

Association between spoligotype-VNTR types and virulence of *Mycobacterium bovis* in cattle

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Mycobacterium bovis is the causative agent of bovine tuberculosis, a disease that affects approximately 5% of Argentine cattle. The aim of this research was to study if it is possible to infer the degree of virulence of different *M. bovis* genotypes based on scorified observations of tuberculosis lesions in cattle.

In this study, we performed association analyses between several parameters with tuberculosis lesions: *M. bovis* genotype, degree of progression of tuberculosis, and animal age. For this purpose, the genotype was determined by spoligotyping and the degree of bovine tuberculosis gross lesion was quantified with a score based on clinical observations (number, size, and location of granulomas along with histopathologic features). This study was performed with naturally infected cattle of slaughterhouses from three provinces in Argentina.

A total of 265 *M. bovis* isolates were obtained from 378 pathological lesion samples and 192 spoligotyping and VNTR (based on ETR sequences) typing patterns were obtained. SB0140 was the most predominant spoligotype, followed by SB0145. The spoligotype with the highest lesion score was SB0273 (median score of 27 ± 4.46), followed by SB0520 (18 ± 5.8). Furthermore, the most common spoligotype, SB0140, had a median score of 11 ± 0.74 . Finally, the spoligotype with the lowest score was SB0145 (8 ± 1.0). ETR typing of SB0140, SB0145, SB0273, and SB0520 did not subdivide the lesion scores in those spoligotypes.

In conclusion, SB0273 and SB0520 were the spoligotypes with the strongest association with hypervirulence and both spoligotypes were only found in Río Cuarto at the south of Córdoba province. Interestingly, there is no other report of any of these spoligotypes in Latin America.

Introduction

Bovine tuberculosis (BTB) is an infectious disease that affects a wide range of mammals, including humans.¹ This disease is caused by *Mycobacterium bovis*, a member of the *M. tuberculosis* complex (MTBC) that also includes *M. tuberculosis*, *M. africanum*, *M. canetti*, *M. microti*, *M. caprae*, *M. mungi*, *M. orygis*, and *M. pinnipedii*. The population of cattle in Argentina has been estimated in 51 million heads.² In 2012, 9 258 541 bovines were slaughtered and 0.3% of these animals presented tuberculosis lesions.³

Several methods, such as RFLP^{3,4} and VNTR,⁵ have been described for genotyping *M. bovis* and other *M. tuberculosis* complex species. However, up to now, spoligotyping is the best option for large-scale screening studies on the distribution of *M. bovis* strains.⁶ However, for a proper discrimination of the isolates other typing methods such as VNTR typing have to be

performed additionally; especially in settings where one spoligotype is largely predominant.

In this study, we analyzed whether a relationship exists between the genotype of *M. bovis* and the degree of virulence that it causes in naturally infected cattle. While this topic has been already elucidated for other pathogenic bacteria, this question remains unclear for *M. tuberculosis* complex bacteria. For this purpose, we assessed possible associations between the genotype of *M. bovis*, the degree of tuberculosis progression and the age of the animals. For these analyses, the genotypes were determined based on the spoligotyping and VNTR typing and the degree of bovine tuberculosis lesion was quantified with a score based on clinical observations. These observations were based on the number and location of tuberculous granulomas as well as the histopathologic features. These data were subsequently stratified by the approximate age of the animal, within the following categories: calves, steers, heifers, etc. Six veterinary groups

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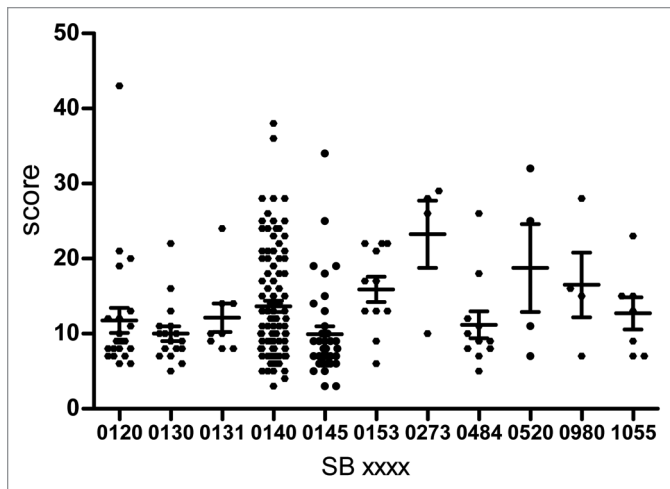


Figure 1. Macroscopic median (\pm SEM) of lesions according to spoligotypes.

were involved in this study. They all performed the collection of the bovine tuberculosis lesions and studied the virulence in the infected cattle through a detailed analysis of the lesions found in the slaughterhouses.

