Short communication: Occurrence and differentiation of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) strains from milk of cows from herd with low prevalence of MAP

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ABSTRACT

Mycobacterium avium ssp. *paratuberculosis* (MAP) is an important pathogen responsible for the chronic progressive granulomatous enteritis known as paratuberculosis. None of the detection methods of MAP infection based on isolation of the bacterium is 100% sensitive or specific. In this article, we describe the comparison of 2 MAP detection methods: direct isolation of genetic material and culture, in individual and pooled milk samples. The genetic types of MAP detected in the samples were also identified. The study was performed in a herd of 321 cows; apparent herd seroprevalence was 3.43%. Seven of 11 individual milk samples from seropositive cows were positive by culture (and confirmed by PCR), whereas all 11 were positive by direct PCR. Of the 62 milk pools from seronegative animals, 15 were positive by culture (and confirmed by PCR) and 13 were positive by direct PCR. Using multiplex PCR and PCR-restriction enzyme analysis (PCR-REA) methods, C (cattle) and S (sheep)-types of mycobacteria were identified. Most of the genetic material tested belonged to C-type. Detection of the MAP type occurring in an infected herd can help track the source of infection. We suggest using genetic material isolated directly from pooled milk samples for quick diagnosis, identification of MAP type, and tracking of infection, without the need to sequence the entire genome.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, type, IS900, IS1311

Short Communication

Mycobacterium avium ssp. *paratuberculosis* (MAP) belongs to the *Mycobacterium avium* complex and is responsible for paratuberculosis (Johne's disease). It

occurs endemically in all regions of the world (Nielsen and Toft, 2009; Fecteau, 2018). The presence of MAP in milk and dairy products is perceived as a potential food safety issue because of the possible link with various human diseases, such as Crohn's disease (Feller et al., 2007; Waddell et al., 2015), ulcerative colitis (Pierce, 2010), and type 1 diabetes (Sechi and Dow, 2015). Thus, detection of MAP in milk and culling of animals excreting MAP can help improve consumer safety. This can be achieved by detecting infected animals in the herd with a specific, sensitive, and economically viable test (Collins, 2011). None of the currently available detection methods of MAP infection are 100% sensitive or specific. Thus, identification of cows that excrete MAP must be based on a combination of methods.

Restructuring of dairy farms after Poland joined the European Union (EU) in 2004 increased the demand for dairy cows. The free movement of goods and services that is guaranteed in the EU accession agreement made it possible to purchase animals from other EU member states. Because of the lack of national requirements for the movement of animals with unknown paratuberculosis status, uncontrolled transfer of MAP-infected cows is possible. A relationship has been previously observed between the number of imported animals and the infected herd seroprevalence (Szteyn and Wiszniewska-Łaszczych, 2012). For herds in which cows come from different countries, it is important to determine whether the MAP infection occurred in the herd before or after the introduction of new animals. Identifying genetic MAP types can help to determine the source of infection.

The aim of this study was to investigate whether the use of direct DNA isolation to detect MAP genetic material in pooled milk samples performed similarly to the culture method. We also determined the occurrence and differentiation of MAP types isolated from milk samples from a low seroprevalence herd.

The research was conducted in a herd of 375 dairy crossbreed cows in an area with endemic paratuberculosis (Żuławy Wiślane in the northern part of Poland).

