Detection of Mycobacterium avium subsp. paratuberculosis in a sheep flock in Tuscany

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Abstract

Paratuberculosis is an infectious disease which affects ruminants. In this work, a sheep flock in Garfagnana district (Tuscany, Italy) was examined by agar gel immunediffusion (AGID) tests, culture, and PCR from feces, milk, tissue samples, and cheeses. At the first AGID test, 7/280 (2.5 %) animals were positive. From these animals, feces and milk samples were collected: 4/7 feces (57.14 %) and 2/7 milk samples (28.57 %) were positive to culture and 7/7 (100 %) feces samples and 1/7 milk samples (14.28 %) were positive to PCR; 2/4 (50 %) cheeses ripened for 14 days and 1/3 (33.33 %) cheese ripened for 20 days were positive to PCR, from which no viable microorganisms were isolated. Then, the AGID-positive animals were slaughtered and tissue samples were taken from one sheep with PCR-positive feces and milk: the liver, intestine, mesenteric lymph nodes, but not the spleen and mammary lymph nodes were positive to culture; all these samples were instead PCR-positive. After 1 year, a second AGID survey was performed on the remaining animals: 6/244 (2.45 %) subjects were positive to this test. Data obtained revealed the presence of paratuberculosis in a sheep population in Garfagnana.

Keywords

Sheeps . Mycobacterium avium subsp. paratuberculosis . Garfagnana district . Culture . Polymerase chain reaction . Genotyping

Introduction

Paratuberculosis or Johne's disease is a chronic infectious granulomatous enteritis caused by Mycobacterium avium subspecies paratuberculosis (MAP), which affects ruminants, especially dairy cattle and a variety of domestic species (Beard et al. 2001; Manning and Collins 2001). Several studies have associated M. avium subsp. paratuberculosis with Crohn's disease, and recently, MAP has also been proposed as a risk factor in genetically susceptible individuals for the development of autoimmune diseases like type-1 diabetes, sarcoidosis, multiple sclerosis, and Hashimoto's thyroiditis (Sechi and Dow 2015). This has focused a spotlight on the presence of MAP in animal-derived foods, particularly in ewe's milk and cheese samples. Not only has MAP been detected by PCR but also it has been isolated (Ikonomopoulos et al. 2005; Dimareli-Malli 2008; Williams and Withers 2010). To our knowledge, only two studies (Nebbia et al. 2006; Attili et al. 2011) have investigated the presence of paratuberculosis in Italian sheep population and none of them was carried out in Tuscany. The aim of this study was not only to evaluate the presence of paratuberculosis in a sheep flock in Garfagnana district (Tuscany, Italy) but also to carry out two AGID tests, with an interval of 1 year after slaughter, to study the spread of this disease in the flock.

Materials and methods

Population study A sheep flock consisting of 280 lactating animals, located in Garfagnana district, in which there were symptomatic subjects with loss of weight and decrease in milk production, was studied. Experimental design Firstly, agar gel immune-diffusion (AGID) test was carried out on the whole flock and then individual milk and feces were taken from animals serologically positive to evaluate the presence of MAP in milk and feces samples by PCR and culture. Then, serologically positive as well as fecal and milk PCRpositive animals were slaughtered, and at necropsy, tissue samples were collected and submitted for PCR and culture. Seven cheeses made of unpasteurized bulk tank milk were collected and analyzed by PCR and culture from serologically positive sheep. After 1 year, a second AGID assay was performed. Serological