Results

A total of 192 cultures of *M. bovis* were obtained from the 378 analyzed samples that were collected from individual animals. In addition, their spoligotyping data were also analyzed. These data showed that SB0140 is the most predominant spoligotype. This spoligotype grouped 33% of these isolates and displayed the highest prevalence (39%) in Santa Fe province and the lowest (26%) in Buenos Aires province. The second most prevalent spoligotype was SB0145 (18%).

Furthermore, clinical and epidemiological data from the 192 animals with typical TB lesions were analyzed, verifying the infection with *M. bovis* through bacteriological confirmation. The collected data were categorized according to sex, slaughterhouse location, and farm or fair origin, as well as macroscopical and microscopical score. No significant differences were observed in the macroscopical score distribution assigned by the different veterinary groups participating in this study (data not shown). For all animals, the visible lesions were photographed and macroscopical observation data were recorded on data entry sheets (data not shown). Representative lesions are shown in **Figure S1**. The macroscopical score was more informative (**Fig. 1**) than the microscopical data, since most of the histopathological lesions had the top score 4. This score means advanced lesions with caseonecrotic granuloma, peripheral fibrosis and central mineralization. In contrast, the macroscopical scores went from 3 to 43, with 7 being the most frequent value (28 times) in the data set; which describes multifocal and small granulomas (1 cm diameter).

SB0273 showed the highest score with a median score of 27 ± 4.46 (several affected organs with granulomas and beaded forms) and was followed by SB0520 (18 ± 5.8). Additionally,

SB0140, the most common spoligotype, had a median score of 11 ± 0.74 . On the other hand, SB0145 displayed the lowest score (8 ± 1.0) (**Table 1**; **Fig. 1**), even though it is predominant in south Buenos Aires province. The statistical comparison analysis performed through the Mann–Whitney test showed significant differences between the values of the first highest macro scores (which is the scores from SB0273 vs. those from SB140) ($P < 0.05$). Furthermore, the differences of SB0273 and SB0520 compared with the lowest score (SB0145) are also statistically significant ($P < 0.01$ and $P < 0.05$, respectively); thus both spoligotypes showed a correlation with virulence.

In conclusion, the spoligotypes that showed the highest association with virulence were SB0273 and SB0520, suggesting a possible connection between the disseminated infection and disease activity. SB0273 and SB0520 were only found in the south of Córdoba province. There is no other report of any of these spoligotypes in other region of Latin America.

To obtain more recent data of the infection, we excluded the category “cow” in a new assessment, keeping younger animals for this particular analysis. In this case, not only did SB0273 show the highest macroscopical median score, but this score even increased without the older animals (28.5). SB520 (17) and SB0153 (17) followed those values.

Occurrence of SB0273, SB0520, and SB0145 at world level

There are reports of SB0273 in Ireland,⁸ Argentina,^{9,10} Australia, and the UK (<http://www.mbovis.org/spoligodatabase/return-singleinfo.php> and <http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/souchesParSpoligotype>). SB0520 has been described only in Argentina.¹¹ SB0145 has been described as the most extensively distributed genotype, found in Argentina,^{9,10,12,13} Australia,¹⁴ Brazil,¹⁵ France,⁵ Northern Ireland,^{16,17} and other countries (as seen at <http://www.MBovis.org>).

Subtyping of spoligotypes by VNTR

The highly predominant spoligotype SB0140 was divided in 25 VNTR types, where the profile 6–5–5–4–5 grouped 14 out of the 104 isolates. The 36 isolates belonging to SB0145 were divided in 7 VNTR types.

