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Molecular typing of *Mycobacterium avium* subspecies *paratuberculosis* strains from different hosts and regions

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Summary
The IS*1311* polymerase chain reaction-restriction endonuclease analysis was used to detect genetic differences among 38 *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) isolates from cattle, sheep, goats and bison from distinct regions of Spain, India and the United States of America (USA). In Spain, all eight bovine isolates, three out of six caprine isolates and one of ten ovine isolates were of the C type, while the other nine ovine isolates and three caprine isolates were of the S type. In India, all five ovine isolates and six caprine isolates were of the B type, and so were all three isolates from bison (*Bison bison*) from the USA. These results show that there are genetic differences between *Map* isolates related to geographic and host factors that have a potential use in the epidemiological tracing of new paratuberculosis isolates.

Keywords
Bison – Cattle – Goat – India – IS1311 – IS900 – Mycobacterium avium subsp. paratuberculosis – Polymerase chain reaction – Restriction endonuclease analysis – Sheep – Spain – United States of America.

Introduction

Genotypic differences have been used to characterise isolates of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), the agent of paratuberculosis in ruminants. According to Collins *et al.* (2), these differences make possible a classification of isolates into two groups: cattle-type strains and sheep-type strains.

Although the most widely used typing methods are IS *900* restricted fragment length polymorphisms (RFLP) (10) analysis and restricted genome on pulsed field gel electrophoresis (6), Marsh *et al.* (8) developed a polymerase chain reaction-restriction endonuclease analysis (PCR-REA) based on polymorphisms in IS*1311*, an insertion sequence present in *Map* and in *Mycobacterium avium* subsp. *avium (Maa)* in seven to ten copies. This technique provides an easy and rapid method of

distinguishing between *Maa* and both cattle and sheep *Map* strains. In a later study, Whittington *et al.* (15) discovered a new IS*1311* restriction band pattern in bison isolates from Montana in the United States of America (USA). In that study, this IS*1311* PCR-REA typing method was applied to a series of mycobacterial isolates in order to detect any genetic differences related to host or geographical origin.

Materials and methods

The identity and origin of each isolate is shown in Table I. Sample processing for culture was performed as described by Aduriz *et al.* (1). While the Indian ovine and caprine samples were cultured on Herrold's egg yolk medium (HEYM) without sodium pyruvate, the Spanish bovine paratuberculosis isolates were grown on HEYM supplemented with sodium pyruvate. The Spanish ovine and caprine paratuberculosis strains were grown on Middlebrook 7H11 medium supplemented with Oleic acid-albumin-dextrose-catalase (OADC) (7H11) (Difco, Detroit, Michigan, USA) and on Löwenstein-Jensen (L-J) (Difco). Bison *Map* isolates grew on L-J or on 7H9, and the remaining mycobacteria were isolated on Coletsos (Bio-Mérieux, Marcy-l'Etoile, France). All media except Middlebrook and Coletsos were supplemented with mycobactin J (Allied Monitor, Inc., Fayette, Missouri, USA). *Mycobacterium avium* subsp. *paratuberculosis* was identified on the basis of time of incubation to visible colonies, colony and bacilli morphology including acid fastness, mycobactin dependance on egg-based media, and IS *900* PCR.

For DNA extraction, colonies were suspended in 500 µl of TE-Triton X100 (Calbiochem, Bad Doden, Germany), subjected to three cycles of freezing and boiling, and treated according to the protocol described by Garrido *et al.* (5). Final DNA concentration was measured in a NanoDrop, ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, Delaware, USA).

Five microlitres of the resuspended DNA were used in a IS*1311* PCR mix containing 0.8 µM of primers M-56 and M-119 (8), 200 µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 20 mM of Tris-HCl, 50 mM of KCl,