testing After cleansing with alcohol, 5 ml of blood per sheep (n=280) were collected from the jugular vein into Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ, USA) without EDTA; the sera were separated after clotting by centrifugation at 200g for 10 min and frozen at −20 °C. All the sera collected were analyzed for specific serology by AGID: one central and six surrounding wells were made in an agar plate with a punch (OIE 2014). Thirty-five microliters of paratuberculosis protoplasmic antigen (Allied Monitor, The Paratuberculosis Laboratory, Fayette, MO) were placed in the central well. One positive control serum (50 µl) was placed in one of the six outer wells, and the testing sera (50 µl) were placed in the other five outer wells. The plate was placed in a sealed humid chamber. Test samples were incubated for 48 h with two readings taken at 24 and 48 h. After 1 year, a second serological survey was performed on 244 animals. Milk, feces, tissue, and cheese sample collection and processing Before collecting milk, the teats were cleaned with alcohol to avoid sample contamination from the skin and the first milk jets were discarded. Each milk sample (70–80 ml) was collected in two sterile 50-ml centrifuge tubes by hand milking. Fifty milliliters of milk was collected with a sterile pipette, transferred to a centrifuge tube, and centrifuged for 15 min at 2500×g. The resulting pellet was decontaminated in 10 ml of 0.75 % (w/v) hexadecylpyridinium chloride (HPC; SigmaAldrich, Milan, Italy). After incubation at room temperature (21 °C) for 5 h and a further centrifugation, the pellet was resuspended in 1 ml of phosphate-buffered saline (PBSTween) (Grant et al. 2002). Individual feces samples were collected directly from the rectal ampulla from each AGID-positive animal, placed into sterile containers, and subjected to culture and DNA extraction. Culture was performed using a sedimentation protocol (Taddei et al. 2004): 10 g of fresh feces were suspended in 100 ml of sterile solution of 0.75 % (w/v) hexadecylpyridinium chloride (Sigma-Aldrich); the suspensions were shaken by horizontal shaking for 30 min at room temperature; then, each suspension was left to settle for 5 min, and 35 ml of the upper portion of the supernatant was transferred to a new 50-ml sterile tube; then, the samples were decontaminated overnight at room temperature. At necropsy, up to a 2.5-cm section of the proximal duodenum, distal ileum, ileocecal valve, mesenteric lymph node, spleen, liver, and mammary lymph node were aseptically collected from one sheep positive to AGID and to PCR carried out on feces and milk. For the intestine, a pool of sections of the ileocecal valve, proximal duodenum, and distal ileum were analyzed. Briefly, 5 g of tissues were added to a sterile stomacher bag (Sto-circular-bag, PBI International, Milan, Italy) with 2 ml sterile normal saline and homogenized for 30 s with a stomacher blender (Stomacher 400 circulator, PBI International). After adding 25 ml of 0.75 % HPC, the homogenates were left at room temperature for 48 to 72 h. For each sample, the sediment from the base of the tube was collected (Whittington et al. 1999). Cheeses manufactured according to traditional procedures with unpasteurized bulk tank milk and produced without commercial starter culture were analyzed: four cheeses ripened for 14 days and three ripened for 20 days. Five grams of cheese were aseptically transferred into a sterile stomacher bag containing 45 ml of 0.75 % HPC. The mixture was homogenized in a stomacher blender and incubated for 5 h in a dark room, at room temperature (Donaghy et al. 2004). MAP culture and colony characterization from milk, feces, tissues, and cheeses Each sample (200 µl) was inoculated on three slopes, one containing Herrold's egg yolk medium supplemented with Mycobactin J (ID vet, Grabels, France), one containing HEYM without Mycobactin J and one with Middlebrook 7H11 medium with mycobactin (Mm). Tubes were incubated at 37 °C for up to 8 months and examined weekly for bacterial growth. The primary cultures, colonies resembling M. avium subsp. paratuberculosis were stained with Ziehl-Neelsen (ZN) method for the presence of acid-fast bacilli. DNA extraction from colonies and PCR DNA was extracted from primary colonies. Briefly, a small loop of bacterial biomass was dissolved in 50 µl of distilled water in a tube and heated at 100 °C for 20 min. For each sample, DNA extracted was added to the amplification mixture; PCR was performed with EconoTaq DNA Polymerase Trop Anim Health Prod Author's personal copy with Mg++ master mix kit (Lucigen, Lucigen Corporation 2905 Parmenter Street Middleton, WI 53562 USA) with primers TJ1 (5'-GCTGATCGCCTTGCTCAT-3') and TJ2 (5'-CGGGAGTTTGGTAGCCAGTA-3') (Bull et al. 2003) with a final concentration of 0.6 µM; these primers amplify a region of IS900 sequence. The cycling conditions were 94 °C for 5 min and then 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 3 min, followed by a final extension at 72 °C for 7 min. A volume of 10 µl of PCR product was run on a 1.5 %