The ETR typing was applied to the most relevant spoligotypes: SB0140, the most predominant; SB0273 and SB0520, which seem to be associated with higher virulence; and SB0145, which is related to a lower virulence. With this typing, the four strains of SB0273 were grouped in three ETR types. One ETR type contained two strains with a high macroscopical pathology score. The four SB0520 isolates consistently had the 7–5–5–4* ETR type. However, the low number of SB0273 ETR-subtypes makes it impossible to validate any analysis of scores. SB0140 was divided into 56 types, but only 4 of these types grouped more than 2 isolates. The 4 major clusters did not show significant differences between them (**Table 2**; **Fig. 2**). Besides, the SB0145 isolates were subdivided in three ETRAD clusters. A cluster (7–5–5–4*) of 10 isolates showed a median score of 13.1. This value was larger than that from SB0145 (9.9) and also of that from the cluster 7–9*–5–4*, which showed low median scores (7.24). However, the differences between 7–9*–5–4* and every other ETR clusters of SB0145 are not statistically significant ($P = 0.10914$).

Table 1. Spoligotypes showing higher and lower macroscopic scores, and most predominant spoligotypes

Spoligotype	Score (median)	SEM	No. of isolates	Characteristics
SB0273	27	4.46	4	Rio Cuarto south Cordoba province, not observed elsewhere in Latin America. Australia
SB0520	18	8.3	3	South Cordoba not observed elsewhere in Latin America
SB0140	13.7	7.4	102	Eu1 family ⁷
SB0145	9.9	6.3	36	Argentina (France?)

Discussion

The evaluation of field diseased animals for testing differential virulence of bacterial pathogens has advantages and disadvantages compared with the experimental inoculation approach. In experimental inoculation, the onset of infection is known. By contrast, this parameter is unclear in field screening. Special caution has to be taken when different strains are tested in experimental infection regarding the number of previous passages of the strains. For instance, the genes involved in the synthesis of phtiocerol-dimycocerosate (PDIM) are lost from *M. tuberculosis* when this bacterium is kept under culture¹⁹; which, in turn, reduces its virulence. Culture conditions may also alter the virulence.²⁰ While in an experimental infection, animals and pathogenic agents are controlled and uniform, in a field evaluation, the infection occurs by a natural route without any specific control. Thus, more animals have to be evaluated in field studies.

In their review, Nicoll and Wilkinson²¹ claim that an important emerging area of research is the study of the outcome of an infection with *Mycobacterium tuberculosis* depending on the variety of the strain involved in the infection. The first report on virulence variability of *M. tuberculosis* isolates came from the pioneering works of Mitchinson on the lower virulence of *M. tuberculosis* isolates from South India.²² This result was confirmed at cellular level by Rajashree and Das.²³ Additionally, López et al. observed that *M. tuberculosis* isolates of the Beijing lineages were more virulent than other lineages in a mice model.²⁴ On the other hand, other non-Beijing strains were more virulent than Beijing lineage in human macrophages.²⁵ Other authors have also investigated the differential virulence of *M. tuberculosis* complex isolates²⁶⁻³⁰ and we previously demonstrated this differential virulence with *M. bovis* isolates.³¹

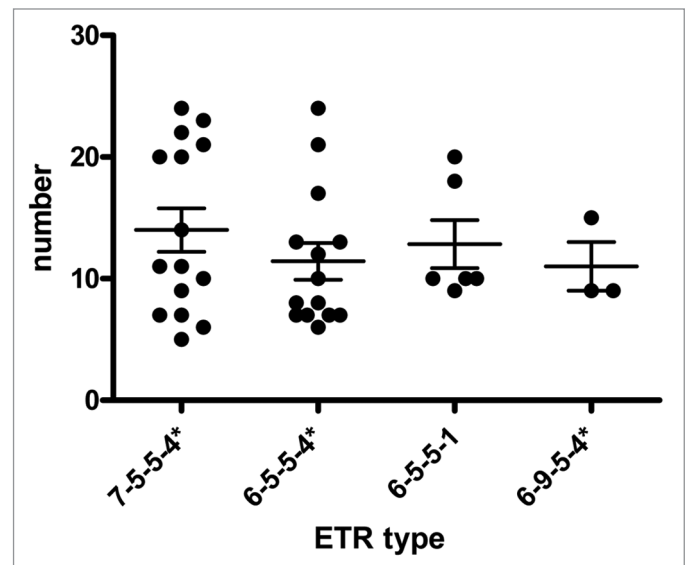
An essential aspect of this kind of study is that the scoring system has to be a reliable correlate of the pathogenesis. With this in mind, in the present study we used the macroscopical scores as a correlate of pathogenesis degree. The score was elaborated taking into account the number, size, and presentation of lesions. Other scoring scales have been proposed. For instance, a shorter scale score was proposed by Buddle.³² This shorter score considers only the total number of lesions (lung lesion score: 0, no lesions; 1, 1 to 9 lesions; 2, 10 to 29 lesions; 3, 30 to 99 lesions; 4, 100 to 199 lesions; and 5, >200 lesions). Palmer et al.³³ assigned different scores for lymph nodes and lungs. In our study, lungs, lymph nodes, and other organs were subjected to semi-quantitative scoring of gross lesions adapted from previous studies.³⁴

