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Evaluation of the discriminatory power of spoligotyping and 19-locus mycobacterial interspersed repetitive unit-variable number of tandem repeat analysis (MIRU-VNTR) of Mycobacterium bovis strains isolated from cattle in Algeria

--Manuscript Draft--

Manuscript Number:	PONE-D-21-26506
Article Type:	Research Article
Full Title:	Evaluation of the discriminatory power of spoligotyping and 19-locus mycobacterial interspersed repetitive unit-variable number of tandem repeat analysis (MIRU-VNTR) of Mycobacterium bovis strains isolated from cattle in Algeria
Short Title:	Mycobacterium bovis from cattle in Algeria
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Keywords:	Mycobacterium bovis; Mycobacterium caprae; molecular characterization; spoligotyping; MIRU-VNTR-typing; cattle; Algeria
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Additional Information:	
Question	**Response**
Financial Disclosure Enter a financial disclosure statement that describes the sources of funding for the work included in this submission. Review the submission guidelines for detailed requirements. View published research	Parts of this work were funded by a travel and stay grant from the High National Veterinary School, Algeria, (ENSV, www.ensv.dz) (grant no. 000592) for Faiza Belakehal. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Evaluation of the discriminatory power of spoligotyping and 19-locus mycobacterial interspersed repetitive unit-variable number of tandem repeat analysis (MIRU-VNTR) of *Mycobacterium bovis* strains isolated from cattle in Algeria

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Abstract

Bovine tuberculosis (bTB) caused by *Mycobacterium* (*M.*) *bovis* and *M. caprae* is a transmissible disease of livestock, notifiable to the World Organization for Animal Health (OIE). BTB particularly affects cattle and small ruminants and can be transmitted to humans thereby posing a significant threat to veterinary and public health worldwide. *M. bovis* is the principal cause of bTB in Algeria. In order to better understand the route of spreading and elaborate an eradication program, isolation and characterization of mycobacteria from Algerian cattle has been performed. Sixty strains belonging to the *M. tuberculosis* complex have been analyzed by spoligotyping, thereof 42 by 19-locus-MIRU-VNTR-typing. Spoligotyping revealed 16 distinguishable patterns (Hunter-Gaston discriminatory index [HGDI] of 0.8294), with types SB0120 (n = 20) and SB0121 (n = 13) being the most frequent patterns, representing 55% of the strains. Analyses based on 19-locus-MIRU-VNTR yielded 32 different profiles, five clusters and one orphan pattern, showing higher discriminatory power (HGDI = 0.9779) than spoligotyping. Seven VNTR-loci [VNTR 577 (alias ETR C), 2163b (QU11b), 2165 (ETR A), 2461 (ETR B), 3007 (MIRU 27), 2163a (QUB11a) and 3232 (QUB 3232)] were the most discriminative loci (HGDI > 0.50). In conclusion, 19-locus-MIRU-VNTR yielded more information than spoligotyping concerning molecular differentiation of strains and better supports the elucidation of transmission routes of *M. bovis* between Algerian cattle herds.

Keywords: Algeria, *Mycobacterium bovis*, *Mycobacterium caprae*, molecular characterization, spoligotyping, MIRU-VNTR-typing, cattle

Short title: *Mycobacterium bovis* from cattle in Algeria

Introduction

Bovine tuberculosis (bTB) represents a veterinary and public health threat of global relevance. Despite the importance of the disease, there is no official data concerning the true prevalence of bTB in many low and middle-income countries. This also holds for Algeria, despite the fact that bTB is a notifiable animal disease [1]. Although multiple disease control plans are implemented by the Algerian official's health services, there are still many obstacles to control and eradicate bTB. Skin test and meat inspection at slaughter represent the main methods in bTB surveillance. Application of culture and molecular diagnostic methods is extremely limited in low-income countries due to high costs and time requirements [2]. Several strategies assessing cost effectiveness and socio-economic factors have been proposed to fight bTB [3]. In Algeria, we suspect that most bTB-infected herds are geographically clustered in the north-east of the country because of the high concentration of cattle compared to other areas. It is assumed that transmission of bTB in Algeria is mainly attributed to cattle movement, although a study conducted in France by Palisson [4] disclosed the key role played by the spatial neighborhood of infected herds. Genotyping tools are crucial for unveiling pathogen transmission routes in bTB and tracing back sources of infections. The main goal of the present study was molecular typing of the Algerian *M. bovis* population in order to improve our understanding of disease epidemiology and bTB spread in Algeria. Spoligotyping [5, 6] and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) analyses were used [7], since the combination of these two genetic markers is known to be a powerful tool to study the molecular epidemiology in a *M. bovis* population [8]. The DR region (direct repeat) analyzed by spoligotyping is a unique region (locus) which contains well-conserved 36 bp direct repeats separated by variable sequences from 35 to 41 bp. These sequences vary from one strain to another by their length, their sequence and their number. The differentiation of the strains is based on the variability of the number of DRs and on the presence

