of *M. tuberculosis* [12, 23], *M. avium* subsp. *avium* [12] and NTM [24]. This method has not yet been assessed for *M. bovis* typing, to the best of our knowledge. In this study we typed *M. bovis* strains by a semi-automated commercial rep-PCR and compared results to those obtained by spoligotyping, 12-locus MIRU-VNTR typing and *embB* gene polymorphism analysis.

Rep-PCR DiversiLab grouped *M. bovis* isolates into ten different clusters. Unexpectedly, no isolate sharing identical spoligotype, MIRU-VNTR profile and *embB* gene polymorphism grouped into a unique rep-PCR DiversiLab cluster. In fact, with the exception of IX, each cluster displayed clearly distinct spoligotypes, MIRU-VNTR profiles and *embB* gene polymorphisms. In cluster IX, by contrast, strains shared identical spoligotype and *embB* SNPs but had



Rep-PCR fingerprint			Spoligotype	MIRU-VNTR (MV) profile		*embB* gene polymorphism			
Key	Sample ID	Cluster		Code	MV type	SNP1	SNP2	SNP3	SNP4
1	3 m		SB0120	5 5 5 3 4 10 4 5 4 3 6 5	E	1	0	0	0
2	12 m		SB0833	4 – – – 3 – 3 3 – – – –	A	1	1	0	0
3	9 m		SB0833	4 – – – 3 – 3 3 – – – –	A	1	1	0	0
4	1 m		SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
5	2 m		SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
6	5 m		SB0841	– – – – 3 – – 4 – – – –	F	1	0	0	0
7	10 m		SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
8	4 m		SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
9	8 m		SB0120	4 – – – 3 – – 3 – – – –	A	1	1	0	0
10	7 m		SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
11	6 m		SB0841	– – – – 3 – – 4 – – – –	F	1	0	0	0
12	13 m		SB0841	– – – – 3 5 – 4 – – – –	I	1	0	0	0
13	41 m		SB0120	4 – – – 3 – – 4 – – 8 –	M	1	1	0	0
14	43 m	I	SB0120	– – 6 – 3 – – 4 – – – –	N	1	0	0	0
15	42 m		SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
16	45 m		SB0841	– – – – 3 – – 4 – – 7 4	P	1	0	0	0
17	46 m		SB0133	– – – – 3 – – 4 – – – –	F	1	0	0	0
18	47 m		SB0120	4 – – – 5 – – 4 – – – –	K	1	1	0	1
19	14 m		SB0120	– – – – 3 – – 4 – – – –	F	1	0	0	0
20	32 m		SB0120	3 3 – – 3 – – 4 – – – –	G	1	0	0	0
21	11 m		SB0120	4 – – – 3 – – 3 – – – –	A	1	1	0	0
22	15 m		SB0018	– – – – 3 – – 4 – – – –	H	1	0	0	0
23	38 m		SB0120	– 4 – – 3 – – 4 – – 4 –	L	1	0	0	0
24	40 m		SB0841	– – – – 3 – – 4 – – 7 –	O	1	0	0	0
25	17 m		SB0120	4 – – – 3 – – 3 – – – –	A	1	1	0	0
26	29 m		SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
27	18 m	II	SB0120	4 – – – 3 – – 3 – – – –	A	1	1	0	0
28	25 m		SB0120	4 – – – 3 – – 3 – – – –	A	1	1	0	0
29	47 m	III	SB0841	– 4 – – 3 – – 4 – – – –	Q	1	0	1	0
30	27 m		SB0120	4 – – – 3 – 3 4 – – 8 –	D	1	1	0	0
31	51 m		SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
32	16 m	IV	SB0120	3 3 – – 3 – – 4 – – – –	G	1	0	0	0
33	19 m		SB0120	4 – – – 3 – – 3 – – – –	A	1	1	0	0
34	30 m	V	SB0120	3 3 – – 3 – – 4 – – – –	G	1	0	0	0
35	44 m		SB0841	– 4 – – 3 – – 4 – – – –	Q	1	0	1	0
36	31 m		SB0120	– – – – – – – – – – – –	E	1	0	0	0
37	28 m	VI	SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
38	39 m		SB0120	– 4 – – 3 – – 4 – – 4 –	L	1	0	0	0
39	50 m	VII	SB0841	– – – – 3 – – 4 – – – –	F	1	0	0	0
40	33 m	VIII	SB0841	– – – – 3 5 – 4 – – – –	I	1	0	0	0
41	48 m	XI	n.d.	n.d.		n.d.			
42	22 m		SB0120	4 – – – 3 – – 3 – – – –	A	1	1	0	0
43	23 m		SB0120	4 – – – 3 4 – 4 – – – 4	C	1	1	0	0
44	52 m	IX	SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
45	24 m		SB0120	4 – – – 3 – – 3 – – – –	A	1	1	0	0
46	35 m		SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0
47	21 m		SB0120	4 – – – 3 – – 3 – – – –	A	1	1	0	0
48	20 m	X	SB0120	4 – – – 3 – – 3 – – – –	A	1	1	0	0
49	36 m		SB0120	– 3 – – 3 – – 4 3 – – –	J	1	0	0	0
50	49 m		SB0120	4 – – – 3 – – 4 – – – –	B	1	1	0	0

