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Culture phenotypes and molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* isolates from small ruminants

Z. Dimareli-Malli [a,*], K. Mazaraki [b], K. Stevenson [c], P. Tsakos [d], A. Zdragas [a], V. Giantzi [a], E. Petridou [e], I. Heron [c], G. Vafeas [a]

[a] *Veterinary Research Institute of Thessaloniki, National Agricultural Research Foundation (NAGREF), Thessaloniki, Greece*
[b] *Technological Educational Institute of Thessaloniki, Department of Animal Production, Greece*
[c] *Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Midlothian, UK*
[d] *Veterinary Institute of Infectious and Parasitic Diseases of Thessaloniki, Ministry of Rural Development and Food, Greece*
[e] *Laboratory of Microbiology, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece*

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ABSTRACT

In this study the suitability of different solid media was investigated for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) in order to identify the optimum single or combination of media to permit the isolation of all strain types from small ruminants. A subset of these *Map* strains was then further characterized by molecular typing methods to assess the genetic diversity of *Map* strains in the study area (Northern Greece). *Map* strains were isolated from tissues and faeces of infected goats ($n = 52$) and sheep ($n = 8$) and were analysed for polymorphisms in IS*1311* to classify the strain type as Type C or S. The study found that M7H11 supplemented with mycobactin j, OADC and new born calf serum (M7H11+Mj) is the best single choice of medium for the primary isolation of *Map* of both Type C and S from small ruminants. The combination of M7H11+Mj and Herrolds egg yolk medium supplemented with mycobactin j and sodium pyruvate allowed the detection of all *Map* isolates in this study.

Nineteen *Map* isolates were characterised by pulsed-field gel electrophoresis and the isolates demonstrated significant genetic diversity. Twelve different SnaBI and 16 distinct SpeI profiles were detected of which 25 have not been described previously and are new profiles. The combination of both enzyme profiles gave 13 different multiplex profiles. Ten different multiplex profiles were detected in goats and three in sheep. One ovine isolate gave the same multiplex profile as a caprine isolate and two different profiles were found within a single goat herd.

Paratuberculosis or Johne's disease is an infection of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) and is responsible for considerable economic losses to the livestock industry worldwide. The disease in sheep and goats first appeared in Greece in 1965. Since then, the number of infected herds as well as the within-herd prevalence has grown rapidly and the disease has spread to most parts of the country via movement of infected stock or flock-to-flock transmission. Goats are more susceptible than sheep. During the period 1987–2003, 322 of 777 (41.4%) goat herds and 97 of 458 (21.1%) sheep flocks were found to be infected with *Map* in Northern Greece. Although the disease is widespread in sheep and goats, in cattle it appears to be limited. This significant difference between sheep/goats and cattle could be due in part to different management factors and conditions of flock (Dimareli-Malli et al., 2009).

Cultivation of *Map* from fecal and tissue specimens still remains the most definitive method for detecting animals with paratuberculosis (Whittington, 2010) and is also an essential step for the application of most molecular typing assays. Despite widespread use, cultivation techniques are not standardized and the ability of different laboratories to cultivate varies considerably. It has been documented since the 1930s that there are differences in the ease of culturing *Map* from sheep compared with cattle and that phenotypic differences exist between isolates (Dunkin and Balfour-Jones, 1935). More recently with the advent of molecular typing, it has been shown that small ruminants can be infected with more than one strain type of *Map* and that these strain types have different growth characteristics (Stevenson, 2010). There are two major strain types of *Map*, which were initially named after the host species from which they were first isolated; 'Sheep' or Type 'S' and 'Cattle' or Type 'C'. However, there is not a direct correlation between strain type and host provenance and some

* Corresponding author. Address: Veterinary Research Institute of Thessaloniki, National Agricultural Research Foundation (NAGREF), NAGREF Campus of Thermi, 57001 Thermi, P.O. Box 60272, Thessaloniki, Greece. Tel.: +30 2310365372; fax: +30 2310365371.
E-mail address: dimareliz@hotmail.com (Z. Dimareli-Malli).

