Paratuberculosis asymptomatic cattle as spillovers of *Mycobacterium avium* subsp. *paratuberculosis*: consequences for disease control

Prevalência de *Mycobacterium avium* subsp. *paratuberculosis* nas fezes de bovinos sem sinais clínicos de doença: consequências para o controlo da paratuberculose

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Resumo: *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) é o agente causador da paratuberculose, uma das mais importantes doenças em bovinos e com um impacto econômico relevante na indústria pecuária. Esta doença estará subdiagnosticada em Portugal e a sua real prevalência em bovinos é desconhecida. O objetivo deste estudo foi avaliar a presença de *Map* em fezes de bovinos aparentemente saudáveis e sem sinais clínicos de paratuberculose. Foram analisadas amostras de fezes de vinte e quatro bovinos, colhidas no Norte de Portugal. As amostras foram rastreadas utilizando um ensaio de PCR visando a amplificação da IS900, presente no genoma de *Map*, e por isolamento desta bactéria em meios de cultura específicos. A genotipagem dos isolados foi realizada por uma abordagem MLVA. Vinte e duas das 24 amostras de fezes originaram resultados de IS900-PCR positivos para a presença de *Map*. O agente foi também isolado a partir de 12 destas amostras, sendo que todos os isolados partilharam um mesmo perfil de MLVA correspondente ao genótipo INMV2. A paratuberculose pode ser mais prevalente em Portugal do que o inicialmente esperado e animais sem sinais clínicos da doença libertam o agente nas suas fezes, perpetuando o ciclo de infecção.

Summary: *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is the causative agent of paratuberculosis, one of the most important diseases in cattle worldwide, imposing a relevant economic impact for the livestock industry. Paratuberculosis is considered an underdiagnosed disease in Portugal and the real prevalence in cattle is unknown. The aim of this study was to assess the presence of *Map* in apparently healthy and asymptomatic Portuguese cattle. Fecal samples from twenty-four bovines were analysed. The samples were screened for the presence of *Map* by an IS900-targeted PCR assay and by culture in specific media. Further characterization of the isolates was performed by an MLVA approach. From the 24 fecal samples 22 were IS900-PCR positive and from these 12 yielded positive *Map* cultures. The 12 *Map* isolates shared an identical MLVA profile, also corresponding to the INMV2 genotype. Paratuberculosis may be more widespread in Portugal than initially expected and asymptomatic animals are shedding the agent in their faeces, perpetuating the cycle of infection.

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Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is the causative agent of paratuberculosis, or Johne’s disease, a chronic granulomatous enteritis affecting a wide range of animals, especially ruminants (OIE, 2008). Other animals like rabbits, foxes, birds, domestic and wildlife animals can also be infected (Timms et al., 2011; Salem et al., 2012). Paratuberculosis is considered a widespread and very relevant disease in the livestock industry due to its considerable economic impact, triggered by a progressive and fatal weight loss of the animals and a diminution in milk production (Englund, 2002). *Map* infection has also become a highly contentious issue regarding its potential implication in the etiology of human inflammatory bowel disease, known as Crohn’s disease (Grant, 2005; Juste and Perez, 2011). This mycobacterium is an intracellular parasite that infects and multiplies inside macrophages in the animals gut. The transmission of *Map* usually occurs by the fecal-oral route during the animals first months of life, being newborn animals the most susceptible ones. The infection can be triggered by stressful conditions such as giving birth and overcrowding. The clinical signs of the disease are exacerbated between the first two to five years of life, after a long period without clinical symptoms, including the diminution of milk production, infertility, oedema and progressive and fatal weight loss with diarrhoea, initially intermittent but becoming progressively more severe. Nevertheless, not all animals exposed to the agent become infected and some can also remain asymptomatic without developing the disease (Timms et al., 2011). The diagnosis of paratuberculosis is difficult and time consuming due to the characteristics of the agent and is also influenced by the disease stage. The culture remains the gold standard method for the detection of *Map* in biological samples, requir-
ing the supply of an exogenous mycobactin source, and may take up to six months due to the extreme fastidious grow of this agent. The introduction of molecular diagnostic techniques has contributed to a more rapid, sensitive and specific detection and characterization of Map (Timms et al., 2011). The multi-copy IS900 and the single-copy F57 elements are the most important Map-specific genomic targets used in molecular assays (Behr and Collins, 2010).

