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Original article

Isolation and molecular typing of *Mycobacterium avium* subsp. *paratuberculosis* from faeces of dairy cows

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the cause of paratuberculosis mainly in domestic and wild ruminants; paratuberculosis is also known as Johne's disease. This disease is endemic all over the world generating significant economic losses, especially in dairy herds, although, MAP is the cause of infection in many other species including primates. Currently, MAP mycobacteria are recognized as pathogens transmitted by food. They are a potential threat to animal and human health. Infected animals excreting mycobacteria with faeces are the main source of MAP. The development of control strategies and disease control are based on determination of the genetic diversity of the MAP strains causing Johne's disease. This study describes 43 strains isolated from a herd of dairy cows located in northern Poland. The types of MAP were determined based on the polymorphism analysis of two insertion fragments: IS900 and IS1311. The polymorphism of IS900 was analyzed with the use of a PCR multiplex according to Collins' method and the IS1311 polymorphism with the use of the PCR-REA method. Based on the differences observed, the strains isolated were classified into two MAP types, cattle (C-type) and sheep (S-type), with the predominance of the cattle type.

Key words: *Mycobacterium avium* subsp. *paratuberculosis* (MAP), insertion sequences polymorphism, C-type, S-type

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a Gram positive, acid-resistant bacterium belonging to the *Mycobacterium avium* complex (MAC). DNA-MAP is > 99% identical with *Mycobacterium avium* DNA, which makes it necessary to use molecular techniques to distinguish these mycobacteria (McFadden et al. 1987). The use of molecular tech-

niques and sequencing of the K-10 MAP genome enabled the division of MAP into two main groups of strains: sheep (S-type) and cattle (C-type) (DeZoete and Cavaignac 2002, Shankar et al. 2010). MAP is an etiological factor of paratuberculosis, also known as Johne's disease (JD). This disease occurs mainly in domestic and wild ruminants, although MAP is also isolated from monogastric animals (Nielsen and Toft

2003, Ghadiali et al. 2004, Hutchings et al. 2010, Fox et al. 2018). Due to primarilis faecal-oral route of spread, contaminated faeces are the major source for MAP transmission. It is endemic all over the world generating significant economic losses, especially in dairy herds. A report published by Nielsen and Toft (2009) indicated that between 3 and 68% of cattle herds in Europe are infected with MAP and in some regions reach up to 80% seroprevalence. Based on data collected 10 years later from 48 countries around the world, Whittington et al. (2019) showed that about 20% of herds are infected with MAP, and in some developed countries the percentage reaches 40%. Currently, MAP mycobacteria are recognized as foodborne pathogens (EFSA Regulation EU, No 2016/429, Davidson et al. 2016, *Frontiers in Public Perspective* 2017). Humans are exposed to MAP after consumption of milk and meat obtained from infected animals (Wynne et al. 2011). The discussion on the potential link between MAP and the occurrence of Crohn's disease in human still ongoing (Naser et al. 2009, McNeese et al. 2015). MAP structure, with a three-layer cell wall made of sugars, lipoarabinate and lipopolysaccharides forming the capsule structure, makes it difficult to isolate genetic material from the cell using commercially available isolation kits, and the hydrophobic properties of the mycobacteria make it difficult to isolate and identify this microorganism in samples from infected animals and animals suspected of being infected. Traditional culture, due to the slow growth of this microorganism, requires the presence of johnin, which distinguishes MAP among other MAC (Multiwall et al. 2004). Due to the similarity of phenotypic features, it is numeric taxonomy (Thorel et al. 1990) that provides the basis for further MAC differentiation; however confirmation of MAP identity is faster and easier by detecting the IS900 insertion fragment (Moss et al. 1991). This fragment was discovered and described for the first time by two independent teams of researchers, from New Zealand and from England (Collins et al. 1989, Green et al. 1989). The sequence of 1451 base pairs and 17 copies in the genome is also used for the determination of MAP types. The differences occurring in flanking regions were the basis for distinguishing the sheep type from the cattle type (Collins et al. 2002, Shankar et al. 2010). There are many techniques that can be used for molecular typing of MAP strains (Castellanos et al. 2012). Marsh (1999) described a fast, easy, polymorphism-based IS 1311 fragment technique for the differentiation of cattle and sheep strains by polymerase chain reaction and restriction endonuclease analysis (PCR-REA). IS1311 was described for the first time in 1995 (Roiz et al. 1996). It is smaller than IS-900, size 1317 bp, and occurs not only in the MAP genome