or absence of the particular spacers [5]. Because of its simplicity, its binary result format and its high reproducibility, spoligotyping is widely used for the molecular epidemiology of members of the *M. tuberculosis* complex (MTC) [9]. However, this method is less resolving than either VNTR typing or restriction enzyme analysis, limiting its usefulness in forensic investigations [10]. The VNTR loci containing a variable number of tandem repeats are amplified by PCR using specific primers and the size of the product is determined by gel electrophoresis. The MIRU-VNTR technique is faster than restriction fragment length polymorphism (RFLP) analysis, easy to interpret, more reproducible between laboratories [11] and gives a higher degree of discrimination than spoligotyping [12].

Materials and Methods

Bacterial strains and molecular identification

A total of 3,848 cattle were inspected for bTB at four slaughterhouses in the north part of Algeria between January and May 2017. The origin of the animals could not be determined due to the lack of an efficient livestock identification system in Algeria. The most important target organs for tuberculosis lesions, i.e. the respiratory tract (lung tissue and lymph nodes), the thoracic cavity, retropharyngeal lymph nodes, liver and kidney, were inspected at slaughter. Tissue samples were taken during inspection of carcasses for the presence of visible lesions suspected of tuberculosis. The cut surfaces of lymph node are examined carefully for the presence of abscess, caseous mass and tubercles. Out of 3,848 animals screened, 184 carcasses presented with lesions suspicious of bTB and samples from single organs of 105 animals were submitted to microbiological examination. Organs predominantly affected were respiratory tract and lymph nodes. The tissue samples were decontaminated according to the modified

Petroff's method [13, 14]. Briefly, an amount of 5 ml NaOH solution (4%) was added to about 10 g of tissue, minced by a mortar in 4 ml of sterile distilled water, and incubated for 15 min. The suspension was diluted by 10 ml of sterile distilled water and then centrifuged at 3000 x g for 20 min. The pellet was used as an inoculum to solid Löwenstein-Jensen medium. Mycobacteria were cultured at Pasteur Institute of Algiers (IPA), Reference Laboratory for Tuberculosis and Mycobacteria for six to eight weeks according to standard protocols.

The molecular characterization of isolates was performed at the National Reference Laboratory for Bovine Tuberculosis at the Friedrich-Loeffler-Institut, Jena, Germany. Using bacterial DNA extracted by the thermal lysis method described by Berg et al. [15], strains were identified as members of the MTC using the RD9 PCR. The PCR was performed using oligonucleotide primers for detection of the RD9 deletion. A PCR reaction mix consisted of 0.5 µl of each primer (100 µM), 0.2 µl of Taq polymerase (Taq PCR Core Kit, Qiagen, Germany), 2 µl 10x PCR buffer, 4 µl of Q solution, 0.4 µl of 0.25 mM $MgCl_2$, 0.4 µl dNTPs (10 mM each) and 1 µl of purified DNA in a final volume of 20 µl. PCR amplification program included one denaturation step (15 min, 96°C) followed by 35 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C. PCR amplification products were separated in 1.5 % agarose gels after electrophoresis at 110 V for 1 h and visualized using ethidium bromide under UV light. As positive control served the strains *M. bovis* BCG (DSMZ 43990, ATCC 27289) and *M. tuberculosis* H37Rv.