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researchers have used a different nomenclature. The Type S group can be divided into Type I and Type III strains (de Juan et al., 2005; Castellanos et al., 2007a, 2007b; Griffiths et al., 2008; Stevenson, 2010; Biet et al., 2012). Type C strains are synonymous with Type II strains. Type C and S strains can also be differentiated using polymorphisms in IS*1311* (Marsh et al., 1999). In general, Type S strains are more fastidious regarding culture media, grow much more slowly compared to Type C strains and have a host preference for sheep and goats. Strain differentiation of pathogens is essential to obtain important information for tracing sources of infection and for epidemiological studies. The first objective of this study was to identify the optimum single or combination of media to permit the isolation of all *Map* strain types from small ruminants. The second objective was to undertake molecular characterisation of a subset of strains to assess the genetic diversity among *Map* isolates from Northern Greece.

Tissue (mesenteric lymph node and intestine) and faecal samples were collected from 39 goat herds, ten sheep flocks and two cattle herds from different areas of Northern Greece between 2005 and 2010. Tissue specimens were collected from suspected clinical cases with emaciation and/or diarrhoea and were examined by Ziehl–Neelsen staining. The tissue and fecal samples were homogenized, decontaminated with hexadecylpyridinium chloride and the bacteria pelleted and resuspended in 0.5 ml sterile distilled water as described by (Greig et al., 1999). One hundred microlitres were inoculated onto one slope of six specific media supplemented with Mycobacteria selectatabs, (amphotericin B 10 mg/L, polymixin B 200,000 unit/L, ticarcillin 100 mg/L and trimethoprim 10 mg/L; code MS24: MAST Laboratories, Ltd; Merseyside, United Kingdom) and 2 mg/L of mycobactin j (Institute Pourquier) where required. The six media used in this study were as follows: Herrold's egg yolk medium (HEY [Herrold, 1931]), HEY supplemented with mycobactin j (HEY+Mj), HEY supplemented with mycobactin J and 4.1 g/L of sodium pyruvate (HEY+Mj+P), Löwenstein–Jensen (Kalis et al., 2000) supplemented with mycobactin j (LJ+Mj), Middlebrook 7H11 (Cohn et al., 1968) supplemented with oleic acid-albumin-dextrose-catalase enrichment medium and heat inactivated new born calf serum (M7H11) and M7H11 further supplemented with mycobactin j (M7H11+Mj). Tubes were incubated at 37 °C and examined every week for bacterial growth.

A total of 69 *Map* isolates were cultured. All were confirmed as *Map* by mycobactin dependence, growth characteristics, colony morphology and by PCR to detect the *Map*-specific insertion sequence IS*900* (Green et al., 1989). DNA extraction and IS*900* PCR was carried out using the protocol described by Challans et al. (1994). Briefly, the mycobacteria were extracted with xylene, pelleted and washed to remove traces of xylene and then lysed by shaking them with zirconium beads on a mini-bead beater three times at high speed for 90 s. DNA was extracted with chloroform/octanol and precipitated with ice-cold isopropanol. Oligonucleotide primers were selected to amplify a 388 bp product from the 5′ region of IS*900* and a 252 base-pair product from IS*902* from *Mycobacterium avium* subspecies *silvaticum* (*Mas*) (Moss et al., 1992). PCR amplifications were performed with 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min with a final extension at 72 °C for 2 min in a Techne PHC-3 thermocycler. PCR products were analysed by agarose gel electrophoresis and the DNA fragments were visualized by ethidium bromide.