Paratuberculosis is considered an underdiagnosed disease in Portugal and the real prevalence in cattle is not known. Since shedding can contribute to the silent disease in Portugal and the real prevalence in cattle is estimated and characterized in feces of asymptomatic cattle from the North of Portugal, an important dairy farming region.

**Materials and Methods**

**Fecal samples**

Twenty four fecal samples, collected from four farms A (n = 7), B (n = 10), C (n = 5) and D (n = 2) of distinct geographical regions in the North of Portugal, from bovines with five to ten years old and without clinical signs of disease, were analyzed. The samples were stored at -20°C until processed for the culture and PCR assays.

**Culture and identification of isolates**

Fecal samples were prepared for culture as described in the OIE Terrestrial Manual (OIE, 2008), with minor modifications. Briefly, 20 mL of sterile distilled water were added to 1 g of faeces and stirred at room temperature for 30 minutes. After settling for 30 minutes, 5 mL of the uppermost suspension were transferred to a new tube containing 20 mL of 0.9% hexadecylpyridinium chloride (HPC), inverted several times and allowed to stand undisturbed at room temperature for 18 hours. After this period, the sediment was carefully transferred to a new tube, washed with 10 mL of sterile distilled water and centrifuged at 900 g for 30 minutes. The pellet was resuspended in 500 µL of sterile distilled water and centrifuged at 900 g for 30 minutes. The pellet was resuspended again in 100 µL of TE buffer and incubated for 45 minutes at 95°C. After bacterial inactivation, the culture was centrifuged at 3500 rpm for 1 minute and the supernatant containing the DNA was transferred to a new microtube and used as template for PCR reactions.

**DNA extraction and IS900-targeted PCR amplification**

Total DNA from fecal samples was extracted with QIAamp® DNA Stool mini kit (Qiagen), stool pathogen detection protocol, as described by the manufacturer. The evaluation of the presence of Map in fecal samples was performed using a standard IS900-PCR assay, described by Sanderson et al. (1992). The reaction was carried out in a final volume of 25 µL containing 250 µM of each deoxynucleotide triphosphate (Promega), 2.0 mM of MgCl₂ (Promega), 0.5 µM of each primer (Primer 90, 5'-GTT CGG GGC CGT CGC TTA GGA-3'; and Primer 91, 5'-GAG GTC GAT CGC CCA GTG GA-3'), 1 U of GoTaq® DNA polymerase and 1× of the respective buffer (Promega), and 2.5 µl of the extracted DNA solution. The amplification was performed in a MJmini™Thermocycler (BioRad) with an initial step at 95°C for 5 minutes, followed by 35 cycles at 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute, ending with a step at 72°C for 3 minutes. DNA from *M. avium* subsp. *paratuberculosis* (ATCC 19698T) and ultrapure sterilized water were used as positive and negative controls of amplification, respectively. The amplified products were electrophoretically analyzed in a 1.5% (w/v) agarose gel in 1× Tris-Borate-EDTA (TBE) buffer stained with GelRed®. Gel electrophoresis images were acquired with an UV Transilluminator UVP M-20.

**Characterization of Map isolates by Multilocus VNTR Analysis (MLVA)**

A set of ten VNTR loci was selected for the molecular discrimination of Map isolates: VNTR-3, VNTR-7, VNTR-10, VNTR-47, MIRU-2, MIRU-3 (alias X3), VNTR-25, VNTR-32, VNTR-259 and VNTR-292 (Thibault et al., 2007; Castellanos et al., 2010). Briefly, PCR reactions were carried out in a total volume of 25 µL containing 1× buffer (Promega), 200 µM of each deoxynucleotide triphosphate (Promega), 2.0 mM of MgCl₂ (Promega), 1 µM of each forward and reverse primers (for each loci separately; see Table 1), 2.5 U of GoTaq® DNA polymerase and 1 µl of extracted DNA solution. Amplification was performed in a MJmini™Thermocycler (BioRad) with an initial step at 95°C for 10 minutes, followed by 38 cycles at 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 1 minute, ending with a step at 72°C for 7 minutes. DNA from *M. avium* subsp. *paratuberculosis* (ATCC 19698T) was used as positive control and amplicon size reference. PCR products were analyzed in a 2.5% (w/v) agarose gel in 1× Tris-Borate-EDTA (TBE) buffer, stained with GelRed®, at 45 V for 2 h, using a 100 bp molecular ladder to estimate the size of the PCR amplicons. Allele
calling tables available by Castellanos et al. (2010) and the INMV database (http://mac-inmv.tours.inra.fr) were used to assign the hypothetical size of each PCR product, for loci VNTR-3, VNTR-7, VNTR-10, VNTR-47, MIRU-2, MIRU-3, VNTR-25, VNTR-32, VNTR-259 and VNTR-292, to their correspondent tandem repeat copy number.