Molecular typing

Spoligotyping was performed using the DNA microarray format of the ArrayStrip platform (Alere Technologies GmbH [now Abbott], Jena, Germany) according to the manufacturer's instructions as described by Ruettger et al. [6]. Briefly, after DNA extraction with the thermal lysis method, DR regions were amplified using 5'-biotinylated primers DRa/DRb [5], and then

allowed to hybridize on the microarray (hybridization at 60°C, wash steps at 55°C). Finally, arrays were scanned and analyzed by the ArrayMate™. The Alere Technologies software measures the signal intensity of each probe and compares the profiles automatically to those available at the *M. bovis* Spoligotype Database (www.mbovis.org [16]) and the SITVIT1 database (www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/index.jsp [17]).

MIRU-VNTR typing of the *M. bovis* strains was carried out by in-house technique [18], according to Supply et al. [7] with slight modifications, as for VNTR locus 2461-PCR the primers described by Frothingham et al. [19] and for VNTR locus 2163a-PCR the primers of Skuce et al. [20] were used. DNA was purified using the High Pure PCR Template Preparation Kit, Roche Life Science (USA). The PCRs were performed using 0.8 µl of each primer (20 µM; MWG Eurofins, Germany), 0.1 µl of Taq polymerase (HotStarTaq DNA Polymerase, Qiagen, Germany), 2 µl 10x PCR buffer, 4 µl 5x Q solution, 0.4 µl 0.25 mM $MgCl_2$, 0.8 µl dNTPs (10 mM each) and 1 µl of purified DNA in a final volume of 20 µl. PCR amplification program included one denaturation step (15 min, 95°C) followed by 35 cycles of 1 min at 94°C, 1 min at 59°C and 120 s at 72°C. The PCR for VNTR locus 2163a differed with a 25 µl reaction mix (0.5 µl of each primer, 0.1 µl of Hotstar Taq polymerase, 2.5 µl 10x PCR buffer, 1.25 µl 5x Q solution, 0.5 µl dNTPs and 2 µl of purified DNA) and an annealing temperature of 55°C during amplification [20]. PCR amplification products were separated in 1.5 % agarose gels after electrophoresis at 100 V for 2 h 15 min and visualized using ethidium bromide under UV light.

Allelic diversity estimations were calculated using the Hunter-Gaston discriminatory index (HGDI) [21]. The HGDI was used to determine the allelic diversity within each MIRU-VNTR locus and the genotypic diversities (discriminatory power) of the spoligotyping assays, the 19-locus MIRU-VNTR and the combination of both methodologies, using the website application

http://insilico.ehu.es/. The sample profiles were analyzed by BioNumerics (version 7.6.2, Applied Maths, The Netherlands) to assess the overall genetic similarity of the strains.

Results and Discussion

Bacterial strains and molecular identification

Of a total of 105 samples submitted to microbiological examination, 60 samples showed bacterial growth on Löwenstein-Jensen medium. With a first screening by analysis for acid-fast bacteria, all 60 samples yielded a positive signal and were subsequently subjected to DNA extraction. Subsequent RD9 PCR showed that all 60 strains belonged to *M. tuberculosis* complex (MTC) whereas non-tuberculous mycobacteria were not isolated.

Spoligotyping

Of the 60 MTC positive strains, 59 were identified as *M. bovis* by spoligotyping, one as *M. caprae.* Among the 60 strains, 16 different spoligotypes were identified (Table 1). Four new spoligotypes have been found in the present study and were subsequently submitted to the *M. bovis* spoligotype database (www.mbovis.org). Those new spoligotypes received the ID numbers SB2520 (n = 1), SB2521 (n = 5), SB2522 (n = 1), and SB2523 (n = 1). Two of the four new spoligotypes, SB2520 and SB2521, have been described recently in Algerian cattle and human samples respectively [22].

Table 1. Spoligotype patterns of 59 *M. bovis* and one *M. caprae* strains and their relative frequencies.