Sixty *Map* isolates (52 from goats and eight from sheep) were cultured from 86 tissue samples (72.2% and 57.1% of the goat and sheep samples, respectively). Among 86 tissue samples, 76 (88.37%) were positive by ZN staining. Acid fast bacilli were constantly found, characterized by morphology and density (in clumps). A total of four isolates were cultured from 41 faecal samples (9.4% and 6.3% of the goat and sheep samples, respectively). Additionally, three isolates were obtained from faecal samples originating from the soil of goat, sheep and mixed (sheep and goats) farms (3/37, 8.1%). Two isolates were cultured from the two cattle herds, one from an intestinal sample and the other from a faecal sample. Five mycobactin dependent isolates, two from goats, one from a sheep, two from soil samples tested negative for IS900, positive for IS*902* by PCR and were therefore not *Map*.

The *Map* isolates were classified into Type C and S strains according to previous criteria using the IS*1311* PCR-REA described by Marsh et al. (1999). Forty-one of the 52 *Map* isolates from goats belonged to Type C (78.9%), seven isolates were Type S (13.5%) and the remainder ($n = 4$) were not identified (7.7%). These results are consistent with those of earlier studies in which Type C strains were more commonly isolated from goats (Djønne et al., 2005; Thoresen and Olsaker, 1994; de Juan et al., 2006). The two *Map* strains originating from cattle were also identified as Type C strains. Type C is the predominant strain type isolated from cattle and has also been isolated from wildlife species including non-ruminants (Stevenson, 2010). Regarding the *Map* isolates from sheep, five were classified as Type C (62.5%), two as Type S strains (25%) and one strain was not identified (12.5%). Three out of the six *Map* isolates from fecal samples from soil were Type C *Map* strains and the rest of them were not identified. Possible reasons for the inability to identify the strain type of eight isolates could have been the presence of PCR inhibitors, insufficient DNA or that the DNA was of poor quality.

The results of primary isolation of the *Map* Type C and S strains on the different media are shown in Table 1. None of the *Map* isolates grew on HEY without the addition of mycobactin j as expected. Only two Type C strains (4.9%) isolated from goats grew on M7H11 without mycobactin j, a phenomenon that has been observed previously on Middlebrook-based media in Type S strains from sheep (Aduriz et al., 1995). A total of 92.7% (38/41) and 75.6% (31/41) of the caprine *Map* Type C isolates grew in M7H11+Mj and HEY+Mj+P, respectively and 68.3% (28/41) grew in both media. The incorporation of these two media allowed the detection of all *Map* isolates from goats in this study. All of the Type C strains isolated from sheep grew on M7H11+Mj and 60% (3/5) and 20% (1/5) grew on HEY+Mj+P and LJ+Mj, respectively. Of the Type C strains from cattle, those derived from intestinal tissue grew on M7H11+Mj and on HEY+Mj+P, while the fecal isolate grew only on M7H11+Mj medium. The *Map* Type S strains were more difficult to isolate, slower growing and more fastidious with respect to the culture medium. None of the *Map* Type S strains grew in LJ+Mj. All of the Type S strains from both goats and sheep grew on M7H11+Mj, making this the medium of choice for isolation of *Map* Type S strains. Therefore, based on the findings of the present study it can be concluded that M7H11+Mj is the best single choice of medium or a combination of M7H11+Mj and HEY+Mj+P for the primary isolation of *Map* of both Type C and S from small ruminants. The incorporation of sodium pyruvate into the media has been reported to enhance the growth of most *Map* strains and reduce the incubation period for growth (Merkal, 1984; Merkal and Curran, 1974; Whitlock et al., 1991). In our study the addition of sodium pyruvate to HEY increased the number of colonies of Type C caprine strains, which were larger (1–2 mm) and easily observed macroscopically. The colonies in HEY without sodium pyruvate were very small (0.5–1.0 mm), fewer in number and more difficult to observe macroscopically. However, two Type S caprine isolates failed to grow in HEY+Mj+P but grew in HEY+Mj without added sodium pyruvate suggesting that sodium pyruvate may be inhibitory to the growth of some strains.

The results of the study are in agreement with Whittington et al. (2011) who reported that most Type S strains grew poorly on HEY and that a number of field isolates from small ruminants