Table 1 - Sequences of the primers used for the MLVA characterization of the isolates

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer forward (5’-3’)</th>
<th>Primer reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VNTR-3</td>
<td>CAT ATC TGG CAT GGC TCC AG</td>
<td>ATC GTG TTG ACC CCA AAG AAA T</td>
</tr>
<tr>
<td>VNTR-7</td>
<td>GAC AAC GAA ACC TAC CTC GTC</td>
<td>GTG AGC TGG CCG CCT AAC</td>
</tr>
<tr>
<td>VNTR-10</td>
<td>GAC GAG CAG CTG TCC GAG</td>
<td>GAG AGC GTG GCC ATC GAC</td>
</tr>
<tr>
<td>VNTR-47</td>
<td>CTT TGC GAT TGC GTC GTA GC</td>
<td>GGT GAT GTG CGT GGT CAT</td>
</tr>
<tr>
<td>MIRU-2</td>
<td>GAA CGA AGA TCC TGG GAC TG</td>
<td>CGA CGA CGA ACA CCT CAA C</td>
</tr>
<tr>
<td>MIRU-3 (alias X3)</td>
<td>AAC GAG AGG AAG AAC TAA GCC G</td>
<td>TTA CGG AGC AGG AAG GCC AGC GGG</td>
</tr>
<tr>
<td>VNTR-25</td>
<td>GTC AAG GGA TCG GCG AGG</td>
<td>TGG ACT TGA GCA CGG TCA T</td>
</tr>
<tr>
<td>VNTR-32</td>
<td>CCA CAG GGT TTT TGG TGA AG</td>
<td>GGA AAT CCA ACA GCA AGG AC</td>
</tr>
<tr>
<td>VNTR-259</td>
<td>GGG TGT GGA GCT ACG ACT TC</td>
<td>GAG CTG CTT GAC CAG GTG AT</td>
</tr>
<tr>
<td>VNTR-292</td>
<td>CTT GAG CAG CTC GTA AAG GCT</td>
<td>GCT GTA TGA GGA AGT CTA TTC ATG G</td>
</tr>
</tbody>
</table>

Results

From the twenty four fecal samples from asymptomatic Portuguese bovines analysed, twenty two were IS900-PCR positive (7 from farm A, 9 from farm B, 4 from farm C and 2 from farm D) (Table 2), exhibiting a Map-specific 400 bp amplified product. Twelve cultures were obtained after 60 days incubation, from the 24 inoculated samples (1 from farm A, 7 from farm B and 4 from farm C) (Table 2). All 12 culture positive samples yielded IS900-PCR positive results. The 12 isolates were confirmed to be acid-fast bacilli by auramine-rhodamine staining and were identified as Map by PCR, showing the specific 400 bp and 424 bp products for the IS900 and F57 PCR-amplified genomic targets, respectively. The isolates were further characterized by a ten loci MLVA analysis approach, showing that all shared the same profile (Table 2), also identical to the control Map strain ATCC 19698T. When considering only the eight VNTR loci set analysed by Thibault et al. (2007) (VNTR 292, MIRU 3, VNTR 25, VNTR 47, VNTR 3, VNTR 7, VNTR 10 and VNTR 32), the Map isolates show to belong to the INMV2 type (Table 2), similarly to the control strain ATCC 19698T. When using the six loci set proposed by Castellanos et al. (2010) (MIRU 2, MIRU 3, VNTR 25, VNTR 32, VNTR 292 and VNTR 259), the respective profile 323832 was not found among their 70 Map Spanish isolates.

Table 2 - Results obtained for the culture-based and molecular detection of Map in bovine faeces samples

<table>
<thead>
<tr>
<th>Origin</th>
<th>Number of samples</th>
<th>Number of IS900-PCR positive samples</th>
<th>Number of culture positive samples</th>
<th>MLVA profile*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A</td>
<td>7</td>
<td>7</td>
<td>1</td>
<td>323322832</td>
</tr>
<tr>
<td>Farm B</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>323322832</td>
</tr>
<tr>
<td>Farm C</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>323322832</td>
</tr>
<tr>
<td>Farm D</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>323322832</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>22</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

*Number of tandem repeat copies in the order VNTR292 - MIRU3 - VNTR25 - VNTR47 - VNTR3 - VNTR7 - VNTR10 - VNTR32 - MIRU2 - VNTR259; The profile corresponding to the first eight loci match the Map INMV2 type, according to Thibault et al. (2007).

