Arch Med Vet 47, 97-100 (2015) DOI: 10.4067/s0301-732x2015000100016

COMMUNICATION

Identification of contaminating bacteria when attempting to isolate *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from bovine faecal and tissue samples using the BACTEC MGIT 960 system#

Identificación de bacterias contaminantes al aislar *Mycobacterium avium* subsp. *paratuberculosis* (MAP) desde muestras de material fecal y tejido de bovinos, utilizando el sistema de cultivo BACTEC-MGIT 960

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RESUMEN

El diagnóstico de la infección por *Mycobacterium avium* subsp. *paratuberculosis* (MAP) al utilizar un sistema de cultivo líquido resulta en una mayor sensibilidad, rapidez y automatización. Sin embargo, tiene como desventajas una mayor tasa de contaminación en relación con los sistemas convencionales y también es menos específico. El presente estudio identificó algunas bacterias contaminantes del sistema de cultivo BACTEC-MGIT 960 al procesar muestras clínicas de ganado bovino del sur de Chile. No se detectaron micobacterias en las muestras falsas positivas a MAP mediante la técnica Reacción en Cadena de la Polimerasa-Análisis con Enzimas de Restricción (PRA)-*hsp*65. Por otra parte, el Análisis de los Espaciadores Intergénicos Ribosomales (RISA) seguido de un análisis de secuenciación, reveló la presencia de *Paenibacillus* sp., Enterobacterias y *Pseudomonas aeruginosa* como contaminantes comunes. Los protocolos de eliminación de contaminantes deberían considerar esta información para mejorar los resultados diagnósticos de los sistemas de cultivo líquido. Además se requieren estudios adicionales que consideran un mayor número de muestras para establecer conclusiones más representativas de la población bovina.

Key words: Mycobacterium avium subsp. *paratuberculosis*, liquid culture, contaminant bacteria, RISA, PRA-*hps*65.

SUMMARY

Diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* infection by liquid culture is sensitive, faster than conventional solid culture and automated. However, a disadvantage of these culture systems is the potential for high frequency of culture contamination. Contaminant bacteria were identified as a step toward better contaminant control. No mycobacteria were detected by mycobacterial Polymerase Chain Reaction-Restriction Enzyme Analysis (PRA)-*hsp*65. Ribosomal Intergenic Spacer Analysis (RISA) followed by sequence analysis identified *Paenibacillus* sp., Enterobacteriaceae and *Pseudomonas aeruginosa* as common contaminants. The present study aimed to identify a representative sample of contaminants encountered when culturing clinical faecal samples from Chilean cattle. Further studies involving a larger and more representative sample of animals are required to extrapolate the results to a broader population.

Palabras clave: Mycobacterium avium subsp. *paratuberculosis*, cultivo líquido, RISA, PRA-*hps*65.

INTRODUCTION

Paratuberculosis, also known as Johne's disease, is a chronic intestinal infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). This infection has a worldwide distribution and affects many animal species (Daniels *et al* 2003, Manning and Collins 2010) but is principally found in domestic and wild ruminants (Lombard 2011). Highest infection prevalence is found in dairy cattle where it has a significant economic impact (Sweeney 2011). Additionally, a potentially causal relationship between MAP and Crohn's disease (CD) in humans has been suggested (Lee *et al* 2011).

Diagnosis of MAP infections can be accomplished by multiple methods but culture of clinical samples in liquid media followed by confirmation using nucleic acid amplification (PCR) techniques has the highest sensitivity and specificity (Collins 2011, Gumber and Whittington 2007). The biggest disadvantage of liquid culture is the high

Accepted: 06.08.2014.

This work was supported by FONDECYT grant (11100200) and FIC De Los Ríos grant (2011). Pamela Steuer was the recipient of a Master Studentship from the Chilean government (CONICYT) during the present study and it was part of a Master Thesis from the Universidad Austral de Chile.

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frequency of contaminated cultures (Whittington 2010). However, it has been demonstrated that contamination of cultures by irrelevant microorganisms is highly clustered within submissions and often within farms, which is suggestive of seasonal and environmental factors such as the type of ration fed to livestock, which may influence the microbial flora in faeces (Whittington 2009). To limit this problem, clinical specimens must be processed using harsh disinfectants and antibiotics incorporated into the culture medium, both of which compromise mycobacterial growth and thus lower both analytical and diagnostic sensitivity (Reddacliff *et al* 2003, Gumber and Whittington 2007, Foddai *et al* 2011). The aim of the present study was to characterize contaminant bacteria as a first step toward better methods to control their growth when using liquid culture methods for MAP infection diagnosis.