but also in *Mycobacterium avium* subsp. *avium* (MAA) in 7 to 10 copies. Our earlier studies using the PCR-REA method (Szteyn et al. 2017) showed the polymorphism of IS1311 strains isolated from milk. Insertion sequences have been shown to be important in differentiating mycobacterial species and subspecies. Determining the genetic diversity of MAP strains causing Johne's disease, and obtaining information on the differences in the traits and properties of mycobacteria causing infection are the basis for the development of disease's control strategies for this disease. The true level of infection of dairy herds in Poland, the distribution of MAP mycobacteria in cattle, and especially their genetic diversity, allowing for precise determination of the source of their origin, are very poorly understood. Complete diagnosis is hindered by the significant fragmentation of ruminant farms, including those that breed dairy cattle. Based on data published by the Main Statistics Office in Poland (Statistical information farm animals in 2017), 6 035 500 cattle are registered in the country, 2 341 000 of them are dairy cows kept on over 266 000 farms - 116 000 small farms with 1-2 cows and 74 500 farms with over 10 cows. The seropositive reaction to Johne's disease was found in large herds with imported heifers (Szteyn and Wiszniewska-Łaszczych 2012). Considering the threat that MAP may pose to humans and animals, we carried out this study to isolate and identify the main types of MAP obtained from a herd consisting of animals of different origin.

Materials and Methods

Ethics statement

This study was approved by the Local Committee on the Ethics of Animal Experiments of the Warmia and Mazury University (Permit Number: 89/2010/DTN).

Collection and preparation of samples for MAP detection

Studies were conducted in a herd of 375 animals, located in the Żuławy Wiślane region (northern Poland), and was approved by the Local Committee on the Ethics of Animal Experiments of the Warmia and Mazury University (Permit Number: 89/2010/DTN).

Blood samples of 10 ml were taken from 321 cows over 2 years old. The presence of antibodies was tested using the Pourquier ELISA Paratuberculosis Screening / Paratu Serum-S test, according to the manufacturer's instruction. The results of the absorbance measurement were read in an ELISA UVM 340 reader. Once the ELISA test results were obtained, the faecal samples collected from the animals were divided into two

Table 1. Primers used in study and size of the product obtained by PCR.

Primer	Sequence (5'-3')	Fragment amplified	According to:
P90	GAA GGG TGT TCG GGG CCG TCG CTT AGG	413	Millar et al. 1996
P91	GGC GTT GAG GTC GAT CGC CCA CGT GAC		
M56	GCG TGA GGC TCT GTG GTG AA	608	Marsh et al. 1999
M119	ATG ACG ACC GCT TGG GAG AC		
DMC529	TTG ACA ACG TCA TTG AGA ATCC	162	Collins et al. 2002
DMC 533	CGG ATT GAC CTG CGT TTC AC		
DMC 531	TCT TAT CGG ACT TCT TCT GGC	310	Collins et al. 2002
DMC533	CGG ATT GAC CTG CGT TTC AC		

groups. The first group included 11 individual samples from seropositive animals. The second group included 62 pooled samples from animals with a negative serological response. Faecal pooled samples were prepared by mixing 5 samples of 10g faeces collected from individual animals.

