Presence of Mycobacterium avium subs. paratuberculosis DNA in milk used to feed calves in Portugal

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Supplementary file

Questionnaires

A questionnaire was performed on 37 Portuguese dairy farms in 3 geographic locations It included questions related to possible risk factors for transmission of *Map* in dairy cattle including animal replacement policy, type of calving area, waste milk use and size of the lactating herd (Table 1). Other questions were included in the questionnaire to evaluate awareness of paratuberculosis by the farmers.

Milk samples

Waste milk fed to calves was sampled on three different days on each farm, separated at least one week between collections, to increase the likelihood that the source of the milk included different animals. Whenever waste milk was not fed to calves a single bulk tank milk sample was collected. Four milk samples had insufficient amount for processing and therefore 99 milk samples, comprising 95 waste milk samples and 4 bulk tank milk samples were analysed by culture and nested *IS900* real time PCR.

Preparation and culture of samples for Map isolation

Milk samples were prepared for culture according to Dimareli-Malli (2010), with minor modifications. Briefly, 20 mL of milk were centrifuged at 3100×g for 15 minutes at room temperature, the supernatant was discarded and the pellet was resuspended in 10 mL of 0·75% hexadecylpyridinium chloride (HPC) and incubated at room temperature for 5 hours. The mixture was centrifuged at 3100×g for 15 minutes at room temperature, the pellet was resuspended in 2 mL of sterile distilled water and 200 µL were inoculated on Herrold's egg yolk medium slants with and without mycobactin J. The incubation was performed at 37 ºC for up to 6 months.

Treatment of samples for genomic DNA extraction

All the milk samples were submitted to a first treatment procedure, as described by Gao and colleagues (2007) with minor modifications. Briefly, 10 mL of milk were incubated at 95ºC during 10 minutes and cooled on ice for 10 minutes. The samples were centrifuged at 3100×g for 30 minutes at 8ºC and the whey was carefully removed. The pellet and the fat layer were resuspended in 15 mL of 0·75% HPC and incubated at room temperature for 30 minutes under agitation. After the incubation step, the samples were centrifuged at 2000×g for 15 minutes, the fat layer and liquid phase were decanted and the pellet was kept for DNA extraction.

DNA extraction

The DNA extraction was performed with the commercial extraction kit - Invisorb® Spin Tissue Mini Kit - protocol 1 (Stratec) with mechanical disruption. The obtained pellet was resuspended in the lysis buffer provided in the kit, and added to a tube containing zirconium beads. The mechanical disruption was performed twice in a FastPrep FP120 Bio101 bead shaker (Savant Instruments Inc., Holbrook, NY) at 6·5 $msec^{-1}$ for 45

seconds. Disrupted samples were cooled on ice for 15 minutes, 40 µL of proteinase K were added, and the resulting mixture was incubated overnight at 52ºC. The remaining procedure was performed according to the manufacturer's instructions. The genomic DNA was eluted with 100 µL of elution buffer and stored at - 20ºC until being tested.

Amplification assays

A nested PCR approach was used, combining a first amplification step by standard PCR using the extracted DNA as template followed by a second amplification step by real time PCR where the amplified product was used as template. The amplification system was previously optimized for fecal samples (C. Leão, unpublished data) targeting the *IS900* multi-copy region. For the first conventional PCR step external primers EXT-*IS900*-FW and EXT-*IS900*-RV were used, followed by real time PCR, using internal primers *IS900*QF/*IS900*QR and *IS900*QP targeted probe, described by Sidoti and colleagues (2011).

The limit of detection (LOD) of the assay for milk samples was determined with spiked milk samples. Nine tubes with 10 ml each of bovine milk, testing negative for paratuberculosis by standard procedures, were spiked with ten-fold dilutions of a *Map* ATCC19698^{T} suspension in a range of 10^{7} to 0 cells per ml of milk. Tubes were mixed well and stored at -20ºC until being tested. The genomic DNA of the milk spiked samples was extracted and tested with the same procedures as the milk samples. Each sample was tested in triplicate and the LOD corresponded to the highest dilution at which the assay could detect a positive result in at least one of the replicates.

β-actin gene-targeted probe/primers (Costa et al., 2013) were used as internal control in the real time PCR to discard the presence of amplification inhibitors.

Statistical analyses

A multivariable logistic regression was performed using SPSS® version 22, to evaluate if the presence of potential risk factors for the occurrence of paratuberculosis at herd level, had a relation with the detection of *Map* through PCR in milk.