Table I
Characteristics of the isolates studied

Geographic origin		Host	Sample	Species	Primary culture	Number of isolates	Isolate code	IS*900* PCR	IS*1311* PCR-REA type
Spain	Basque Country	Cattle	Faeces	*Map*	HEYM+	5	341/02, 3515/02, 2059/03, 2226/03, 2702/03	Pos	C
	Castilla y León	Cattle	Tissues	*Map*	HEYM+	2	2484/02-7, 2484/02-18	Pos	C
	Madrid	Cattle	Faeces	*Map*	HEYM+	1	181/02	Pos	C
	Andalucía	Goat	Tissues	*Map*	L-J	3	s/n, 43, 52504	Pos	S
	Andalucía	Goat	Tissues	*Map*	L-J	3	242, 392, 485	Pos	C
	Basque Country	Sheep	Tissues	*Map*	7H11	1	P38I	Pos	C
	Basque Country	Sheep	Tissues	*Map*	7H11	8	9G, 12I, 14I, 17G, 18I, 19I, 21I, 269ov	Pos	S
	Andalucía	Sheep	Tissues	*Map*	L-J	1	27	Pos	S
	Basque Country	Cattle	Tissues	*Maa*	Coletsos	1	3321/00	Neg	*Maa* pattern
	Andalucía	Goat	Tissues	*Maa*	Coletsos	1	Cap 120	Neg	*Maa* pattern
	Basque Country	Badger	Tissues	*M. intracellulare*	Coletsos	1	2974/03	Neg	275 + 245 bp
	Basque Country	Cattle	Tissues	*M. bovis*	Coletsos	1	320/03	Neg	Negative PCR
	Cantabria	Cattle	Tissues	*M. chelonae*	Coletsos	1	858/03	Neg	Negative PCR
	Castilla y León	Goat	Tissues	*M. bovis*	Coletsos	1	887/00	Neg	Negative PCR
India	Farah	Goat	Faeces	*Map*	HEYM–	6	S3, S5, T7, S2, S28, S4	Pos	B
	Mathura	Sheep	Faeces	*Map*	HEYM–	5	3812, S9, V95, V16, 43	Pos	B
	Mathura	Sheep	Faeces	*M. asiaticum*	HEYM–	1	V 30	Neg	Negative PCR
USA	Montana	Bison	Tissues	*Map*	L-J	3	6.1, 8.3, 10.3	Pos	B
	Montana	Bison	Tissues	*Maa*	7H9	1	13.1	Neg	*Maa* pattern

7H11: Middlebrook 7H11 with Oleic acid-albumin-dextrose-catalase (OADC)
7H9: Middlebrook 7H9 with OADC
HEYM+: Herrold's egg yolk with Na pyruvate
HEYM–: Herrold's egg yolk without Na pyruvate
L-J: Löwestein-Jensen
Map: *Mycobacterium avium* subsp. *paratuberculosis*
Maa: *Mycobacterium avium* subsp. *avium*
PCR: polymerase chain reaction
REA: restriction endonuclease analysis
USA: United States of America

2.5 mM of $MgCl_2$ and 2 U of Taq polymerase (Invitrogene Ltd, Paisley, UK). The assay was performed in a GeneAmp 9,600 PCR system (Applied Biosystems, Foster City, California, USA) under the following conditions: one cycle of 3 min at 94ºC and 37 cycles of 30 s at 94ºC, 15 s at 62ºC and 1 min at 72ºC. In order to confirm *Map* identification by IS*1311* PCR, an IS*900* PCR was also performed, using primers and conditions described by Garrido *et al.* (5). DNA bands of 608 bp and 389 bp were considered PCR positive results for IS*1311* and IS*900*, respectively, after separation in a 2% agarose gel stained with ethidium bromide. An IS*1311* REA was performed to detect the C/T polymorphism at base pair 223 between *Map* strains. Eight microlitres of the positive IS*1311* PCR solution was digested for 2 h at 37ºC in a 16 µl reaction containing 2 U of each endonucleases *Hinf*I and *Mse*I supplemented with buffers provided by the manufacturer (New England Biolabs, Inc., Beverly, Massachussetts, USA). Band patterns visualised after electrophoresis on 4% agarose gels stained with ethidium bromide were interpreted in the manner indicated by Whittington *et al.* (15): *Map* S strains were defined by two bands of 285 bp and 323 bp (all IS*1311* copies have a C at 223 bp); *Map* C strains were characterised by four bands of 67 bp, 218 bp, 285 bp and 323 bp (some copies with a C and some with a T); *Map* B strains were identified by three bands of 67 bp, 218 bp and 323 bp (T in all copies); and finally, isolates showing three bands of 134 bp, 189 bp and 285 bp were considered as *Maa* (C at base pair 223 and T at base pair 422).

After purification with a GFX™ PCR DNA purification kit (Amersham Biosciences, Buckinghamshire, UK), IS*1311* PCR products of two ovine (3812, S9) and two caprine (S3, S5) isolates from India, and one ovine (269ov.) and one bovine (3515/02) isolates from Spain were sent to the DNA Automatic Sequencing Service of the Centro de Investigaciones Biológicas-Consejo Superior de Investigaciones Científicas in Madrid to confirm the DNA sequence of the amplicons and the point mutations between strains. Both forward and reverse strands were sequenced with M56 and M119 PCR primers, respectively. Vector NTI v 8.0 software (InforMax Inc., Oxford, UK) was used to analyse and compare these sequences with those described earlier for *Maa* and *Map* C, S and B strains (GeneBank U16276, and EMBL AJ223974, AJ223975, AJ308375).