Isolation of MAP from individual and pooled samples by culture method

One-gram faecal samples were distributed in 35 ml sterile deionized water and shaken at room temperature for 30 min. The samples were then set aside for 30 min to sediment out large particles. 5 ml of supernatant was then taken from each sample and 25 ml of 0.9% aqueous solution of cetylpyridine hydrochloride was added in order to decontaminate fast-growing flora. The mixture was incubated for 12 h at room temperature and then centrifuged at 20°C for 20 min. The supernatant was poured away and the pellet was dissolved in 1 ml of aqueous amphotericin B (100 µg/ml). This suspension was cultured on 3 HEYM (Herrold's Egg Yolk Medium) slants with mycobactin J. The incubation was carried out at 37°C for 8 months.

Isolation of genomic DNA-MAP from the colonies obtained on HEYM culture

Bacterial colonies grown on HEYM medium were collected and distributed in 50 µl of Ringer's fluid. Due to the formation of the MAP CAP structure around the cell, the samples were subjected to pretreatment developed in our laboratory to effectively isolate genetic material with commercially available isolation kits. The samples were frozen for 24 h at -20°C and then heated at 98°C for 15 minutes. After this time, they were again frozen for 1h at -20°C. DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Germany) from thawed bacterial suspension (in accordance with the manufacturer's procedure). The genetic material solutions were kept at 4°C for further analysis.

Detection of the presence of the IS 900 fragment by PCR

For the reaction, a mixture was prepared using ready-to-use PCR Master Mix (Hot-Start PCR StartWarm A&A Biotechnology, Poland). The 25 µl reaction mixture contained 12.5 µl PCR Master Mix (0.1U/ µg, Taq DNA polymerase, 0.5mM of dNTPs and 2.5 mM of Mg Cl₂) 2 µl (0.5µM of P90 and P91 mix primer (Table 1), and 9.5 µl of DNase free water (to reach a volume of 24 µl). The 1 µl of DNA isolate was added to the reaction mixture and amplified in a Mastercycler pro thermocycler (Eppendorf, Germany) as follows: initial denaturation for 8 min at 95°C, 40 cycles - 1 min at 94°C denaturation, primer attachment 45 s at 67°C, elongation for 2 min at 72°C, completion of the reaction - final chain synthesis for 2 min at 72°C. The PCR product obtained was visualized in a 2% agarose gel - 8 µl of the products were mixed with 2 µl of loading buffer and applied to the gel for electrophoresis at 100 V for 60 min in the presence of a 1 kb ladder marker (Gibco, Thermo Fisher Scientific, USA). The results were read and archived using a GelDoc-UVP (BioRad, USA) Gel Documentation Kit. The MAP strain from the ATCC-BAA-968 collection was used as a positive control (Fig.1).

DNA-MAP analysis by multiplex PCR according to Collins' method

For the reaction, a mixture was prepared using ready-to-use PCR Master Mix (Hot-Start PCR StartWarm A&A Biotechnology, Poland) and primers: 2 µl (0.5µM) of DMC529 and DMC533 mix for sheep type MAP (product length 162 bp) and DMC531 and 2 µl (0.5µM) of 0,5 DMC533 and DMC533 mix for cattle type MAP (product length 310 bp) (Table 1). The 1 µl of DNA isolate was added immediately after the combination and transferred to the thermocycler for amplification. The reaction was as follows: initial denaturation for 3 min at 95°C, 25 cycles - 30 s at 60°C denaturation, primer attachment 30 s at 72°C, elonga-