SB No.	Spoligotype		No. of strains	Frequency [%]	SIT	Family
	binary code	octal code				
SB0120	11011111011111101111111111111111111111100000	676773777777600	20	33.3	482	BOV_1
SB0121	11011111011111101111011111111111111111100000	676773677777600	13	21.7	481	BOV_1
SB0134	11000111011111101111111111111111111111100000	616773777777600	7	11.7	665	BOV_1
SB2521	11000111011111101110111111111111111111100000	616773577777600	5	8.3	n.k.	n.k.
SB0339	11011111011111101111011110000111111111100000	676773674177600	2	3.3	696	BOV_1
SB0828	11011111011111101111111111111111101111100000	676773777773600	2	3.3	1047	BOV_1
SB1542	11000111000111101111111111111111111111100000	616173777777600	2	3.3	n.k.	BOV
SB0119	11011111011111001111011111111111111111100000	676763677777600	1	1.7	695	BOV
SB0818	11011111011111101111111111111111101111100000	676773777767600	1	1.7	1044	BOV_1
SB0822	11001111011111101111111111111111111111100000	636773777777600	1	1.7	997	BOV_1
SB0848	11011111011101101111011111111111111111100000	676733677777600	1	1.7	1010	BOV_1
SB1452	11011110001111001111011111111111111111100000	674363677777600	1	1.7	1595	BOV
SB2520	11000111011111101111111111101111111111100000	616773777377600	1	1.7	n.k.	n.k.
SB2522	11011111011111101111100111111111011111100000	676773717757600	1	1.7	n.k.	n.k.
SB2523	11011111011111101001111111111111111111100000	676772377777600	1	1.7	n.k.	n.k.
SB0835	01000000000000001111111111101110111111100000	200003777357600	1	1.7	978	BOV_4 Caprae
	Total		60	100		

n.k.: not known; **SIT:** spoligo-international type according to the SITVIT1 nomenclature; **BOV:** Bovine.

The binary code is represented by 43 digits. The number 0 indicate the absence of signal and the number 1 indicate the presence of signal.

The predominant spoligotypes found were SB0120 (n = 20) and SB0121 (n = 13), both belonging to the family BOV_1 and differing in only one spacer (no. 21), followed by SB0134 (n = 7), SB2521 (n = 5), and SB0828 (n = 2). The currently most prevalent spoligotypes SB0120, SB0121, and SB0134 were already detected by a study conducted 10 years earlier in Algeria [23]. Of note, the two new types SB2522 and SB2523 as well as three additional spoligotypes, namely SB0119 (n = 1), SB0848 (n = 1), and SB1542 (n = 2) have not been reported in Algeria before.

Presence of spoligotypes SB0121, SB0120, and SB0134 was also reported for the neighboring countries Morocco and Tunisia as well as France [24-26]. Interestingly, the second most frequently found spoligotype in Morocco, type SB0265, was not detected in our study in Algerian cattle although in silico analysis of whole genome sequence (WGS) data from four Algerian human *M. bovis* isolates [27] could assign two of the strains to SB0265 implying that the spoligotype is present in Algeria, too. The BCG cluster associated spoligotypes SB0120 and SB0121 are circulating nearly worldwide in Europe, Africa, and America [28, 29]. Spoligotype SB0134 is also frequent in many African countries (e.g., Tunisia, Morocco, Mali and Ethiopia) [30-33]. The new type SB2520 identified in our study has been recently found in Ethiopia [33]. The spoligotypes SB0339 and SB0119 are common on the Iberian peninsula, thereof SB0339 frequently reported for Spain and SB0119 in Portugal, and both were also reported for Morocco [24, 34, 35]. The spoligotype SB0818 has been reported in Italy [36] and France [26, 37], SB0848 in the neighboring country Tunisia [31] and in Portugal [38]. According to the database www.mbovis.org, spoligotype SB1542 was previously detected in Italy and *M. caprae* SB0835 in France. *M. caprae* has been identified mainly in continental Europe in the alpine region [39] and Spain [40]. In the study presented here only one *M. caprae* isolate with the spoligotype SB0835 was detected. Only few data exist on the detection of *M. caprae* in Africa. In Algeria

two isolates of spoligotypes SB1451 and SB0835 were described before [22, 23], pointing to a rare occurrence of *M. caprae* in this country.