The DNA from cultures of eight mycobacteria other than *Map* (*Maa, M. intracellulare, M. bovis, M. chelonae, M. asiaticum*) was used as a negative control for IS*900* and IS*1311* PCR tests. Identification of non-*Map* mycobacteria was performed by the National Reference Laboratory at the Centro Nacional de Microbiología (Instituto de Salud Carlos III, Madrid, Spain) by bacteriological and molecular methods.

Results

Both IS*900* and IS*1311* PCR and REA results are given in Table I. As expected, all *Map* isolates yielded positive results in both PCR assays, and all *Maa* isolates were also positive in IS*1311* PCR. None of the other isolates was positive in any of the PCR assays performed, except *M. intracellulare*, which yielded a band of approximately 520 bp in the IS*1311* PCR. Although the 67 bp band of C and B type digests is difficult to see, all samples submitted to restriction analysis gave unmistakable band patterns (Fig. 1) which corresponded to the four possibilities described above, except for the *M. intracellulare* isolate which produced two bands of approximately 275 bp and 245 bp. All *Map* isolates from India were identified as B type strains regardless of whether they were of ovine or caprine origin. With regard to the Spanish cultures, while all the bovine isolates gave a type C REA pattern, only one (P38I) of the ten ovine isolates analysed was of C type, while all the others were of S type. Three of the six caprine paratuberculosis isolates were S type and three were C type. Finally, the three bison *Map* isolates from the USA were classified as B strains. *Mse*I endonuclease recognised the T at base position 422 of the two *Maa* isolates (substituted by a C in paratuberculosis strains), which produced *Maa* type REA patterns.

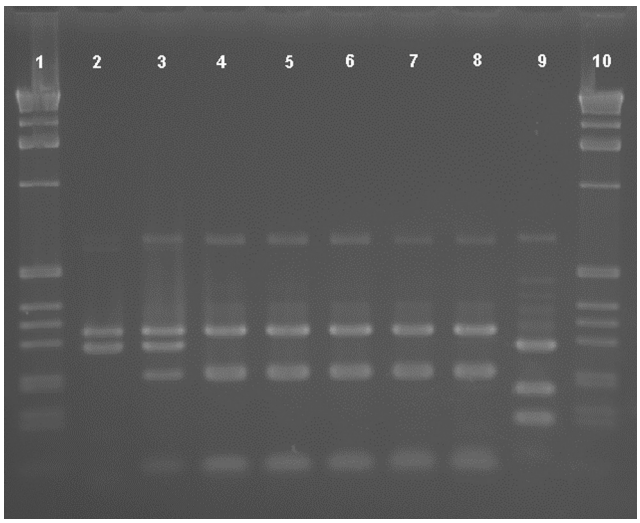

Fig. 1
Photograph of IS*1311* polymerase chain reaction-restriction endonuclease analysis electrophoresis on 2% agarose gel stained with ethidium bromide

Lanes 1 and 10, 1Kb DNA size marker. Lane 2 shows an S pattern from a Spanish ovine isolate (269ov). Lane 3 shows a C type pattern from a Spanish bovine isolate (3515/02). Lane 4 shows a type B pattern from an American bison isolate (8-3). Lanes 5 and 6 show type B pattern from Indian caprine isolates (S5 and T7). Lanes 7 and 8 show type B pattern from Indian ovine isolates (3812 and S9). Lane 9 corresponds to an avian type isolate from a Spanish goat (Cap120).

The DNA sequences obtained from the amplified products matched exactly with those published previously for each strain type. The ovine *Map* isolate from Spain, which was S type in restriction analysis, had a C at base position 223. A mixed C/T signal was detected at the same base position in the sequence of the Spanish bovine paratuberculosis isolate, classified as C type by REA. And finally, all four Indian ovine and caprine PCR products sequenced possessed a T in all copies at this position, which had only been found before in bison isolates from the USA.

Discussion

Results of this study confirm previous findings (14) that both IS*900* and IS*1311* PCR techniques are suitable for use in *Map* confirmation and typing. The unique amplification observed in *M. intracellulare* IS*1311* PCR, is easily distinguishable and can be confirmed by REA.

The cultural characteristics and restriction patterns of Spanish paratuberculosis isolates analysed in the authors' laboratory to date are in accordance with part of the literature (8, 15). These results indicate that cattle are mainly infected with type *C Map*, which is readily isolated on HEYM, although the presence of S type strains cannot be ruled out in this species since primary cultures of bovine samples are usually performed only on this medium. Most of the ovine *Map* isolates (nine out of ten) were of the S type and difficult to grow in primary cultures, but a C type strain (in one case out of ten) was also detected in sheep. Goats appeared to be affected by both C and S types, much as has been found in other studies (10, 12, 15).