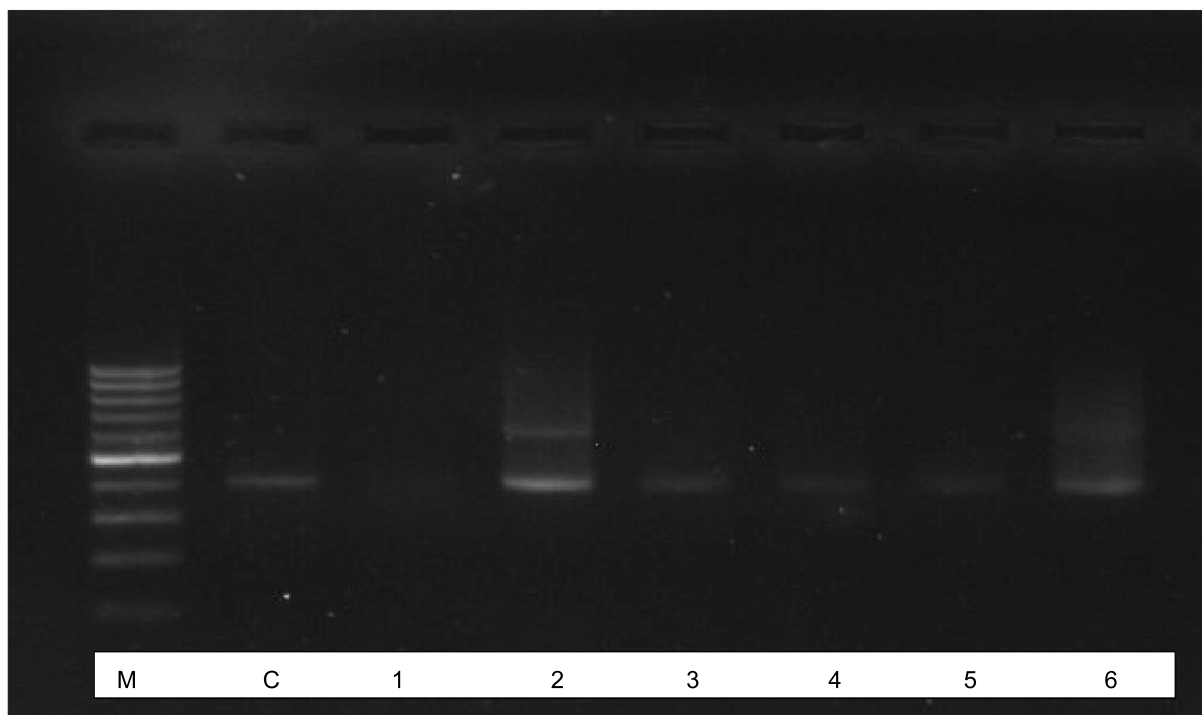


Fig. 1. Electrophoretic image presence of IS900 fragment in tested material. Individual lines: M – Marker; C – Control; 1 – Negative sample; 2-6 – MAP – presence of IS900 fragment.

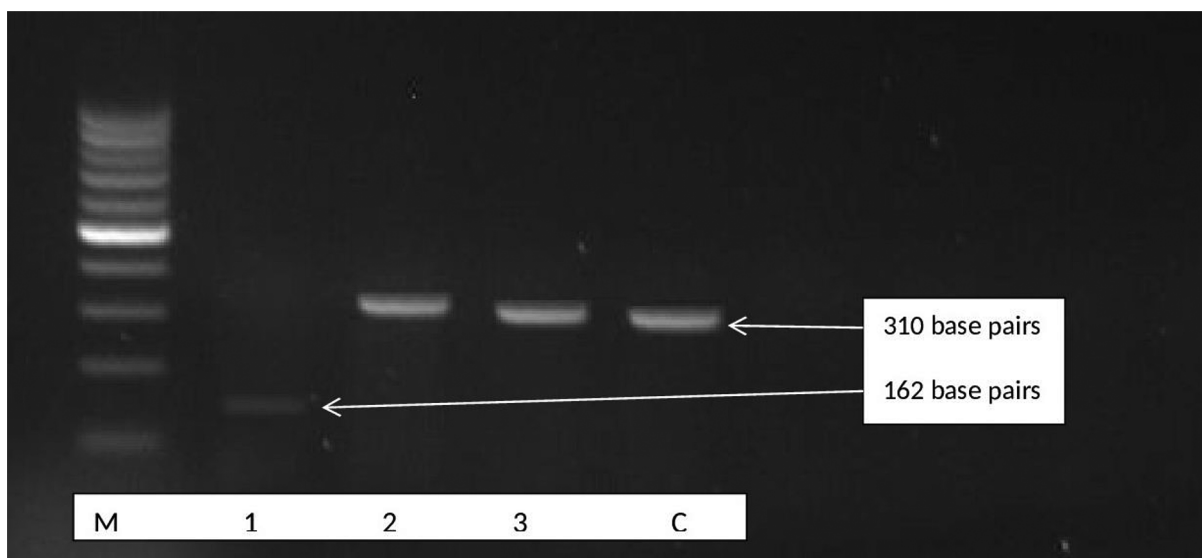


Fig. 2. Electrophoretic image presence of Multiplex PCR products. Individual lines: M-Marker; 1 – S-type; 2 and 3 – C-type; C- Control.