Nowadays, the composition of the spoligotypes in North Africa (Morocco, Algeria, Tunisia) is dominated by spoligotypes SB120, SB121 and SB0134 possibly having evolved from common ancestral strains introduced since decades from Europe [23-25]. Indeed, this can be explained by recent imports, given the incomplete eradication of bTB in Europe. France represents the country from which the majority of cattle in Algeria were imported. According to WAHIS interface, France is declared "officially tuberculosis free" (OTF) by the EU but bTB still exists and single outbreaks are repeatedly reported for cattle herds and wildlife in some areas of the country. Thereby, SB0120, SB121 and SB0134 are the major spoligotypes distributed in animals in France, too [26]. Moreover, a study conducted in Tunisia by Siala and colleagues analysed human *M. bovis* isolates and found 13 diffferent spoligotypes, thereof, SB0120 and SB0121 as the dominating types [41].

MIRU-VNTR typing

Out of 60 strains of this study, VNTR profiles could be generated for 42 *M. bovis* strains. Missing success in the remaining cases might have been due to low DNA quality or quantity. A panel of 19 loci was chosen for conducting the MIRU-VNTR (Table 2).

Table 2. Allele diversity of the 19-locus MIRU-VNTR in 42 Algerian *M. bovis* strains.

Locus		Number of strains with the respective number of copies													Allele diversity (HGDI)
VNTR	**Alias**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12-15**	**16**	
2165	ETR-A	0	0	**1**	**7**	**11**	**15**	**8**	0	0	0	0	0	0	0.7573
3232	QUB3232	0	0	**2**	**1**	0	**17**	**13**	**8**	**1**	0	0	0	0	0.7178
2163a	QUB11a	**2**	0	0	0	**1**	0	0	0	**15**	**17**	**5**	0	**1**	0.6927
2163b	QUB11b	0	**16**	**8**	**16**	**1**	0	**1**	0	0	0	0	0	0	0.6887
2461	ETR-B	0	0	**1**	**20**	**19**	**2**	0	0	0	0	0	0	0	0.5796
3007	MIRU27	**3**	**17**	**22**	0	0	0	0	0	0	0	0	0	0	0.5703
577	ETR-C	**1**	**1**	**13**	**2**	**25**	0	0	0	0	0	0	0	0	0.5598
4052	QUB26	**3**	**1**	**2**	**5**	**30**	**1**	0	0	0	0	0	0	0	0.4785
3192	MIRU31/ETR-E	0	**3**	**33**	**6**	0	0	0	0	0	0	0	0	0	0.3659
580	MIRU04/ETR-D	**2**	0	**38**	**1**	0	0	0	0	**1**	0	0	0	0	0.1823
2687	MIRU24	**2**	**40**	0	0	0	0	0	0	0	0	0	0	0	0.0929
154	MIRU02	**1**	**41**	0	0	0	0	0	0	0	0	0	0	0	0.0476
802	MIRU40	**1**	**41**	0	0	0	0	0	0	0	0	0	0	0	0.0476
2059	MIRU20	**1**	**41**	0	0	0	0	0	0	0	0	0	0	0	0.0476
2996	MIRU26	0	0	0	**1**	**41**	0	0	0	0	0	0	0	0	0.0476
4348	MIRU39	0	**41**	**1**	0	0	0	0	0	0	0	0	0	0	0.0476
960	MIRU10	0	**42**	0	0	0	0	0	0	0	0	0	0	0	0
1644	MIRU16	0	0	**42**	0	0	0	0	0	0	0	0	0	0	0
2531	MIRU23	0	0	0	**42**	0	0	0	0	0	0	0	0	0	0

ETR: Exact Tandem Repeat; **MIRU:** Mycobacterial Interspersed Repetitive Unit; **QUB:** Queen's University Belfast.

Evaluated loci were selected according to the 12-locus panel proposed in the MIRU-VNTR plus web site (www.miru-vntrplus.org) in combination with the panel proposed by the European Reference Center for Bovine Tuberculosis (EU-RL bTB, VISAVET Health Surveillance Centre, Universidad Complutense de Madrid) for the molecular typing of *M. bovis* in Europe. Among them, the ETR loci (VNTR580, 2165, 2461, and 3192) had been evaluated by Sahraoui et al. [42] for Algerian strains previously. Additionally, the QUB loci (VNTR2163a, 2163b,

3232, and 4052) and VNTR2996 were included as these are regarded highly discriminative for *M. bovis* strains.