Fig. 1. Comparison of rep-PCR results with spoligotypes, MIRU-VNTR profiles and *embB* gene polymorphisms. First column: rep-PCR-generated dendrogram and virtual gel images representing rep-PCR DiversiLab fingerprints. A cut-off value of 95 % similarity was used for the interpretation of relatedness. Clusters from I to XI are indicated. The control *M. bovis* ATCC 19210 clustered separately from all the *M. bovis* isolates (cluster XI). Second column: spoligotypes. Third column: MV profiles – codes and types. Codes derived from 12 genomic loci previously selected by Boniotti and colleagues [19]. Spoligotypes and MV profiles were previously characterized by our group [25, 26]. Dashes indicate that the MV code does not differ from the code written in full. Fourth column: *embB* gene polymorphisms. Four single-nucleotide polymorphisms (SNP1-SNP4) were previously characterized by Marianelli and colleagues [26]. The presence or absence of a mutation on each SNP was represented as 1 or 0, respectively. ND, not determined.

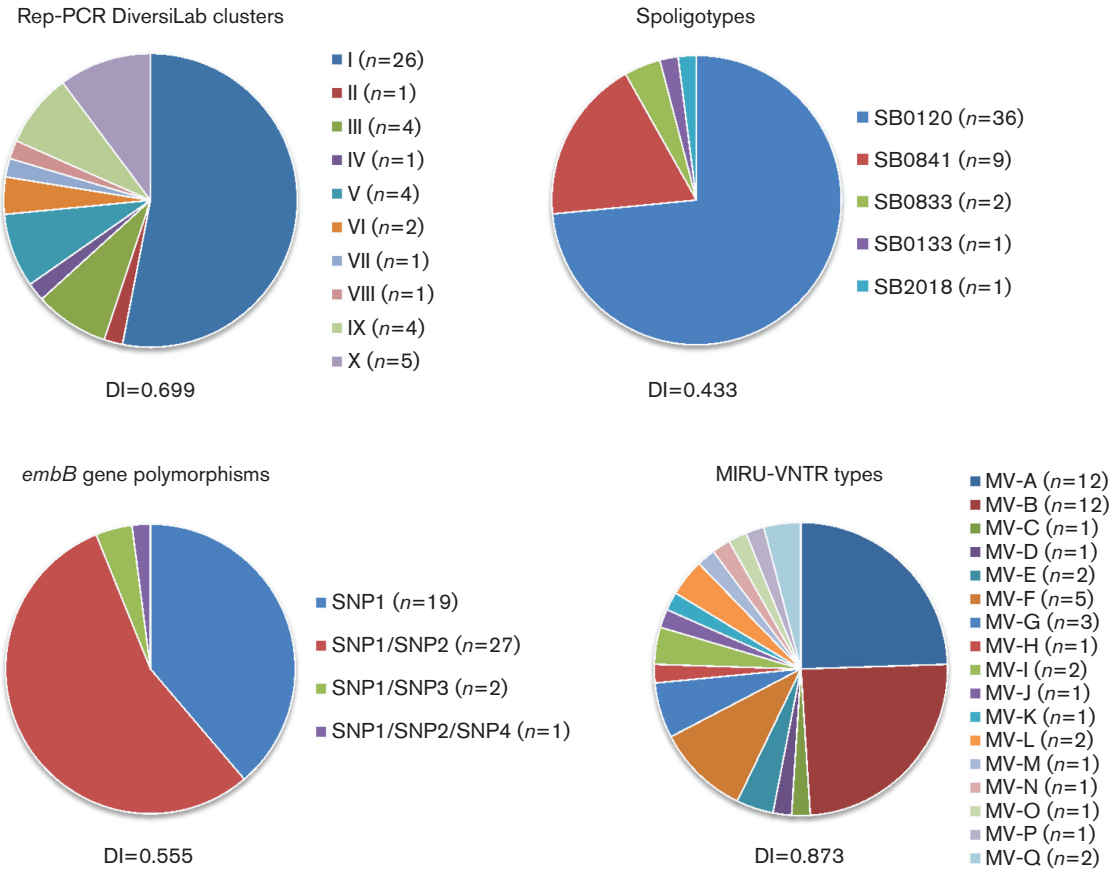


Fig. 2. Discriminatory ability of each genotyping method (*N*=49 *M. bovis* isolates).

different MIRU-VNTR profiles. Rep-PCR DiversiLab displayed greater discriminatory power than spoligotyping and *embB* gene polymorphism analysis but less than 12-locus MIRU-VNTR typing. Varying results on the resolution power of rep-PCR in *Mycobacterium* typing were found in the literature. Some studies showed that rep-PCR was at least as good as MIRU-VNTR analysis for typing *M. tuberculosis* [12, 23] and NTM [24]. Other studies, in agreement with ours, highlighted the limitations of this method compared to MIRU-VNTR for *M. tuberculosis* typing [29, 30].

SNP analysis of the *embB* gene was included in our comparative typing study. Our group has recently characterized four new SNPs – one synonymous mutation (SNP1) and three nonsynonymous mutations (SNP2–4) – within one of the known genes linked to ethambutol resistance, the *embB* gene. These four SNPs were not related to antibiotic resistance but rather to *embB* gene polymorphisms [26]. In this current work, we compared the *embB* SNP to spoligotypes and MIRU-VNTR types. We found that most isolates displaying different spoligotypes and MIRU-VNTR profiles clustered into two main groups, SNP1 and SNP1/SNP2. Three strains with unique genotypes, on the other hand, were linked into separate clusters, the SNP1/SNP3 and SNP1/SNP2/SNP4 groups. Calculating the discriminatory ability of this method, we found that SNP analysis of the *embB* gene showed a greater DI than that obtained with spoligotyping. These results suggest that, at least in our setting, the *embB* gene sequencing might be used as an additional *M. bovis* typing strategy.