tion 30 s at 94°C, reaction completion – 7 min at 72°C. Electrophoresis was performed on 2% agarose gel. MAP types of mycobacteria were determined based on the size of the bands obtained in the agarose gel (Collins et al. 2002) (Fig. 2).

Determination of the polymorphism of IS 1311 fragment by PCR- REA

Detection of the IS1311 fragment was performed using ready-to-use PCR Master Mix (Hot-Start PCR

StartWarm A&A Biotechnology, Poland) and 2 µl (0.5 µM) of primers mix, for the fragment IS1311: M56 and M119 (Table 1). 1 µl of DNA isolate was added to the reaction mixture and amplified in a Mastercycler-Eppendorf thermocycler. The reaction was as follows: initial denaturation 3 min 94°C; 40 cycles - 30 s, 94°C denaturation, primer attachment 15 s 62°C, elongation 1 min 72°C; completion of the reaction 2 min at 72°C. The PCR product obtained was subjected to electrophoresis in a 1.5% LSI agarose gel in TAE buffer at 100v for 60 min

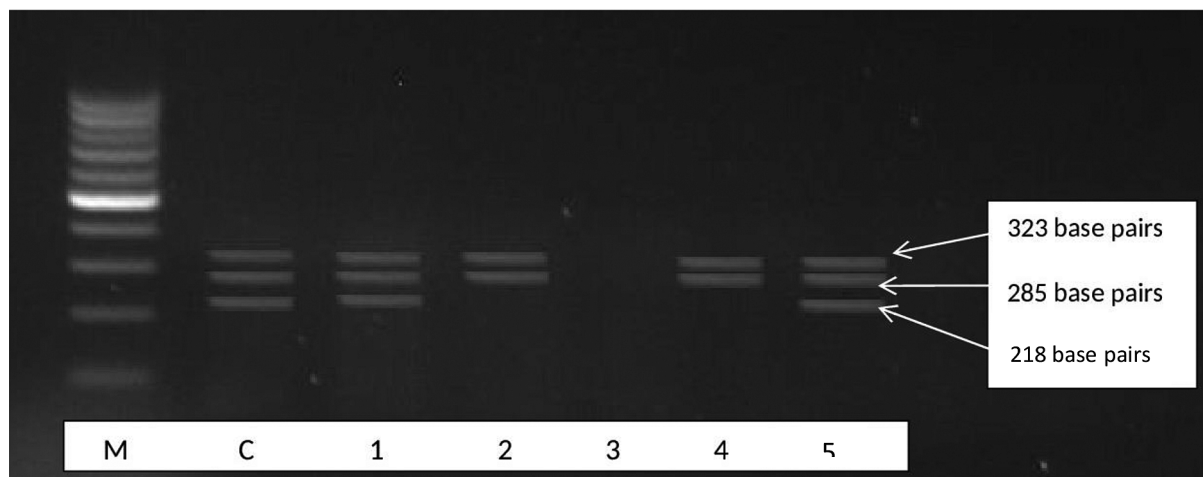


Fig. 3. Electrophoretic image presence of PCR-REA products. Individual lines: M-Marker; C – Control; 1 and 5 – C-type; 2 and 4 – S-type; 3- negative sample.

Table 2. Number of isolated bacterial colonies of *Mycobacterium* sp. from fecal samples and the presence of IS900 in the analyzed isolates.