MATERIAL AND METHODS

SAMPLE DESCRIPTION

From an ongoing study, 17 presumptively contaminated cultures for detection of MAP in bovine faecal and/or tissue samples were selected for molecular typing. These cultures were considered contaminated based on a positive signal in the MGIT 960 instrument and a negative real-time IS*900* PCR. The decontamination of the specimen plus the use of supplement and antibiotics were performed as recommended by the manufacturer (MGIT ParaTB supplement, Becton Dickinson, Sparks, MD; vancomycin, nalidixic acid, and amphotericin, Sigma-Aldrich). The culture and PCR methods have been previously described (Salgado *et al* 2013).

Identification of mycobacteria other than MAP was attempted by PCR-Restriction Enzyme Pattern Analysis (PRA) - *hsp*65 protocol (Telenti *et al* 1993). Additionally, a broader approach for bacterial identification was done using Ribosomal Intergenic Spacer Analysis (RISA) (Baudoin *et al* 2003). Starting material for both assays was DNA extracted from the 17 presumptively contaminated cultures by the cethyltrimethylammonium bromide (CTAB) method described by van Soolingen and coworkers (van Soolingen *et al* 1991).

POLYMERASE CHAIN REACTION-RESTRICTION ENZYME ANALYSIS (PRA)-HSP65

DNA from the 17 cultures was submitted to restriction enzyme analysis according to methods described by Telenti *et al* (1993), with some modifications. Briefly, 5 µL of the extracted DNA was added to each reaction tube. The PCR mixture was composed of 18 µL of MiliQ water; 5 µL of saccharose; 2 µL of dNTPs (deoxy nucleoside triphosphate, 10 mM each); 5 µL of $MgCl_2$ (25 mM); 5 µL of buffer 10X; 0.25 µL of *Taq* Polymerase (5U/µL); and 5 µL (each) of primers TB11 pmol/µL (5'-ACCAACGATGGTGTGTCCAT) and TB12 pmol/µL (5'-CTTGTCGAACCGCATACCCT), that amplified a 439-bp fragment. The reactions were carried out in a Mastercycler (Eppendorf AG, Hamburg, Germany). The reaction was subjected to a denaturation step (94ºC x 3 min); followed by 35 cycles of amplification (94ºC x 30 s, 55ºC x 30 s and 72ºC x 30 s); and an extension cycle (72ºC x 5 min). Negative and positive (*Mycobacterium avium* subsp. *paratuberculosis* ATCC 19698) PCR controls, as well as DNA extraction negative and positive controls, were included. Digestion of the amplification product was carried out with *Bst*EII and *Hae*III enzymes. Digested products were evaluated by electrophoresis using 2 µL of gel loading buffer, 25 µL of digestion product and a 3% agarose gel at 100 V for 50 min. The PRA pattern was visualized under UV light and banding patterns were compared with those published at PRA-site[1].

RIBOSOMAL INTERGENIC SPACER ANALYSIS (RISA)

Using DNA from each of the 17 cultures the bacterial 16S-23S intergenic spacer region was amplified by PCR with universal primers 1490-72, 5'-TGCGGCTGGATCCCCTCCTT-3' (Normand *et al* 1996) and 21-38, 5'-TGCCAAGGCATCCACCGT-3' (Acinas *et al* 1999). Amplification was performed with GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA). The PCR mixture, containing 0.25 µl of GoTaq® Flexi DNA Polymerase, 20 pmol of each primer, 6 µl of 25 mM $MgCl_2$ solution, 5 µl of 2 mM of each deoxyribonucleotide triphosphate, and 10 µl of 5x Green GoTaq® Flexi Buffer, was made up to 50 µl with DNA-free water. The PCR program was: hot-start at 96ºC for 3 min, 25 cycles of denaturation at 94ºC for 1.5 min, annealing at 52ºC for 1 min, and extension at 72ºC for 1.5 min. A final extension step was carried out at 72ºC for 10 min. PCR products were analyzed using 2% agarose gel electrophoresis; 4 h at 100 V. Representative RISA bands were carefully excised, reamplified, and run again in RISA gels to ensure that the excised bands did not contain multiple PCR products. The re-amplified product were purified with a E.Z.N.A® Cycle-Gel Kit (Omega Bio-Tek, Doraville, GA, USA), and the 16S-23S intergenic spacer region amplicons were sequenced by Macrogen (Seoul, Korea). More than one sample per band was sequenced to ensure correct taxonomic assignment. The consensus nucleotide sequences obtained in this study were deposited and compared with those present in GenBank database from National Center for Biotechnology Information (NCBI) by using BLAST tools[2].

[1] http://app.chuv.ch/prasite/index.html

[2] http://blast.ncbi.nlm.nih.gov/Blast.cgi

RESULTS AND DISCUSSION

PRA of highly conserved sequences in mycobacterial *hsp*65 (Telenti *et al* 1993, Devallois *et al* 1997, Brunello *et al* 2001) revealed no mycobacteria, even after re-analysis with a different batch of restriction enzymes. RISA followed by sequence analysis identified three groups of bacteria: *Paenibacillus* sp., Enterobacteria and *Pseudomonas aeruginosa* (table 1).