Our scoring takes into account the size and pathological presentation of lesion and the total number because individual lesion score are added up. Also, this scoring method yields a larger

Table 2. Macroscopical scores of VNTRs profiles among predominant spoligotypes

Spoligotype	Score	SD	VNTR	No. of isolates	Score
SB0140	13.7	7.4	7-5-5-4*	15	11 ± 1.8
			6-5-5-4*	13	8 ± 1.51
			6-5-5-1	6	10 ± 2.0
			6-9-5-4*	3	9 ± 2.0
SB0145	9.9	6.3	7-5-5-4*	10	13.1 ± 7.4
			7-9*-5-4*	10	7.24 ± 1.76
			7-5-5-3	6	7.6 ± 3.8

The variants of an integer were marked by an asterisk (*) as previously suggested.¹⁸

**Figure 2.** Association between macroscopic score median (± SEM) according to VNTRs types only from the major spoligotype SB0140.

linear rank. The specificity and sensitivity of scores is a matter of further research and analysis. The microscopical score was not considered in the analysis because it failed to provide more information to the global results (data not shown).

In this cohort, the slaughtered animals presented severe visible lesions with varying degrees of gross pathology scoring. The bovines with SB0273 and SB0980 isolates showed the highest macro scores and these values were significantly different from the others. Interestingly, these spoligotypes were present in the provinces with the highest prevalence of bovine tuberculosis. Furthermore, if individual scores are considered, 26, 28, and

Table 3. Macroscopical scoring for individual lesions

Characteristic	SCORE				
	0	1	2	3	4
Red Halo	Absent	Present	//	//	//
Capsule	Non visible	Visible	//	//	//
Color	//	White–yellow	Yellow	//	//
Calcification	Absent	Present	//	//	//
Presentation	//	Focal (1 granuloma)	Multifocal (> 2)	Miliary	Disseminated
Size (diameter)	//	<0.5 cm	0.5–1 cm	1–5 cm	>5 cm

29 are the most represented with a genotype associated to the largest number of macroscopic lesions observed in the inspected animals. Other high scores, such as 32, 34, 38, and 43, were obtained in only one case each. Therefore, they were exceptional findings among different genotypes with regular averages of score.

The spoligotype SB0273 was also described in Ireland⁸; however it had been first identified in Australia. In the present study, this spoligotype was detected at south of Córdoba province. There are no other reports of SB0273 in Latin America.¹⁰ Remarkably, the two spoligotypes with the highest score (SB0273 and SB0520) represent less than 0.7% of the total isolates of *M. bovis* from Argentina. The spoligotype SB0273 is a close relative of SB0140 showing difference in only one spacer (spacer #37). SB0520 is less related to SB0140 differing in various spacers. ETR types prevailing in SB0140 also appear in SB0273 but not in SB0520. These results suggest that SB0520 is more distantly related to SB0140 than SB0273. Furthermore, the largely prevalent SB0140 appears to have a moderate virulence. The ETR subtypes of SB0140 do not differ much in the macroscopical lesion media. The link between success in infection and transmission and a moderate virulence has been debated and analyzed and the Trade-off hypothesis of virulence evolution has been proposed.³⁵

The molecular and cellular basis of the differential virulence of the isolates identified here is yet unknown. Krishnan et al.³⁶ observed that the *M. tuberculosis* strains that showed differential virulence in macrophages, dendritic cells, and mice had different compositions of cell envelope lipids. Because the molecular and cellular basis of the differential virulence of the isolates identified here are not known, we plan to sequence the genome of the strains with the highest and lowest virulence and look for differences in those genes related to lipids.