Faecal samples	The number of samples		
	Analyzed	Suspected based on the morphology of the colonies	Confirmed by the presence of IS900
Individual	11	11	11
Pooled	62	42	32
total	73	53	43

in the presence of a 1kb ladder marker (Gibco). In order to determine the type of MAP mycobacteria IS1311 (608 bp) fragments obtained in the amplification reaction, size 413bp and IS1311, size 608bp, were digested with restriction enzymes BspPI (A1wI), MseI and HinfI. For this purpose, a reaction mixture was prepared: 10 µl of PCR product + 18 µl water + 2 µl buffer + 1 µl restriction enzyme. For the IS900 fragment of 413 bp, the reaction was carried out using the BspPI (A1wI) enzyme under the following conditions: initial digestion 3 h 55°C, reaction stopping 20 min 80°C. For the IS1311 fragment, the reaction was carried out using 2 enzymes; MseI under the following conditions: initial digestion 3h 65°C, reaction completion 20 min 80°C, and HinfI with the initial digestion for 3 hours at 37°C and the reaction was being stopped for 20 min at 65°C. The products obtained were subjected to electrophoresis on a 2% agarose gel for products obtained by digestion with the BspPI enzyme and 4% for products obtained by digestion with HinfI and MseI enzymes. Determination of MAP type was based on the size and number of the bands obtained: 285 bp and 323 bp for type S and 67 bp, 218 bp, 285 bp and 323 bp for type C (Marsh et al.1999) (Fig. 3).

Results

Based on serological tests of cattle using the commercial Pourquier ELISA test Paratuberculosis Screening/Paratub. serum-S, seroprevalence of the herd was determined at 3.4%. The presence of antibodies was detected in 11 out of 321 serum samples tested. Of the eleven positive results, seven came from cows imported into the farm from other European Community countries. True prevalence as estimated based on the formula proposed by the Rogan and Gladen (1978) was 9.2246%.

Out of 11 faecal samples from single seropositive animals and 62 faecal pooled samples from seronegative cows, the number of colonies exhibiting characteristic phenotypic traits for the *Mycobacterium* species was 11 and 42, respectively, observed on HEYM (Herrold's Egg Yolk Medium), (Table 2). Detection of the IS-900 fragment in the genome of 11 strains from the seropositive animals confirmed that the isolates were MAPs. Examination of the genetic material of 42 strains of mycobacteria isolated from pooled faecal samples showed the presence of the IS-900 fragment in 32 samples (Table 2). Collins multiplex PCR analysis of DNA isolates of strains obtained from individual faecal samples confirmed type C in 10 samples and type S in one sample. The DNA of MAP strains

Table 3. Determination of type of MAP isolated from individual and pooled fecal samples.

Material		Method			
MAP strains isolated from fecal samples	IS900 / MAP	PCR- REA IS - 1311		Multiplex PCR According to Collins' method IS - 900	
		MAP Type - C	MAP Type - S	MAP Type - C	MAP Type - S
Individual	11	10	1	10	1
Pooled	32	29	3	29	3
Total	43	39	4	39	4

obtained from pooled samples showed the presence of type C in 29 samples and type S in 3 samples. (Table 3). A total of 43 strains were tested for fragment IS-1311 polymorphism. Analysis of the IS-1311 fragment of strains isolated from seropositive animals by PCR-REA showed that 10 out of 11 strains had fragment features characteristic for MAP type cattle and 1 for the sheep type. Analysis of the IS-1311 fragment in 32 strains isolated from pooled faecal samples, indicated features typical for type C were noted in 29 cases, and type S in 3 cases (Table 3). The sheep type of mycobacterium was isolated from single samples from animals imported to the herd from other European countries. Similarly, the strains defined as a sheep type were isolated from pooled samples that included faeces from cows from other countries.

