Research in Veterinary Science 103 (2015) 44–50

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Bovine tuberculosis in domestic pigs: Genotyping and distribution of isolates in Argentina

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ARTICLE INFO

Article history:
Received 6 April 2015
Received in revised form 7 September 2015
Accepted 13 September 2015

Keywords:
Mycobacterium bovis
Pigs
Spoligotyping
VNTR

ABSTRACT

Bovine tuberculosis is caused by *Mycobacterium bovis* and affects primarily cattle, among many other mammal species. In this study, 250 isolates of *M. bovis* collected from pigs slaughtered in Argentina were typed by spoligotyping. Over half of the isolates (66%) grouped into two spoligotypes.
Moreover, SB0140 was the most frequent spoligotype detected in the three performed samplings. In addition, 195 isolates were typed through variable number of tandem repeats (VNTR) by selecting 7 loci (MIRU 16–26–31 and ETR A–B–C–D). The relationship among the patterns was performed using a goeBURST algorithm and the main clonal complexes grouped 110 isolates (56%). Although pigs shared genotypes with cattle (n = 21), some patterns were detected only in pigs (n = 14). These findings suggest the pig as a source of *M. bovis* infection to cattle.

1. Introduction

Bovine tuberculosis (BTB) is caused by *Mycobacterium bovis* (*M. bovis*), a member of *Mycobacterium tuberculosis* complex (MTBC). *M. bovis* infects a wide range of wild and domestic mammals, which play different epidemiological roles in the transmission and persistence of the disease; its epidemiology depends on several factors such as genetics, population density, disease prevalence and ethological characteristics (Nugent et al., 2011; Pesciaroli et al., 2014). Pigs are susceptible to different mycobacteria, including *Mycobacterium avium*, which is the main species identified in countries where BTB is very low or is eradicated (Agdestein et al., 2012; Eisenberg et al., 2012). By contrast, in developing countries, where the disease is endemic in cattle, *M. bovis* is the most frequently isolated species in pigs (O'Reilly and Daborn, 1995; Parra et al., 2003; Muwonge et al., 2012).

The prevalence of BTB is estimated by detection of macroscopic tuberculosis lesions during the slaughterhouse inspection and, in Argentina, is 0.3% (SENASA, 2014). Usually, pigs are considered a final host or spillover in the transmission of the BTB; however, no molecular study has described the function and distribution of the disease in this species (Nugent et al., 2011). In Argentina, we have studied the diversity of spoligotypes of *M. bovis* from pigs (Barandiaran et al., 2011). In other work we have assessed tuberculosis produced by *M. avium* complex and also by *M. bovis*. We have was detected a significant number of animals coinfected with these two species (Barandiaran et al., 2015).

The genotyping of *M. bovis* in different hosts has contributed to a better understanding of the transmission of BTB and to the identification of wild reservoirs contributing with BTB control (de Lisle et al., 2001).

The molecular typing methods most commonly used for the members of the MTBC are the spoligotyping and the VNTR (McLernon et al., 2010). Spoligotyping has demonstrated to be a rapid and profitable cost-effective first-line typing method of *M. bovis* (Zumárraga et al., 2013). Furthermore, typing by VNTR is feasible and has a good power of discrimination for the strains (Roring et al., 2004; Allix et al., 2006; McLernon et al., 2010).

Because the discriminatory power of spoligotyping is moderate, a group of strains with the same type are not necessary identical. In this context, the aim of this study was to perform the molecular typing using spoligotyping and VNTR (MIRU–ETR) typing to assess the genetic diversity and geographical distribution of *M. bovis* isolates from pigs in Argentina. This study will contribute with the knowledge of the epidemiology of the BTB in pigs from our country, where these data are still limited.

2. Materials and methods

2.1. Samples

The *post-mortem* inspection of pig carcasses was performed in three slaughterhouses located at the Buenos Aires province; two samplings

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http://dx.doi.org/10.1016/j.rvsc.2015.09.013
0034-5288/

were made: 2007–2009 and 2011–2013. Thirty eight isolates obtained between 1994 and 1996 were also studied.

The pigs came from Argentinian provinces: La Pampa, Santa Fe, Córdoba, Entre Ríos, Buenos Aires and Mendoza. These areas centralized most part (80%) of livestock production in the country. The presence of macroscopic granulomatous lesions was considered as a criterion for selection of samples. The head and mesenteric lymph nodes as well as the liver and the lung were routinely examined; gross lesions in other locations were also recorded. Approximately 10 g of the lymph nodes with gross pathological lesions was ground in a sterile bag and transported to the laboratory in insulated boxes with ice packs at 4 °C.

2.2. Bacterial cultures

The tissue sample was ground with sterile sand and water in a mortar, subsequently decontaminated according to Petroff's method using 4% NaOH, and cultured in Stonebrink media at 37 °C for 60 days (Jorge et al., 2005). The developed colonies were subjected to Ziehl–Neelsen stain for detection of acid-fast bacilli (AFB).

2.3. Molecular identification of M. tuberculosis complex

The DNA was obtained from colonies by suspending them in 250 μL of distilled water and then heated at 96 °C for 45 min. After centrifugation at 12,000 *g* for 5 min, 10 μL of the supernatant was used as template for PCR. The positive Ziehl–Neelsen micobacteria were analyzed by IS*6110*-PCR to identify the MTBC as described by Hermans et al. (1990).

