Veterinary Microbiology 144 (2010) 118–126

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Molecular characterization of *Mycobacterium avium* subspecies *paratuberculosis* Types II and III isolates by a combination of MIRU–VNTR loci

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ARTICLE INFO

Article history:
Received 10 July 2009
Received in revised form 16 December 2009
Accepted 21 December 2009

Keywords:
MIRU
VNTR
Mycobacterium avium subspecies *paratuberculosis*
Type II
Type III

ABSTRACT

Mycobacterial interspersed repetitive units and variable number tandem repeats typing (MIRU–VNTR) is a useful technique that has been recently applied to characterize members of the *Mycobacterium avium* complex (MAC). The aim of this study was to examine the genetic variability among a collection of Spanish *M. avium* subspecies *paratuberculosis* (*M. a. paratuberculosis*) isolates with a combination of MIRU–VNTR loci. For this purpose we tested six MIRU–VNTR loci (MIRU-2, MIRU-3, VNTR-25, VNTR-32, VNTR-292 and VNTR-259) in 70 *M. a. paratuberculosis* isolates of Types II and III that were recovered from 22 Spanish localities during a nine-year period (1998–2007). The combination of five loci (MIRU-2, MIRU-3, VNTR-25, VNTR-32 and VNTR-259) enabled the differentiation of 12 allelic profiles, with a resulting Hunter and Gaston discriminatory index (HGDI) of 0.84. Moreover, we obtained MIRU–VNTR patterns that were unique for each of the *M. a. paratuberculosis* types analyzed (II and III); other patterns were host-related or restricted to geographic areas. Therefore, this MIRU–VNTR approach could be a useful sub-typing molecular tool in order to get a better sense of the epidemiology of Johne's disease.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (*M. a. paratuberculosis*), the etiological agent of Johne's disease or paratuberculosis is difficult to isolate, often hindered by long-time incubation periods and culture growth requirements (Cocito et al., 1994; de Juan et al., 2006a,b).

Tandem repeats (TRs) are DNA elements dispersed throughout the genome and are divided into microsatellites (sequence repeats of 1–13 bp) and minisatellites (10–100 bp sequence repeats) (Supply et al., 2000). Included within the minisatellites are the variable number tandem repeats (VNTRs) and the mycobacterial interspersed repetitive units (MIRUs). MIRUs were first described in the genome of *Mycobacterium tuberculosis* (*M. tuberculosis*) (Supply et al., 1997). MIRUs are characterized by the presence of small open reading frames (ORFs) that overlap and are oriented in the same direction as the contiguous ORFs. In recent studies, these VNTR and MIRU have been used to sub-type the members of the *M. avium* complex (MAC) (Bull et al., 2003; Overduin et al., 2004; Romano et al., 2005; Thibault et al., 2007, 2008; Möbius et al., 2008, 2009).

M. a. paratuberculosis has been classified into Types I, II and III according to the differences observed at the genetic level (Dohmann et al., 2003; de Juan et al., 2005; Castellanos et al., 2008; Griffiths et al., 2008; Turenne et al., 2008; Castellanos et al., 2009a,b). Regarding MIRU–VNTR typing

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doi:10.1016/j.vetmic.2009.12.028

on *M. a. paratuberculosis* Type III, there is only one report where three Type III isolates were analyzed (Möbius et al., 2009). The main purpose of this study was to evaluate a set of MIRU–VNTR loci in a varied collection of *M. a. paratuberculosis* Types II and III isolates of Spanish origin to observe the differences present at the number of repeats and also at the nucleotide level.

2. Materials and methods

2.1. Isolates

We selected a panel of *M. a. paratuberculosis* isolates ($n = 70$) from goats, cattle, and wild animals collected from different Spanish locations over a period of nine years (1998–2007). Isolates from domestic animals representing 22 different herds [nine from cattle (*Bos taurus*) and 13 from goats (*Capra aegagrus hircus*)] and two isolates from wild animals included in the study [fallow deer (*Dama dama*) and mouflon (*Ovis orientalis musimon*)] were obtained from different locations in Spain (Table 1). Every isolate was processed, cultured and classified into *M. a. paratuberculosis* Types I, II or III as formerly described (de Juan et al., 2006a,b; Castellanos et al., 2008).