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Most animals (72%) in the herd were imported; 28% were bred on the farm (Wiszniewska-Łaszczych et al., 2009). Blood (10 mL) and milk (100 mL) samples were collected aseptically from 321 cows at least 2 yr old. In the laboratory, the serum was poured into sterile tubes without anticoagulants and centrifuged, followed by the Pourquier ELISA Paratuberculosis Screening/Paratub. Serum-S test (Pourquier, France), according to the manufacturer's instructions. Based on ELISA test results, the milk samples were divided into 2 groups: (1) samples from seropositive animals (11 individual samples); (2) samples from seronegative animals (these were combined in groups of 5, resulting in 62 pooled samples). Milk samples were tested for the presence of MAP (by culture method) and the MAP-characteristic IS900 sequence (by direct isolation of genetic material from milk). Before culture, the milk samples were pre-treated to decontaminate fast-growing microflora: 50 mL of milk was centrifuged at $9,803 \times g$ for 20 min at 20°C, the pellet was resuspended in 20 mL of 0.75% aqueous solution of cetylpyridinium chloride, and incubated for 3 h at room temperature. Then, the solution was centrifuged at $9,803 \times g$ for 20 min at 20°C and the pellet resuspended in 1 mL of aqueous solution of amphotericin B (100 µg/mL). Then, 100 µL of the suspension obtained was cultured in 3 Herrold's egg yolk medium (HEYM) tubes with the addition of mycobactin J and incubated at 37°C for up to 8 mo. The growth of bacteria was evaluated once a week. Colonies with morphology typical of *Mycobacterium* spp. were collected to isolate the genetic material necessary to confirm the presence of the MAP-characteristic IS900 fragment by PCR. The bacterial colonies were suspended in 50 µL of Ringer's solution, frozen for 24 h at -20°C, heated at 98°C for 15 min, and refrozen for 1 h at -20°C. DNA was isolated from the prepared suspension with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Bacterial suspension (50 µL) was diluted with 180 µL of Buffer ATL (Qiagen), shaken for 15 s, and then 20 µL proteinase K was added to increase the efficiency of isolating genetic material from MAP cells. The suspension was incubated at 56°C for 3 h and shaken 3 times during incubation. Further procedures were performed according to the manufacturer's instructions.

DNA was isolated directly from previously homogenized whole-milk samples by QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The PCR detection of IS900 in DNA directly isolated from milk samples and from cultured MAP-suspected colonies was performed in accordance with the method described by Szteyn et al. (2008). Determination of the MAP type was carried out by multiplex PCR (Collins et al., 2002) and detection of polymorphisms in the

IS1311 fragment by using of restriction endonuclease analysis (REA; Marsh et al., 1999).

Animal serum testing defined herd seroprevalence for paratuberculosis at 3.43% (11/321). The presence of suspected colonies was observed in 46 milk samples, including 11 (100%) from individual samples and 35 (56.4%) from pooled samples. The IS900 fragment was confirmed by PCR in DNA of colonies isolated from 7 (63.6%) individual and 15 (24.15%) pooled milk samples. The presence of IS900 in the genetic material isolated directly from the samples was confirmed in 11 (100%) individual samples and 13 (21%) pooled samples. Testing pooled milk samples from seronegative cows by the culture method revealed more infected cows than detecting the IS900 fragment in the genetic material obtained directly from the same milk samples: 24.19% (15/62) and 20.96% (13/62), respectively.

The multiplex PCR and PCR-REA method indicated the presence of 2 types of MAP: C-type (isolated mainly from cattle) and S-type (isolated mainly from sheep). Analysis of the genetic material obtained from confirmed MAP culture by multiplex PCR showed the presence of C-type MAP strains in all 7 individual milk samples and in 13 pooled milk samples. Two individuals from the pooled milk samples were classified as S-type MAP. When PCR was performed after culture, the MAP type results were the same for PCR-REA and multiplex PCR. The results were different when the type of mycobacterium was determined in genetic material obtained by direct isolation from milk samples. Using multiplex PCR, genetic material belonging to C-type was found in all 11 milk samples from seropositive cows and in 11 pooled milk samples; S-type was found in 2 pooled samples. The PCR-REA method revealed C-type MAP in 10 individual and 11 pooled milk samples; S-type was found in 1 individual and 2 pooled milk samples. The obtained results were placed in Table 1.