agarose gel in Tris-acetate-EDTA (TAE) running buffer; ATCC 19698 was included as positive control. The expected size of the amplification product was 356 bp. To genotype the isolates, the DNA extracted from each sample was also used in a DMC-PCR assay (Collins et al. 2002) using EconoTaq DNA Polymerase with Mg++ master mix kit (Lucigen, Lucigen Corporation 2905 Parmenter Street Middleton, WI 53562 USA); the products were analyzed by electrophoresis on a 1.5 % agarose gel. Direct DNA extraction from milk, feces, tissues, and cheese samples and PCR A commercially available DNA extraction kit (Adiapure, Adiagene, bioMérieux Company, France) was used to extract MAP DNA from 10 ml of individual milk. For the cheese samples, 5 g were aseptically transferred into a sterile stomacher bag containing 45 ml of cheese diluent (2 % w/v sodium citrate). The mixtures were stomached (Donaghy et al. 2011). Ten milliliters of each cheese resultant suspension was extracted with the same kit used for milk as described above, using the manufacturer's instructions. The resultant DNA extract was immediately stored at −20 °C. DNA was extracted from feces and from tissue samples with commercial kits QIAamp DNA Stool Mini Kit and DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer instruction. After DNA extraction, 5 μl of template was used for PCR (Bull et al. 2003). The quality of the DNA extract was evaluated with regard to purity and integrity by submerged gel electrophoresis. The PCR reaction was carried out as previously described. A volume of 10 μl of PCR product was run on 1.5 % agarose gel by electrophoresis in TAE running buffer; the expected length of the amplification product was 356 bp.

Results

From the serological studies, 7/280 (2.5 %) animals were positive to the first AGID test and 6/244 (2.46 %) to the second. MAP was detected by culture methods in 4/7 (57.14 %) fecal samples and by PCR in 7/7 (100 %) fecal samples. The isolated strains grew on M7H11 with Mycobactin J, and two of them grew also in Herrold's egg yolk medium supplemented with Mycobactin J. These isolates were confirmed as MAP by IS900 PCR as described above. None of the MAP isolates grew on Herrold without Mycobactin J. MAP was detected in the individual milk samples by culture in 2/7 (28.57 %) samples and by PCR in 1/7 (14.28 %) samples. The two isolates grew on M7H11 with Mycobactin J, and only one of these grew also in Herrold's egg yolk medium supplemented with Mycobactin J; the isolation was confirmed by IS900 PCR. None of the MAP isolates grew on Herrold without Mycobactin J. The cultures overgrown by contaminants were 5/21 (23.8 %); these contaminants on slopes may have masked the presence of MAP colonies or prevented the growth of MAP. MAP was not isolated by culture methods from cheese samples; however, it was detected by PCR in 2/4 (50 %) cheeses ripened for 14 days and in 1/3 (33.33 %) cheese ripened for 20 days. All tissue samples were MAP PCR-positive. However, of these, only the liver, intestine, and mesenteric lymph node were positive by culture. The spleen and mammary lymph node tissues were culture-negative. DMC-PCR (Collins et al. 2002) carried out on DNA extracted from colonies cultured from four fecal, two milk, and three tissue samples revealed that a S (sheep) strain was present in this flock.