Overall, seven loci (VNTR577, 2163a, 2163b, 2165, 2461, 3007, and 3232) presented a high discriminatory power (HGDI > 0.50, Table 2). Our findings corroborate therefore earlier data obtained in Tunisia [25] which showed a high resolution of VNTR2163a, 2163b, 2461, 2165, and 3232, while the four loci VNTR577, 2165, 2461, and 3007, showed a high discriminatory power for Algerian strains also in previous data sets [42]. Furthermore, this is consistent with data reported for *M. bovis* strains from Mexico and Germany [43, 44]. Rodriguez-Campos et al. [45] found only a very low allele diversity for locus VNTR2163b (HGDI = 0.08), but analyzed only SB0121 strains. The two loci VNTR3192 and 4052 showed moderate allelic diversity (HGDI = 0.36 and HGDI = 0.47, respectively) and locus VNTR580 a low allelic diversity (HGDI = 0.18). Very low allele diversity (HGDI <0.15) was observed for six loci (VNTR154, 802, 2059, 2687, 2996, and 4348). By contrast, Yang et al. [46], found a high allele diversity in the loci VNTR580 (HGDI = 0.607) and 802 (HGDI = 0.495) but no resolution for loci VNTR2059, 2687 and 2996. This difference may be related especially to the geographic region from which the strains have been isolated, as we analyzed exclusively bovine strains from Algeria whereas Yang et al. typed strains from Sika deer in Northern China [46]. Finally, there is a difference in the resolution of loci between MTC species. For example, loci VNTR960 and 2531 are reported very discriminatory for *M. tuberculosis* [47], but performed less well for *M. bovis* in the present study. VNTR2996 demonstrates a very low allele diversity in the present study (HGDI = 0.047). A possible explanation could be, that the number of strains analyzed in the present study was smaller and therefore less variance found. No allele diversity (HGDI = 0) was observed with the loci VNTR960, 1644, and 2531 which suggests that these loci are not suitable for typing Algerian *M. bovis* strains. Overall, analysis of 19-locus MIRU-VNTR in 42

244 strains revealed 32 different profiles among which 27 were found only in a single strain (Table
245 3).

246

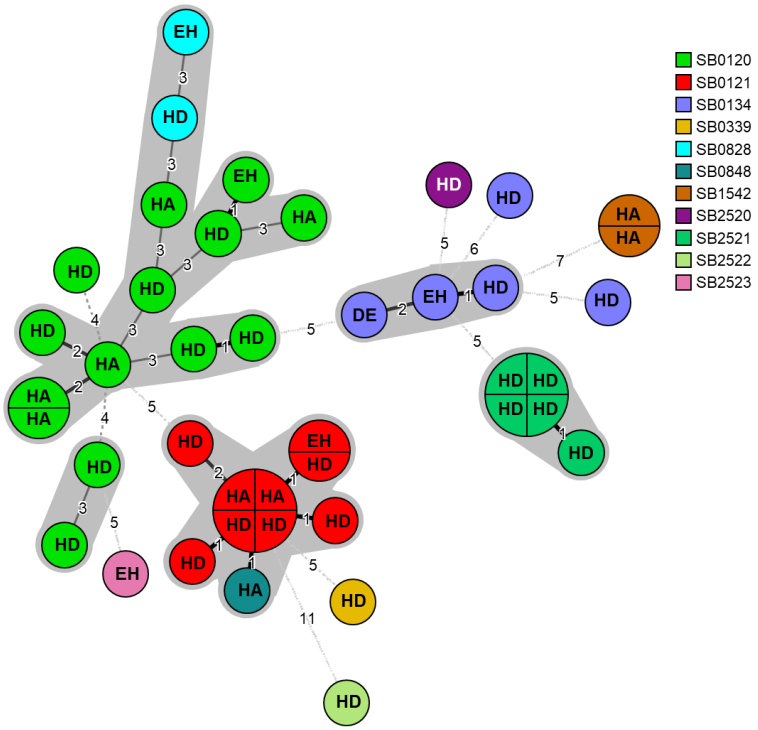
247 **Table 3. Comparison of the discriminatory power of spoligotyping, 19-locus MIRU-VNTR**
248 **typing and the combination of both methods.**