In accordance with the World Organisation for Animal Health (OIE) guidance, cases of paratuberculosis must be registered. Information on registered cases of paratuberculosis in Poland is gathered by the Chief Veterinary Inspectorate; however, there is no program for disease control at the country level. Only partial results of seroprevalence evaluations in dairy cattle herds are available (Wiszniewska-Łaszczych et al., 2009; Szteyn and Wiszniewska-Łaszczych, 2011). These results indicate significant differences in terms of MAP incidence in cows depending on herd size and location, as well as the origin of the animals (Szteyn and Wiszniewska-Łaszczych, 2012). Beaver et al. (2016) showed a similar relationship between the occurrence of PCR-positive results of bulk-tank milk and herd size, location, and

Table 1. The occurrence of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in milk samples and determination of the MAP type by PCR-restriction enzyme analysis (PCR-REA) and multiplex PCR¹

Method of MAP isolation	Type of sample	Milk samples, n	Samples suspected positive by culture, no.	Samples confirmed by IS900 PCR, no.	Multiplex PCR ²		PCR-REA	
					C-type	S-type	C-type	S-type
Culture	Individual	11	11	7	7	0	7	0
	Pooled	62	35	15	13	2	13	2
Direct isolation of DNA	Individual	11	—	11	11	0	10	1
	Pooled	62	—	13	11	2	11	2

¹C-type mainly found in cattle; S-type mainly found in sheep.²Collins et al. (2002).

breeding methods. Likewise, considerable differences in MAP prevalence have been observed in 13 European countries (Nielsen and Toft, 2009).

High sensitivity and fast detection of MAP in milk have been shown in previous studies (Szteyn et al. 2014). Two methods were used in this study: culture and detection of the IS900 fragment of the MAP genome in genetic material isolated directly from milk. The presence of IS900 has been reported in other mycobacterial species (Englund et al., 2002). It is therefore recommended to determine the presence of IS*Mav2* (Marsh et al., 1999) and the F57 locus (Coetsier et al., 2000) in the genome for more specific results. In our study, the use of IS900 was considered sufficient to determine the presence of MAP because of our additional analysis of polymorphisms in the MAP-specific IS1311 fragment.

For pooled milk samples, more positive results were obtained by culture than by direct DNA isolation. A higher number of seropositive samples was also identified by culture compared with PCR by Giese and Ahrens (2000) and Shankar et al. (2010), whereas Stabel et al. (2014) identified more MAP-positive samples in direct quantitative PCR. Previous evaluation of the methods used in this study revealed sensitivity of 63.6% for culture and 75% for PCR, and specificities of 89.4 and 74.3%, respectively (Szteyn et al., 2014). The sensitivity of MAP detection in milk is high and the amount of MAP excreted in milk increases until clinical symptoms appear (Stabel et al., 2014; Beaver et al., 2017).

When developing a strategy for detecting MAP-infected cows, attention should be paid to the origin of the animals, which may result in different types of MAP responsible for infection. *Mycobacterium avium* ssp. *paratuberculosis* can be identified and its type determined by different techniques. Two methods were compared in this study: (1) analysis of the insertion fragment IS1311 polymorphism by PCR-REA: in the MAP genome, C-type strains have thymine in position 223, whereas S-type strains have one or more copies of cytosine and thymine (Whittington et al., 1998); (2) the analysis of differences in individual nucleo-

tides of terminal sequences in IS900: the 5' terminus of S-type is not homologous with the 5' terminus of the C-type (Castellanos et al., 2009). The presence of mutations and differences in the nucleotide sequence can be detected by electrophoretic separation of the PCR product. In our study, we found 2 types of MAP (cattle and sheep) in the milk samples. The C-type was present in 89.1% of milk samples, and only 10.9% of strains were identified as S-type. This corresponds with research by Sevilla et al. (2007) and Gastaldelli et al. (2011), which showed that most strains isolated from cattle herds in Europe were of the C-type. Detecting genotypically different MAP strains in a herd in connection with cattle origin suggests different sources of infection. Differences in the occurrence of MAP types in samples from seropositive animals depending on the origin of genetic material may suggest the occurrence of mixed infections caused by both types of mycobacteria. The S-type MAP was detected only in genetic material isolated directly from milk samples. Because S-type MAP is difficult to grow on HEYM agar (Whittington et al., 2011), we suspect that genetic material belonging to the more easily growing C-type MAP substrate predominated in the DNA obtained from cultures.

Information on the types of MAP found in herds with low seroprevalence can help to determine the source of mycobacteria, which is important if animals from different regions or countries are present in the herd. Because of difficulties in culturing S-type MAP for identification tests, we suggest the use of DNA derived from direct isolation of genetic material by PCR-based methods.

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