Discussion

In our studies, an AGID test was initially applied as a diagnostic screening tool to establish the infection status of the flock. This serological method was used because it was reported that in small ruminants, the AGID test has a slightly higher specificity than ELISA (Gwozdz et al. 2000; Hope et al. 2000; Sergeant et al. 2003; Whittington et al. 2003; Gumber et al. 2006; Robbe-Austerman et al. 2006). The sensitivity of AGID and ELISA is a controversial subject: on one hand, Gwozdz et al. (2000) and Hope et al. (2000) highlighted that AGID has higher sensitivity than ELISA for ewes; on the other hand, Sergeant et al. (2003), Gumber et al. (2006), and Robbe-Austerman et al. (2006) highlighted that ELISA has a higher sensitivity than AGID test. Our research has been developed within an ASL's (public and local authority which deals with animal health) monitoring plan of Lucca district aimed to ensure the safety of pastures. The main objective was to establish, with a screening test, which animals were true negatives; for this reason, we have chosen AGID test which has a higher specificity than ELISA. Data obtained highlighted the presence of MAP in the flock which showed a low infection prevalence (2.5 %). Despite the Trop Anim Health Prod Author's personal

copy fact that the animals, positive in the first AGID survey, had been slaughtered, the infection continued to spread in the flock, attaining a level of prevalence of 2.46 % after 1 year. As the laboratory diagnosis of this disease in subclinical animals is extremely difficult (Stabel 1998; OIE 2014), especially with serological tests, control of Johne's disease by surveillance and slaughter, based solely on serological tests, is not recommended to control this disease. To our knowledge, only two studies (Nebbia et al. 2006; Attili et al. 2011) have investigated the presence of paratuberculosis in sheep flocks in Italy and none of them have investigated the presence of this disease in ewes in Tuscany, where only one study has detected MAP in a goat flock (Cerri et al. 2002). Moreover, there are few studies about the survival of MAP in cheeses from small ruminants (Ikonomopoulos et al. 2005; Botsaris et al. 2010; Williams and Withers 2010), and none was carried out in Italy. On one hand, our study confirmed, in accordance with Dimareli-Malli (2008), that sheep milk can be a route of human exposure to MAP, which is a potential zoonotic agent for the development of Crohn's disease and autoimmune diseases like type-1 diabetes, sarcoidosis, multiple sclerosis, and Hashimoto's thyroiditis (Sechi and Dow 2015). On the other hand, MAP was not isolated from cheese samples ripened for 14 days or from cheeses ripened for 20 days; however, the practical difficulties in culturing MAP may have caused an underestimation of its presence, especially in food samples (Donaghy et al. 2008). Moreover, because the survival of MAP in cheeses could be influenced by cheese ripening, it could be advisable to carry out more studies on presence and survival of MAP with respect to different ripening times, as has already been done for bovine dairy products (Donaghy et al. 2004). Regarding cheese samples, the culture-negative and PCRpositive results recorded could be due to the incubation of cheese samples with hexadecyl-pyridinium chloride carried out for 5 h, which is the time usually used (Ikonomopoulos et al. 2005; Williams and Withers 2010); this decontamination process could be responsible for the death of MAP in these samples (Gao et al. 2005). Moreover, not only in food samples but also in tissues and fecal samples, the presence of MAP could have been underestimated, even though in this study, we used not only Herrold's egg yolk medium but also M7H11, which is the medium specifically recommended for MAP isolation from small ruminant specimens, and the incubation period was increased up to 8 months. In fact, it has been demonstrated that sheep strains are usually more difficult to cultivate (De Juan et al. 2006). Furthermore, contaminants on slopes may have masked the presence of MAP colonies or prevented the growth of MAP. We have demonstrated that in this flock, a S (sheep) type strain is present, according to other molecular studies, which have highlighted that cattle are generally infected with C (cattle) strains, which are also commonly found in wildlife, while ewes are generally infected with S strains and goats, by both. This finding is important also because a recent study which has evaluated the pathogenicity of bovine and ovine strains in experimentally infected lambs highlighted that lesions induced by ovine strains were more severe than those caused by bovine strains (Verna et al. 2007). In our opinion, more research must be conducted to determine the presence of MAP in Italian sheep flocks and on ewe milk and cheeses. Statement of Animal Rights The manuscript does not contain clinical studies or patient data. Conflict of Interest The authors declare that they have no competing interests.