Materials and Methods

Animals, lesions, and *M. bovis* isolates

Around 35 000 animal carcasses were inspected in slaughterhouses between 2008 and 2011. The isolates came from bovines from Buenos Aires, Córdoba, and Santa Fe provinces. These three provinces hold 60% of the whole cattle population in the country. A convenience non-probabilistic sampling was performed at each slaughter plant with the assistance of the local animal health

service staff (SENASA). The selected categories, which depend on the age of bovines and their weight, and the corresponding percentages were as follows: calves (<12 mo, <220 kg), 13%; young steers (12 to 18 mo, <350 kg), 5%; steers (>18 mo, >350 kg), 11%; heifers (12 to 30 mo), 8.3%; cows (>30 mo, >350 kg), 61%; and bulls, 0.5%.

The anatomical dissemination of the visible gross pathological lesions in organs and tissues were scored according to Table 3. The individual score of each animal was calculated based on the total score of all lesions. For example, if an animal has a granuloma with red halo (1), of yellow color (2), no calcification (0), multifocal (2), and with a size of 1–5 cm (3), it gives a score of 8 for this individual lesion, and so on up to complete all lesions. The microscopical score was calculated according to Wangoo et al.³⁷

A total of 378 samples from individual animals were collected and submitted to histopathology and culture. A total of 192 *M. bovis* isolates coming from Buenos Aires ($n = 63$), Córdoba ($n = 67$), and Santa Fe ($n = 62$) provinces were included in this study.

Histopathology

Tissues with granulomatous macroscopic bovine tuberculosis-like lesions from 378 adult bovines obtained during the slaughtering were processed according to routine histopathological technique. The lesions consisted in different-sized granulomas, encapsulated, with caseous necrosis and calcification. After 24 h fixation, samples were embedded in paraffin, cut in 4- μ m sections, and stained with hematoxylin–eosin (H&E) and Ziehl–Neelsen acid-fast stain. Microscopic lesions were observed and granulomas classified in stages 1 to 4 according to previously described criteria.³⁸ The presence of acid-fast bacilli was classified in scores 1 to 4 as suggested also by Wangoo et al.³⁸ All data were registered in individual ad hoc spreadsheets.

Spoligotypes

A total of 192 isolates were typed by spoligotyping, according to Kamerbeek et al.⁶ The spoligotypes were collected in a binary format in an excel database and the scanned films were analyzed using BionumericsR (Version 3.5, Applied Maths, Sint-Martens-Latem, Belgium).

The spoligotypes of this study were compared with the *M. bovis* spoligotypes contained in the <http://www.mbovis.org> database from the University of Sussex, UK and were named according to a code composed by SB of four digits.

VNTR typing

The 192 isolates typed by spoligotyping were further analyzed by VNTR typing, using the six VNTR loci originally identified by Frothingham and Meeker-O'Connell.³⁷ The analysis was limited to the exact tandem repeat ETR-A to -D loci, because the ETR-E and -F loci were monomorphic within this data set. The VNTR genotype of a strain, representing the number of repeat elements at each locus, is presented as a series of four integers that ranges between 1 and 12 according to the different number of alleles and separated by hyphens. The variants of an integer were marked by an asterisk (*) as previously suggested.¹⁸

Multiplex PCRs were used combining primer pairs of ETR-A/B and ETR-C/D (Table 4). The PCR mix was prepared in 96-well plates with the Hot Start Mastermix kit (Qiagen). Five nanograms of DNA were added to a final volume of 20 μ L containing 0.4 μ M of each primer. For each multiplex mixture, one primer of each oligonucleotide pair was tagged with a different fluorescent dye (Table 4). The thermocycler programs for the two multiplex reactions were identical. The PCRs were performed using an initial denaturation of 15 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 1 min and extension at 72 °C for 2 min, and with a final extension at 72 °C for 10 min.

Statistics

Lesion and histopathological scores were analyzed by the Mann-Whitney test and represented as median \pm SEM. Statistical analyses were performed using GraphPad prism 5.03 software (GraphPad Software).

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Table 4. Primers sequences of VNTRs locus

VNTR locus	Primer sequence (5-3)- (labeling)
ETR-A	AAATCGGTCC CATCACCTTC TTA-(FAM) CGAAGCCTGG GGTGCCCGCG ATTT
ETR-B	GCGAACACCA GGACAGCATC AT (JOE)-GGCATGCCGG TGATCGAGTG G
ETR-C	GTGAGTCGCT GCAGAACCTG CAG-(HEX) GGCGTCTTGA CCTCCACGAG TG
ETR-D	CAGGTCACAA CGAGAGGAAG AGC-(FAM) GCGGATCGGC CAGCGACTCC TC

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found at:

www.landesbioscience.com/journals/virulence/article/27193/

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