Discussion

The existence of phenotypically distinct MAP strains has been observed since the 1930s. The use of restriction endonuclease analysis and DNA hybridization to study a group of strains from New Zealand, Australia, Canada and Norway gave the basis for distinguishing between “bovine” and “sheep” MAPs (Collins et al. 1990). Since then, various PCR-based tests have been used to determine these two main types. Depending on the technique used, different types of changes in the genome were detected, such as the polymorphism of large fragments, insertional fragments, tandem repeats, or single nucleotide polymorphisms. The majority of molecular techniques may be used for MAP typing, but it is recognized that it is necessary to use more than one locus to qualify the isolates into one of two groups (Collins et al. 2010, Bannantine et al. 2013). Therefore, out of 19 different types of insertion fragments present in the MAP genome, the most characteristic, i.e. IS900 and IS 1311, were chosen. Among MAP strains isolated in this study from the faecal samples from dairy cows, of the 43 MAP strains tested, 39 belonged to type C, which is 90.7% and only 4 (9.3%) to the type S. Studies on MAP strains from

different regions of Europe and the USA carried out in Spain showed that type C was more frequently isolated in Europe than type S (Sevilla et al. 2005). Moreover, these studies confirm the observations of other authors that C- type strains are not host specific and can be isolated not only from farm animals but also from wild animals (O'Brien et al. 2006). S-type strains were most often isolated from sheep and goats (de Juan et al. 2006). Experimentally MAP was also a cause of John's disease in cattle (O'Brien et al. 2006). Moreover, there have also been documented cases of natural infection with type S in cattle (Collins et al. 1989). Detection of sheep type MAP in Poland is interesting because cases previously registered by the Main Veterinary Inspectorate, and confirmed cases of paratuberculosis, concerned only cattle. The breeding of cattle in Poland is very fragmented. In the last three years, the disease was confirmed in 34 outbreaks in 117 animals. In Poland, there are no comprehensive studies on the occurrence of paratuberculosis in dairy herds, and there is no program for disease control. Previously published research (Szteyn et al. 2005, Wiszniewska-Łaszczych et al. 2010) and data provided by the Main Veterinary Inspectorate in Poland, show that paratuberculosis occurs in the country in dairy herds. Studies on the prevalence of MAP infections in dairy cattle in Poland have demonstrated the endemic nature of the disease, as well as its occurrence mainly in herds that were reinforced with animals from other European countries (Szteyn et al. 2005). Investigation of the polymorphism of the IS1311 insertion fragment of MAP strains isolated from milk samples from a herd located in the Żuławy Wiślane region, showed the presence of two genetic types: C and S (Szteyn et al. 2017). The current study shows that all strains classified as S-type have been isolated from animals imported from outside Poland. Every year, large numbers of cattle are imported to Poland. In 2016 it was over 193 000, and in the two subsequent years, over 224 000 per year. The animals are imported from many European Union countries, eg.: Estonia, Lithuania, Latvia, Germany, Slovakia and the Netherlands. In the herd examined in the current

study there were cows from Germany, Belgium and the Netherlands.

Knowledge of the types is important for understanding the differences that exist between MAP strains, the impact they have on the development and transmission of the disease, and consequently on the diagnosis and control of the disease (Stevenson 2015). This information is necessary to develop better diagnostic methods, and effective vaccines against John's disease. Epidemiological studies carried out in other countries indicate the possibility of mixed infections with different MAP genotypes in dairy herds, which affects the course and development of the disease in the herd (Davidson et al. 2016). In order to study the genetic diversity of MAP isolates in Poland and the dynamics of transmission, it is necessary to use molecular techniques that will allow the source of isolates to be traced and phylogenetic analysis to be carried out. Identification of the main types of strain provides limited information but may serve as the introduction to further research. Whole genome sequencing is the most accurate method to determine the types of MAP (Li et al. 2005); however, due to the costs, time, and diagnostic possibilities, and the need to analyze many isolates, a significant number of laboratories have practically limited possibilities of using this method.

Conclusion

MAP strains were isolated from animal faeces of both seropositive and seronegative animals. Diagnosis of an animal infection should not only be based on serological tests. The pooling of faeces samples for MAP detection may partially reduce the cost of diagnostics. The use of the multiplex PCR technique to analyze polymorphism of the IS-900 fragment and the PCR-REA technique for the IS-1311 fragment indicated that the herd could be infected by more than one type of mycobacterium. This information may be useful for MAP diagnosis in herds with cows of different origins.

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