There is a certain discrepancy in the distribution patterns according to host species that were found in this study and the results reported by other authors (9, 12). The latter conclude that C type strains are commonly isolated from sheep in Europe, while in the present study only 10% of the sheep isolates were found to be of C type. This could be for the same reason that was discussed above with regard to the fact that only type C strains were isolated from cattle in the present study. It is likely that our isolates might represent a less biased sample of strains infecting Spanish sheep since we used L-J and 7H11 for primary isolation. According to Juste *et al.* (7), L-J might improve primary isolation of ovine strains by about 90%, but decrease primary isolation of bovine strains by approximately 37% in relation to HEYM. The other authors either did not report the media used for primary isolation (12) or used HEYM (9). A larger, more specifically designed study that avoids primary isolation bias is needed in order to draw any definitive conclusions on this subject.

It has been reported that culture of *Map* from bison samples is more difficult than from cattle but easier than from sheep, and that it can be improved by the use of HEYM without sodium pyruvate (13). In the case of the Indian isolates, such differences were not noticed because only HEYM without added pyruvate was used for primary isolation. The observation of *Map* colonies produced from sheep samples cultured in HEYM (3, 4, 11) contradicts the findings of some other authors, who reported that the culture of MAP colonies from sheep samples was unrewarding (7, 13). These findings led to the suspicion that a strain different from S could be involved in the Indian cases in the present study, probably a C strain. However the molecular methods used in this study proved that they were B type strains, which had previously only been found in bison from Montana (USA). Nevertheless, the preliminary results from RFLP analysis of genomic DNA indicate that these isolates are not the same as those from bison and probably represent a new biotype, as yet not seen outside India (Whittington, unpublished observations).

In summary, this study confirms the existence of *Map* genetic differences related to geographic and host factors that would help to explain the variable success rates in the primary isolation of *Map* from some host species on some culture media in previous reports. These differences could also prove useful for the epidemiological tracing of new paratuberculosis cases.

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Caractérisation moléculaire de souches de *Mycobacterium avium* sous-espèce *paratuberculosis* issues de différents hôtes et régions du monde

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Résumé

Les différences génétiques entre 38 isolats de *Mycobacterium avium* sous-espèce *paratuberculosis* issus de bovins, d'ovins, de caprins et de bisons de différentes régions d'Espagne, d'Inde et des États-Unis d'Amérique ont été mises en évidence au moyen de l'amplification en chaîne par polymérase IS*1311* par analyse de l'endonucléase de restriction. Parmi les isolats provenant d'Espagne, les huit isolats bovins, trois des six isolats caprins et l'un des dix isolats ovins appartenaient au type C, tandis que les neuf isolats ovins et les trois isolats caprins restants étaient du type S. Les cinq isolats ovins et les cinq isolats caprins prélevés en Inde étaient du type B, de même que les trois isolats à partir de bisons (*Bison bison*) aux États-Unis d'Amérique. Les différences génétiques entre isolats de *M. avium paratuberculosis* mises en évidence dans cette étude, liées tant à la géographie qu'aux espèces hôtes, s'avèrent potentiellement utiles pour déterminer l'origine épidémiologique des nouveaux isolats de l'agent de la paratuberculose.

Mots-clés

Amplification en chaîne par polymérase – Bison – Bovin – Caprin – Caractérisation par endonucléase de restriction – Espagne – États-Unis d'Amérique – Inde – IS1311 – IS900 – Mycobacterium avium sous-espèce paratuberculosis – Ovin.

Tipificación molecular de cepas de *Mycobacterium avium* subespecie *paratuberculosis* de diferentes huéspedes y regiones

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Resumen

Los autores describen una experiencia en la que se empleó la reacción en cadena de la polimerasa (PCR) y seguidamente un análisis por endonucleasas de restricción para amplificar la secuencia de inserción (IS) *1311* y detectar diferencias genéticas entre 38 cultivos de *Mycobacterium avium* subesp. *paratuberculosis* (*Map*) procedentes de muestras tomadas a bovinos, ovejas, cabras y bisontes de distintas regiones de España, la India y los Estados Unidos de América. En España, las ocho muestras tomadas de bovinos, tres de las seis de cabra y una de las diez de oveja correspondieron al tipo C, mientras que las nueve ovinas y tres caprinas restantes fueron del tipo S. En la India, las cinco muestras de oveja y seis de las de cabra resultaron del tipo B, al igual que todas las muestras (tres) obtenidas en bisontes (*Bison bison*) de los Estados Unidos.

Estos resultados demuestran que existen diferencias genéticas entre las muestras de *Map*, relacionadas con factores geográficos o con el tipo de huésped, que podrían ser útiles para seguir el rastro epidemiológico de las nuevas muestras de paratuberculosis que se vayan aislando.

Palabras clave
Análisis por endonucleasas de restricción – Bisonte – Bovino – Cabra – España – Estados Unidos de América – India – IS1311 – IS900 – Mycobacterium avium subesp. paratuberculosis – Oveja – Reacción en cadena de la polimerasa.

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