Variability	Spoligotyping	MIRU-VNTR	Spoligotyping and MIRU-VNTR
No. of strains included	60	42	42
Total number of profiles (n)	16	32	33
Number of individual profiles (n)	9	27	28
Discriminatory index (HGDI)	0.8294	0.9779	0.9826

249

250 The combination of MIRU-VNTR allele diversity and spoligotyping pattern demonstrated a
251 large genotypic variety resulting in 33 profiles (Fig 1). Thereby spoligotypes were typed in
252 sub-profiles, e.g. SB0120 in 13 sub-profiles, SB0121 in five, and SB0134 in five, reflecting the
253 heterogeneity of strains causing bTB in Algeria.

254



255

256 **Fig 1. Minimum-spanning-tree based on the combination of spoligotyping and MIRU-VNTR**
257 **results of 42 *M. bovis* strains isolated from slaughtered cattle in Algeria.**
258 Each node represents a unique spoligotype-VNTR sub-profile. If more than one strain exhibited the
259 identical profile the node is separated. Nodes closer than three differences are put into one cluster
260 (gray background). The number of differing alleles (repeat number of MIRU-VNTR and
261 absence/presence of spacer for spoligotyping) between two nodes is indicated by the respective
262 number. The letter inside the nodes represent the slaughterhouse the strains came from (**HD**: Hussein
263 Dey, **HA**: Hadjout, **EH**: El Harrach, **DE**: Dellys) (BioNumerics, version 7.6.2).

264

265 Scientific literature describes several routes of transmission of animal tuberculosis [48, 49]. The
266 emergence of tuberculosis in wildlife [50] may constitute a continuous source for reinfection
267 of cattle and could be at the origin of the persistence of bTB worldwide. This particularly holds
268 for wild boar, which represent a new threat for livestock animals and agriculture in Algeria.
269 Hence, the pathway of transmission of this pathogen in Algeria including wildlife needs to be
270 studied. El Mrini et al. [51] described cases of tuberculosis due to *M. bovis* in Eurasian wild
271 boar in Morocco. Without neglecting the role of re-infections within single cattle herds,

mycobacteria may survive decades unrecognized in several biological niches due to the chronic subclinical course of infection, leading to persisting bTB in Algerian livestock. On the other hand, all our *M. bovis* strains were recovered from lungs and associated lymph nodes, suggesting a primarily airborne transmission of bTB to the cattle under study here and, consequently, an intimate contact between the respective animals. Indeed, combination of MIRU-VNTR allele diversity and spoligotyping pattern allowed us to unveil some possible epidemiological links. Constructing a minimum-spanning-tree revealed five clusters (spoligotype and MIRU-VNTR profile differing in 3 allels in maximum) with the largest one comprising of 14 strains. Two sub-profiles (one in the SB0121 cluster and the other in the SB2521 cluster) contained four strains, that were identical in their spoligotype and MIRU-VNTR profiles, two additional sub-profiles were represented by two strains each. The four strains of the SB2521 sub-profile were all isolated at the slaughterhouse Hussein Dey. It is tempting to assume that cross-infection occurred between animals within one herd or the same area. In contrast, both sub-profiles with four and two strains, respectively, within the spoligotype SB0121 contained strains probably from different areas in Algeria as the strains were isolated from cattle slaughtered in different abattoirs. This includes the slaughterhouses of Hussein Dey and Hadjout which are 88 km distant from each other (source of the strains belonging to the four strain sub-profile), as well as slaughterhouses in El Harrach and Hussein Dey which are more proximate to each other. As we consider it unlikely that one owner had delivered his cattle to different slaughterhouses, the occurrence of identical spoligotype/MIRU-VNTR profiles in different geographical areas might be explained by the trade of animals between herds in those areas. In two cluster, two different spoligotypes (SB0848 and SB0121 as well as SB0828 and SB0120) are combined. Both pairs differ from each other only in one spacer (no. 13 is missing in SB0848 compared to SB0121 as well as no. 34 is missing in SB0282 compared to SB0120) it might be possible that spoligotypes SB0828 and SB0848 may have

297 originated from spoligotype SB0120 and SB0121, respectively, by undergoing a genetic
298 mutation on spacer 13 respectively 34. The singular SB0848 strain and four SB0121 strains
299 possess identical MIRU-VNTR profiles, supporting the close relatedness of both spoligotypes.