Discussion

The worldwide herd prevalence of paratuberculosis is estimated to be 7 to 40%, based on serological monitoring tests (Timms et al., 2011). However, an accurate estimation of the prevalence of Map in cattle is difficult since most infected animals are asymptomatic, the diagnosis in the early stages of disease is difficult and the animals with clinical signs of decreased milk production can be slaughtered before the final diagnosis (Behr and Collins 2010). To date, only a few studies tried to assess the prevalence of paratuberculosis in Portugal, namely in the cattle population, where this disease probably runs under-diagnosed. Ferreira et al., (2002) reported 4.8-7.0% of bovines serologically positive to Map in the “Alentejo” region, South of Portugal, with 13-25% of herds positive for the disease. In another more recent study, anti-Map antibodies were detected in 2.3% of milk samples collected from 5294 milking animals with clinical signs of disease in animals, noteworthy in sheep (Amado et al., 1994). Map was also previously isolated in Portugal from the mesenteric lymph nodes.
of wild boars (Sus scrofa) with granulomatous lymphadenitis (Matos et al., 2013a), from kidney samples of wild red deer (Cervus elaphus) (Matos et al., 2013b) and from Eurasian otters (Lutra lutra) (Matos et al., 2013c). The agent was also detected by PCR-based methods in tissues of domestic pigs (Miranda et al., 2011). There are, presently, in Portugal no reliable data about shedding of Map in faeces.

Detection of Map in faeces by IS900-PCR is an efficient and rapid method when compared with the conventional culture-based assays, which take up 8 to 12 weeks to obtain a result. However, bacteriological culture is the gold standard methodology for the diagnosis of paratuberculosis and the isolation of the agent is required if further studies are intended. In this study twenty four faecal samples from asymptomatic Portuguese bovines from the North of Portugal were analysed. Twenty two samples were found to be IS900-PCR positive (91.7%) while only 12 (50%). Our preliminary data suggests that Map infection in cattle may be more prevalent in Portuguese cattle than initially expected, based in the previous surveys referred above, employing mainly serological assays. Even with the absence of clinical signs, our data points out that the animals are shedding the agent in faeces, perpetuating the cycle of infection.

The analysis of the polymorphisms in MIRU/VNTR loci proved to be very useful for the discrimination of Map isolates (Thibault et al., 2007; Stevenson et al., 2009; Castellanos et al., 2012) and the correspondent alleles were found to be very stable after several in vitro subcultivation, on different media, and after in vivo passage (Kasnitz et al., 2013). However, several distinct sets of MIRU/VNTR loci have been used to characterize Map isolates (Thibault et al., 2007; Castellanos et al., 2010; Castellanos et al., 2012), which difficult the comparison between different studies. In this work we used the MIRU/VNTR loci set proposed by Thibault et al. (2007) and Castellanos et al. (2010), which were used before to genotype major collections of Map isolates from different countries, allowing the comparison of the correspondent allelic profiles with the profiles of the Portuguese isolates. The twelve Portuguese Map isolates shared the same MLVA allelic profile, suggesting that they belong to the same clonal lineage. This profile corresponds to the Map INMV2 type, according to Thibault et al. (2007). The INMV2 type seems to be, together with INMV1, most abundant in Europe (Thibault et al., 2007). For example, 35% and 61% of the French and Dutch bovine Map isolates, respectively, analysed by Thibault et al. (2007) represented the INMV2 type. This type is widely disseminated, occurring in many other countries such as Germany, Czech Republic, Finland, Scotland, Greece and Spain (Fritsch et al., 2012). Our preliminary data suggests that INMV2 Map strains are also most abundant in Portugal and potentially circulates in the environment, by the shedding of the agent in the faeces of infected cattle.

This is the first study reporting the isolation and identification of Map from Portuguese asymptomatic cattle, along with its molecular characterization with a MLVA approach. Identification of shedding animals is extremely important for the prevention of the spread of Map infection. We highlight the need of a systematic evaluation for the presence of shedding bovines in subclinical infected dairy cattle herds and this can be accomplished by the use of PCR-based assays that can be applied at the herd or individual level, regardless of animal age or production stage. Testing of additional and different cattle samples - for instance milk - from different geographical regions, are currently underway in order to have a clearer picture of the real situation of paratuberculosis in cattle in Portugal.

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