The 12-locus MIRU-VNTR analysis displayed the greatest discriminatory power and split *M. bovis* isolates into 17 MIRU-VNTR types. The value of MIRU-VNTR analysis has been proven in *M. tuberculosis*, as well as in *M. bovis* typing in different countries and epidemiological scenarios [19, 31–36]. However, a standardized panel of loci assuring high discriminatory power at a global geographical level is not yet achievable in *M. bovis*, as it is in *M. tuberculosis*, since allelic diversity of each locus differs among countries. That could hinder the comparison of results.

We also calculated the discriminatory power of combined PCR-based methods in *M. bovis* typing. Combining the 12-locus MIRU-VNTR typing with the *embB* gene SNP analysis, spoligotyping or rep-PCR, *M. bovis* isolates split into a greater number of genotypes. The ability of the MIRU-VNTR typing to add discriminatory value to groups of *M. bovis* defined by spoligotyping has already been reported

in the literature [19, 32, 34]. The highest degree of diversity among *M. bovis* isolates was reached by combining three PCR-based methods – the 12-locus MIRU-VNTR analysis, spoligotyping and rep-PCR – and 49 *M. bovis* isolates split into 37 different genotypes.

In conclusion, rep-PCR showed greater discriminatory power than spoligotyping and lower resolution power than the 12-locus MIRU-VNTR analysis in *M. bovis* typing. MIRU-VNTR is confirmed to be superior to the other PCR-based methods tested here. In combination with spoligotyping and 12-locus MIRU-VNTR analysis, rep-PCR improved the discriminatory power for *M. bovis* typing.

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Conflicts of interest
At the time the experiments were performed, Dr Serena Selvaggini was employed at bioMérieux, Italy. bioMérieux Company markets the DiversiLab *Mycobacterium* typing kit. Dr Selvaggini helped in analysing rep-PCR results. All authors declare no financial and/or personal relationships with other people or organizations that could inappropriately influence their work.

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