References

Attili, A.R., Ngu Ngwa, V., Preziuso, S., Pacifici, L., Domesi, A., Cuteri, V., 2011. Ovine paratuberculosis: A seroprevalence study in dairy flocks reared in the Marche Region, Italy, Veterinary Medicine International, doi: 10.4061/2011/782875. Beard, P.M., Daniels, M.J., Henderson, D., Pirie, A., Rudge, K., Buxton, D., Rhind, S., Greig, A., Hutchings, M.R., McKendrick, I., Stevenson, K., Sharp, J.M., 2001. Paratuberculosis infection of nonruminant wildlife in Scotland, Journal of Clinical Microbiology, 39, 1517–1521. Botsaris, G., Slana, I., Liapi, M., Dodd, C., Economides, C., Rees, C., Pavlik, I., 2010. Rapid detection methods for viable Mycobacterium avium subspecies paratuberculosis in milk and cheese, International Journal of Food Microbiology, 141, 87–90. Bull, T.J., McMinn, E.J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild, P., Rhodes, G., Pickup, R., Hermon-Taylor, J., 2003. Detection and verification of Mycobacterium avium subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease, Journal of Clinical Microbiology, 41, 2915–2923. Cerri, D., Cantile, C., Ebani, V.V., Montagnese, M.,