300 Beyond disclosing recent transmission events and microevolution of *M. bovis* strains,
301 genotyping of bacteria also may allow for a reconstruction of phylogeography and evolutionary
302 history of these pathogens [52]. Spoligotyping in combination with other genomic markers,
303 such as deletions [52, 53] and single-nucleotide polymorphisms (SNPs) have been used to
304 construct a phylogeography for *M. bovis* strains. The clonal complexes named "African 1" (Af1,
305 characterized by missing of spacer 30) and "African 2" (Af2, missing spacers 3-7) were
306 described as geographically localized in Central-West and East Africa, respectively. The cluster
307 "European 1" (Eu1, missing spacer 11) is found mainly on the British Islands, in New Zealand,
308 Australia, South Africa, and Korea and "European 2" (Eu2, missing spacer 21) on the Iberian
309 peninsula and Western Europe [52-54]. Additional to those four 'classical' phylogenetic
310 lineages recently eight new clonal lineages were defined on the basis of WGS data of more than
311 3,300 *M. bovis* isolates and spoligotypes where affiliated to certain phylogenetic lines [52]. Six
312 out of the 16 spoligotypes detected in our study are also mentioned in this recent publication.
313 The discriminatory capacity of spoligotyping is limited since diversity is measured at a single
314 locus prone to convergent evolution and phylogenetic distances cannot be reliably inferred [55].
315 Despite this limitation our findings might point towards the phylogenetic positions of strains
316 and indicate the multiplicity of clonal lines circulating in Algeria. The signature profile of the
317 *M. bovis* BCG vaccine strain is the absence of spacers 3, 9, 16, and 39 to 43 [29]. The BCG
318 cluster is divided into two groups: the BCG-like group, represented by SB0121, and the ancestor
319 BCG-like SB0120. In the present study, all *M. bovis* strains lacked spacers 3, 9, 16, and 39 to
320 43 and were therefore grouped to the BCG cluster (Table 1). On the African continent, the
321 ancestral BCG-like cluster is predominantly found in Algeria, Zambia [56], and Mozambique

[57]. The cluster Af1, Af2, and Eu1 have not been found in cattle outside of the previous mentioned regions [24]. Further studies, implicating WGS technology, will be required to determine if or to what extend the current bTB epizootic in Algeria originates from the long-term evolution during which animal-adapted MTC, i.e. *M. caprae* and *M. bovis*, might have originally come out of Africa [58] or on a more recent (re)introduction of *M. bovis* from Europe as discussed above.

Conclusion

Until now, tuberculosis still represents a serious burden in Algeria for both, humans and animals. The BCG cluster derived spoligotypes (SB0120, SB0121) are the most frequent types circulating in Algeria and also worldwide. The analysis of four MIRU-VNTR loci [ETR A, VNTR2163b (QUB11b), 2163a (QUB11a), and 3232 (QUB3232)] would be sufficient to characterize Algerian *M. bovis* strains deeply. Nevertheless, the combination of MIRU-VNTR and spoligotyping is highly advisable. The association of those two techniques demonstrates the heterogenic population of Algerian *M. bovis* strains, indicating that different strains are responsible for bTB in Algeria. However, the lack of information concerning the origin of cattle (herd, country) currently impairs the unveiling of transmission routes of bTB in Algeria and must be implemented as part of the control program for the eradication of bovine tuberculosis in Algeria.

Acknowledgments

The author's address special thanks to the technicians Uta Brommer and Josefine Bach at FLI, Jena, Germany, for their excellent support to perform the molecular methods. We are grateful

to Prof. Dr. Dr. h.c. Thomas C. Mettenleiter for providing the opportunity to perform the practical part of this work at the FLI, Jena, Germany.

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