Bacterial endospores from the genus *Bacillus* are the most common cause of contamination from processed bovine faeces, according to the BACTEC-MGIT 960 manufacturer's protocol. *Paenibacillus* sp. is a normal soil inhabitant and an endospore-former making it a plausible organism to occur in faecal samples and survive decontamination with hexadecylpyridinium chloride (HPC). *Enterobacteriacea* are likewise logically found in bovine faeces but should have been killed by HPC or inhibited by the antimicrobials used to supplement the culture medium (Famiglietti 2005). Possibly, organic material in faeces inhibits the HPC or in other ways protects cells from the effects of HPC. Similar to the report by Cornfield *et al* (1997), we also found *Pseudomonas* as a common contaminant in MGIT ParaTB medium. *Pseudomonas aeruginosa* is ubiquitous and produces alginate, a viscous gel that surrounds microcolonies or biofilms of the organism making it less susceptible to certain antibiotics (Anwar *et al* 1992). It is also notoriously resistant to antimicrobials.

Detection of MAP in clinical samples by culture, with PCR verification of MAP identification, provides the most definitive *ante mortem* diagnostic evidence of infection and MAP isolates for molecular epidemiology investigations. Removal or inhibition of the many contaminating microorganisms in clinical samples such as faeces, however, is sometimes a major impediment (Cornfield *et al* 1997, Tortoli *et al* 1999). The present study aimed to identify a representative sample of contaminants encountered when culturing clinical samples from Chilean cattle. Further studies involving a larger and more representative sample of animals are required to extrapolate the results to a broader population.

Table 1. Phylogenetic assignment of RISA bands.
Asignación filogenética de las bandas obtenidas por RISA.

Sample	Band	Taxonomic group	Closest relatives or cloned sequences (accession no.)	Similarity (%)
control	1	Actinobacteria, Mycobacteriaceae	*Mycobacterium avium* subsp. *paratuberculosis* MAP4 (CP005928)	99
2	3	Firmicutes, Bacilli, Paenibacillaceae	*Paenibacillus popilliae* strain Pj 1 (DQ062687)	99
	4	Firmicutes, Bacilli, Paenibacillaceae	Uncultured *Paenibacillus* sp. clone 70FTM-1 from environmental samples (DQ298389)	100
	5	Firmicutes, Bacilli, Paenibacillaceae	Uncultured *Paenibacillus* sp. clone 70FTM-1 from environmental samples (DQ298389)	100
261T	6	Proteobacteria, Pseudomonadaceae	*Pseudomonas aeruginosa* RP73 (CP006245)	99
	7	Proteobacteria, Pseudomonadaceae	*Pseudomonas aeruginosa* RP73 (CP006245)	99
	8	Proteobacteria, Pseudomonadaceae	*Pseudomonas aeruginosa* DK2 (CP003149)	93
325T	9	Proteobacteria, Enterobacteriaceae	*Enterobacter cloacae* subsp. *cloacae* ATCC 13047	87
	11	Proteobacteria, Enterobacteriaceae	*Enterobacter cloacae* subsp. *cloacae* ENHKU01 (CP003737)	97
	12	Proteobacteria, Enterobacteriaceae	Enterobacteriaceae bacterium strain FGI 57 (CP003938)	94
461	13	Proteobacteria, Enterobacteriaceae	*Escherichia coli* O104:H4 str. 2009EL-2050 (CP003297)	89
	14	Proteobacteria, Enterobacteriaceae	*Escherichia coli* strain K-12 substr. MDS42 (AP012306)	98
462	15	Firmicutes, Bacilli, Paenibacillaceae	*Paenibacillus polymyxa* SC2 (CP002213)	81
	16	Proteobacteria, Enterobacteriaceae	*Escherichia coli* ETEC H10407 (FN649414)	82
	17	Proteobacteria, Enterobacteriaceae	*Escherichia coli* O104:H4 str. 2009EL-2071(CP003301)	78
	18	Firmicutes, Bacilli, Paenibacillaceae	*Paenibacillus mucilaginosus* K02 (CP003422)	89
465	21	Firmicutes, Bacilli, Paenibacillaceae	*Paenibacillus mucilaginosus* 3016 (CP003235)	95
	22	Actinobacteria, Micromonosporaceae	*Actinoplanes* sp. N902-109 (CP005929)	96
	23	Uncultured organism clone p401 16S-23S ribosomal RNA intergenic spacer and 23S ribosomal RNA gene, partial sequence (unclassified sequence)	Uncultured organism clone p401 from environmental samples (JN607185)	84
474	25	Firmicutes, Bacilli, Paenibacillaceae	*Paenibacillus mucilaginosus* K02 (CP003422)	90
	26	Firmicutes, Bacilli, Paenibacillaceae	*Paenibacillus mucilaginosus* K02 (CP003422)	76

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