2.4. Molecular typing

2.4.1. Spoligotyping

To detect and type *M. bovis* isolates spoligotyping (Kamerbeek et al., 1997) was carried out by using the spoligotyping kit (Isogen Biosolutions B.V., Ocimum Biosolutions Company, Hyderabad, India) to identify *M. bovis* and to differentiate intraspecies. The scanned images of the films were analyzed using BioNumerics (Version 3.5, Applied Maths, Sint-Martens-Latem, Belgium). The patterns were compared with the *M. bovis* spoligotypes stored at the database of the Institute of Biotechnology, INTA, Argentina, and at the www.mbovis.org database from the Animal and Plant Health Agency (APHA), United Kingdom. The spoligotypes were named according to the APHA code (SB).

2.4.2. VNTR typing

VNTR typing was performed to increase the degree of differentiation of the isolates. We selected MIRU 16, 26 and 31 (Supply et al., 2000) because these MIRUs have shown the highest discrimination power among the *M. bovis* isolates from Argentinian cattle, in accordance with other publications (Roring et al., 2004; Boniotti et al., 2009; McLernon et al., 2010). The MIRUs were amplified using the primers MIRU 16 (F TCGGTGATCGGGTCCAGTCCAAGTA and R CCCGTCGTGCAG CCCTGGTAC), MIRU 26 (F TAGGTCTACCGTCGAAATCTGTGAC and R CATAGGCGACCAGGCGAATAG) and MIRU 31 (F ACTGATTGGCTTCATA CGGCTTTA and R GTGCCGACGTGGTCTTGAT). The PCR thermal profile consisted of an initial denaturation at 96 °C for 3 min and 35 cycles of denaturation, annealing and extension of 96 °C for 1 min, 55 °C for 1 min and 72 °C for 45 s, respectively. Six microliters of the PCR product was subjected to horizontal electrophoresis in a 3% agarose gel (Ultra Pure Agarose 1000, Invitrogen, USA) with ethidium bromide (0.5 μg/mL) in 1× TBE buffer for 4 h at 95 V. A 100 bp DNA Ladder (Promega Corp., USA) was used as a molecular marker. The size of the amplicons was estimated by comparing with the molecular weight marker and by using the image analysis program BioNumerics (Applied Maths, Belgium). We limited the analysis to the exact tandem repeat ETR-A to D (Frothingham and Meeker-O'Connell, 1998). Multiplex PCRs were used combining primer pairs: ETR-A/B and ETR-C/D. The PCR mix was prepared in 96-well plates with the Hot Start Mastermix kit (Qiagen, Germany). For each multiplex mixture, only one primer of each pair was tagged with a different fluorescent dye ETR-A (F; AAAT CGGTCCCATCACCTTCTTA-FAM and R; CGAAGCCTGGGGTGCCCGCGAT TT), ETR-B (F; GCGAACACCAGGACAGCATCAT-JOE and R; GGCATGCC GGTGATCGAGTGG), ETR-C (F; GTGAGTCGCTGCAGAACCTGCAG-(HEX) and R; GGCGTCTTGACCTCCACGAGTG) and ETR-D (F; CAGGTCACAACG AGAGGAAGAGC-FAM and R; GCGGATCGGCCAGCGACTCCTC). The PCR thermal profile for the two multiplex reactions consisted of an initial denaturation at 95 °C for 12 min; 35 cycles of denaturation, annealing and extension of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min, respectively, and a final extension of 72 °C for 10 min.

Polymerase chain reaction amplifications were subjected to capillary electrophoresis analysis on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The molecular marker used was GS500-250 (ROX). The allele was established using the GenMapper software version 3.7 (Applied Biosystems Foster City, CA, USA).

The VNTR genotype of a strain, which represents the number of repeat elements at each locus, is presented as a series of four integers between 1 and 12 separated by hyphens. The variants of an integer were marked by an asterisk (*).

2.5. Discriminatory power

The discriminatory index (D) described by Hunter and Gaston and expressed by the formula of Simpson was calculated to determine the discriminatory power of spoligotyping and VNTR (MIRU and ETR) techniques (http://insilico.ehu.es) (Hunter and Gaston, 1988).

2.6. Genetic relationship of the strains

To analyze and visualize the hypothetical relationship of genetic patterns of the strains, we have applied a goeBURST algorithm using the PhyloViz free software (Francisco et al., 2012). The combination of each spoligotype with the VNTR (MIRU and ETR) was denoted with a type number (ST). For the spoligotyping, each spacer was considered as a character.

3. Results

Three hundred and ten samples with lesions compatible with tuberculosis (LCTB) were collected. The samples were cultured and development of colonies was observed in two hundred fifty samples.

All the isolates were positive for the Ziehl–Neelsen staining and IS*6110*-PCR.

3.1. Spoligotyping

The isolates were obtained from six different provinces, Buenos Aires (n = 132), Córdoba (n = 60), Santa Fe (n = 45), Entre Ríos (n = 8), La Pampa (n = 4) and Mendoza (n = 1) (Fig. 1). All isolates were positive for IS*6110*-PCR and showed the characteristic pattern of the *M. bovis* strains by spoligotyping. The isolates were distributed into 35 spoligotypes. Most (n = 143) of the 250 isolates of *M. bovis* have been previously described by Barandiaran et al. (2011) and were included in this study to complete the typing with VNTR and with a comparison with patterns of cattle and humans.

The novel spoligotypes (SB2192, SB2189 and SB2350) were incorporated into the spoligotype database of the APHA (http://www.mbovis.org).

Most of the isolates (93.2%: 233/250) grouped in 18 clusters, with at least two isolates, whereas a small minority (6.8%: 17/250) were unique (Table 1 and Fig. 1). The main spoligotype was SB0140 and grouped 56.8% (142/250) of the isolates. Seventeen patterns were unique, of which five were described previously in cattle from Argentina and 12 were reported for the first time in the country (SB1788, SB1784, SB1786, SB1600, SB0849, SB0859, SB1247, SB2192 SB2189, SB0121,