2.2. MIRU–VNTR typing

We analyzed five specific loci MIRU-2, MIRU-3 (alias X3 and VNTR-1658) (Bull et al., 2003; Overduin et al., 2004; Thibault et al., 2007), VNTR-25, VNTR-32 and VNTR-292 (Thibault et al., 2007; Möbius et al., 2008), using the same specific primers that were reported. Moreover, we selected one additional locus (VNTR-259) that was identified by using *in silico* analysis software, Tandem Repeats Database, Laboratory of Biocomputing and Informatics, Boston University (https://tandem.bu.edu/cgi-bin/trdb/trdb.exe) on the genome of *M. a. paratuberculosis* K-10 (GenBank accession number. AE016958; reference sequence NC_002944). Specific primers for VNTR-259, F (5′ GGGTGTGGAGCTACGACTTC 3′) and R (5′ GAGCTGCTT-GACCAGGTGAT 3′), targeting flaking genes were designed using Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

PCR reactions were carried out in a final volume of 50 μl, containing 5 μl of DNA template, 1× standard reaction buffer containing 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate (Biotools B&M Labs, S.A, Madrid, Spain), upstream and downstream primer (3 μM), 10% of DMSO (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) and 2.5 U of Hot-Start-Taq-Polymerase (Qiagen GmbH, Hilden, Germany). As suggested before (Möbius et al., 2008), primer concentrations for MIRU-3 were three times higher than for the rest of the loci.

PCR conditions for MIRU-2, VNTR-25, VNTR-32, VNTR-292 and VNTR-259 were denaturation at 95 °C for 15 min, then 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 2 min, and a final cycle of extension at 72 °C for 10 min, as reported by

Table 1
Panel of *Mycobacterium avium* subspecies *paratuberculosis* isolates ($n = 70$) in the study.

Isolate	Map type[a]	Host/breed	Farm	Geographic distribution
CAM 72	II	Guadarrama goat	Herd 1	Madrid (Central)
MI05/03722-2, MI05/03723-2			Herd 2	
CAM 19			Herd 3	
CAM 63			Herd 4	
413, 415, 417, 464, MI05/00483-2, MI05/00484-2, MI05/00490-2, MI05/00494-2, MI05/00503-2			Herd 5	
CAM 20			Herd 6	
574		Murciano-Granadina goat	Herd 7	Ciudad Real (Central-South)
MI05/04734, MI05/04735, MI05/04736, MI05/04737,			Herd 8	Toledo (Central-South)
MI05/02938-2, MI05/02943-2, MI05/02945-2, MI05/02948-2, MI05/02950		Dairy cattle	Herd 9	Canary Islands
25, 27			Herd 10	Cantabria (North)
648, 682			Herd 11	Madrid (Central)
MI07/02713, MI07/02721, MI07/02728, MI07/02730, MI07/02731			Herd 12	Valladolid (Central-North)
MI07/03564			Herd 13	
MI06/06133-2, MI06/06134-2			Herd 14	Zamora (Central-North)
D206		Fallow deer	–[b]	Huelva (South)
1		Mouflon	–[b]	Soria (Central-North)
740, 750, 752, 896, 940, MI05/02685-2, MI05/02686-2, MI05/02693-2, MI06/00304-2, MI06/00305-2, MI06/00306-2, MI06/00307-2		Bullfighting cattle	Herd 15	Salamanca (Central-North)
733, MI05/02547-2	III	Bullfighting cattle	Herd 15	Salamanca (Central-North)
619, 634, 841, MI06/00285-2, MI06/00286-2			Herd 16	Albacete (Central-South)
MI07/06579-2, MI07/06582-2,			Herd 17	Ciudad Real (Central-South)
MI07/01787-2		Guadarrama goat	Herd 18	Madrid (Central)
CAM 38, CAM 40, CAM 42, CAM 86, CAM 87			Herd 19	
CAM 78			Herd 20	
MI07/04010-2			Herd 21	
793, MI05/03132		Murciano-Granadina goat	Herd 22	Toledo (Central-South)

[a] Assigned according to PFGE data for the isolates with enough growth (de Juan et al., 2005), and PCR-REA of *gyrB* (Castellanos et al., 2007) or PCR-REA of *inhA* (Castellanos et al., 2008).
[b] Wild animals did not belong to a particular herd.