Voltini, B., Arispici, M., 2002. Diagnosis of paratuberculosis in naturally infected goats, New Microbiologica, 25, 131–137. Collins, D.M., De Zoete, M., Cavaignac, S.M., 2002. Mycobacterium avium subsp. paratuberculosis strains from cattle and sheep can be distinguished by a PCR test based on a novel DNA sequence difference, Journal of Clinical Microbiology, 40, 4760–4762. De Juan, L., Álvarez, J., Romero, B., Bezos, J., Castellanos, E., Aranaz, A., Mateos, A., Domínguez, L., 2006. Comparison of four different culture media for isolation and growth of type II and type I/III Mycobacterium avium subsp. paratuberculosis strains isolated from cattle and goats, Applied and Environmental Microbiology, 72, 5927–5932. Dimareli-Malli, Z., 2008. Detection of Mycobacterium avium subsp. paratuberculosis in milk from clinically affected sheep and goats, International Journal of Applied Research in Veterinary Medicine, 8, 44–50. Donaghy, J.A., Totton, N.L., Rowe, M.T., 2004. Persistence of Mycobacterium paratuberculosis during manufacture and ripening Trop Anim Health Prod Author's personal copy of cheddar cheese, Applied and Environmental Microbiology, 70, 4899–4905. Donaghy, J.A., Rowe, M.T., Rademaker, J.L.W., Hammer, P., Herman, L., De Jonghe, V., Blanchard, B., Duhem, K., Vindel, E., 2008. An inter-laboratory ring trial for the detection and isolation of Mycobacterium avium subsp. paratuberculosis from raw milk artificially contaminated with naturally infected faeces, Food Microbiology, 25, 128–135. Donaghy, J.A., Johnston, J., Rowe, M.T., 2011. Detection of Mycobacterium avium ssp. paratuberculosisin cheese, milk powder and milk using IS900 and f57-based qPCR assays, Journal of Applied Microbiology, 110, 479–489. Gao, A., Odumeru, J., Raymond, M., Mutharia, L., 2005. Development of improved method for isolation of Mycobacterium avium subsp. paratuberculosis from bulk tank milk: Effect of age of milk, centrifugation, and decontamination, Canadian Journal of Veterinary Research, 69, 81–87. Grant, I.R., Ball, H.J., Rowe, M.T., 2002. Incidence of Mycobacterium paratuberculosis in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom, Applied and Environmental Microbiology, 68, 2428–2435. Gumber, S., Eamens, G., Whittington, R.J., 2006. Evaluation of a Pourquier ELISA kit in relation to agar gel immunodiffusion (AGID) test for assessment of the humoral immune response in sheep and goats with and without Mycobacterium paratuberculosis infection, Veterinary Microbiology, vol. 115, no. 1–3, pp. 91–101. Gwozdz, J.M., Thompson, K.G., Murray, A., Reichel, M.P., Manktelow, B.W., West, D.M., 2000. Comparison of three serological tests and an interferon-γ assay for the diagnosis of paratuberculosis in experimentally infected sheep, Australian Veterinary Journal, vol. 78, no. 11, pp. 779–783. Hope, A.F., Kluver, P.F., Jones, S.L., Condron, R.J., 2000. Sensitivity and specificity of two serological tests for the detection of ovine paratuberculosis, Australian Veterinary Journal, vol. 78, no. 12, pp. 850–856. Ikonomopoulos, J., Pavlik, I., Bartos, M., Svastova, P., Ayele, W.Y., Roubal, P., Lukas, J., Cook, N., Gazouli, M., 2005. Detection of Mycobacterium avium subsp. paratuberculosis in retail cheeses from Greece and the Czech Republic, Applied and Environmental Microbiology, 7, 8934–8936. Manning, E.J.B., Collins, M.T., 2001. Mycobacterium avium subsp. paratuberculosis: pathogen, pathogenesis and diagnosis, OIE Revue Scientifique Et Technique, 20, 133–150. Nebbia, P., Robino, P., Zoppi, S., De Meneghi, D., 2006. Detection and excretion pattern of Mycobacterium avium subspecies paratuberculosis in milk of asymptomatic sheep and goats by nested-PCR, Small Ruminant Research, 66, 116–120. OIE, 2014. Paratuberculosis (Johne's disease), OIE Terrestrial Manual 2014, Chapter 2.1.11. Robbe-Austerman, S., Gardner, I.A., Thomsen, B.V., Morrical, D.G., Martin, B.M., Palmer, M.V., Thoen, C.O., Ewing, C, 2006. Sensitivity and specificity of the agar-gel-immunodiffusion test, ELISA and the skin test for detection of paratuberculosis in United States Midwest sheep populations, Veterinary Research, vol. 37, no. 4, pp. 553–564. Sechi, L.A., Dow, C.T., 2015. Mycobacterium avium ss. paratuberculosis Zoonosis - The Hundred Year War - Beyond Crohn's Disease, Frontiers in Immunology., 6. Sergeant, E.S.G., Marshall, D.J., Eamens, G.J., Kearns, C., Whittington, R.J., 2003. Evaluation of an absorbed ELISA and an agar-gel immuno-diffusion test for ovine paratuberculosis in sheep in Australia, Preventive Veterinary Medicine, vol. 61, no. 4, pp. 235– 248. Stabel J.R., 1998. Johne's Disease: A Hidden Threat, Journal of Dairy Science, 81, 283–288. Taddei, S., Robbi, C., Cesena, C., Rossi, I., Schiano, E., Arrigoni, N., Vicenzoni, G., Cavirani, S., 2004. Detection of Mycobacterium avium subsp. paratuberculosis in bovine fecal samples: Comparison of three polymerase chain reaction-based diagnostic tests with a conventional culture method, Journal of Veterinary Diagnostic Investigation, 16, 503–508.

Verna, A.E., Garcia-Pariente, C., Muñoz, M., Moreno, O., GarcíaMarin, J.F., Romano, M.I., Paolicchi, F., Pérez, V., 2007. Variation in the immuno-pathological responses of lambs after experimental infection with different strains of Mycobacterium avium subsp. Paratuberculosis, Zoonoses and Public Health, 54, 243–252. Whittington, R.J., Marsh, I., Mcallister, S., Turner, M.J., Marshall, D.J., Fraser, C.A., 1999. Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of Mycobacterium avium subsp. paratuberculosis from sheep, Journal of Clinical Microbiology, 37, 1077–1083. Whittington, R.J., Eamens, G.J., Cousins, D.V., 2003. Specificity of absorbed ELISA and agar gel immunodiffusion tests for paratuberculosis in goats with observations about use of these tests in infected goats, Australian Veterinary Journal, vol. 81, no. 1–2, pp. 71–75. Williams, A.G., Withers, S.E., 2010. Microbiological characterisation of artisanal farmhouse cheeses manufactured in Scotland, International Journal of Dairy Technology, 63, 356–369.