Evaluation of methods for *Mycobacterium avium ssp. paratuberculosis* detection in milk samples from the cattle herd showing low seroprevalence of Johne's disease

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Abstract

Mycobacterium avium ssp. paratuberculosis (MAP) is the cause of chronic gastroenteritis in cattle called the Johne's disease (JD). The disease causes significant economic losses in cattle production. MAP is also supposed to be involved in the Crohn's disease and inflammatory bowel disease (IBD) in people. The detection of the cattle infection based on investigations of milk samples and evaluation of the capacity of the methods used to detect the disease was the objective of the present study. Following methods were applied for milk samples testing: detection of MAP in bacterial culture, detection of the specific IS-900 fragment of MAP in the genetic material isolated directly and detection of MAP antibodies. The results obtained were compared with the "golden standard" results, i.e. the isolation of MAP from the faeces. PQStat-the program for diagnostic reliability estimation, was used for evaluation of the sensitivity, specificity and predictive value. The method based on detection of the specific IS-900 fragment of MAP in the genetic material isolated directly from milk samples was found to possess the highest sensitivity. Detection of anti-MAP antibodies on the other hand showed the lowest sensitivity. The method of detecting anti-MAP antibodies in milk was the most specific while detection of the IS-900 fragment in the genetic material was the least specific method. These results obtained may serve as a guide to choose the most appropriate method for diagnosis of MAP infections by milk sample testing.

Key words: *Mycobacterium avium ssp. paratuberculosis*, Johne's disease, diagnostics of paratuberculosis, milk

Introduction

Diagnostics of Johne's disease (JD) in cattle caused by *Mycobacterium avium ssp. paratuberculosis* (MAP) creates numerous difficulties and represents an important issue for both veterinarians and cattle growers. JD, called paratuberculosis, is characterised by slow progress within the herd and long incubation period from 6 months to a few years (Collins 2003). Given its global coverage, it was included by the

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World Organisation for Animal Health (OIE 2004) in the list of diseases with particular economic importance and importance for public health (OIE 2008). Currently, JD occurs in the majority of European countries (Nielsen and Toft 2009), in both Americas (Manning and Collins 2001, Hori-Oshima et al. 2007), Asia (Singh et al. 2008), as well as in Australia and New Zealand (Ridge et al. 2010). Examinations of dairy cattle herds in north-eastern Poland confirmed its occurrence in the country (Wiszniewska-Łaszczych et al. 2009, Szteyn and Wiszniewska-Łaszczych 2012). Milk is one of the paths for excretion of mycobacterium by the infected animals. It can then become a vector for MAP transmission on consumers. The potential role of MAP as zoonotic pathogen of humans is still the subject of numerous studies (Juste et al. 2009 and www.nacc.org.uk). The number of clinically recorded cases of paratuberculosis in cattle in Western Europe and North America was decreasing during the late 20[th]c. However, increasingly frequent and numerous cases of subclinical forms of the disease that are more difficult to detect have become a problem (Hermon-Taylor 2001). Economic losses induced by the occurrence of the subclinical form result from decreased milk yields, decreased protein and fat content in milk, decreased fertility, increased frequency of mastitis, increased susceptibility to secondary diseases, reduction of slaughter weight and early slaughter (Ott et al. 1999). Screening examinations allowing determination of the herd JD status are conducted in numerous countries by applying ELISA testing of blood serum, which allows detecting anti-MAP antibodies. Despite the low sensitivity this test still is the method of choice in epidemiological studies (Böttcher and Gangl 2004). The choice of the method that may and should be applied in further diagnostics of animals in the herd remains an unsolved issue. Given that milk is one of the major MAP transmission vectors and the role of mycobacterium in the pathogenesis of human alimentary system diseases as well as various problems in the diagnostic examinations, the present study was aimed at detecting the cattle infection MAP based on milk samples and evaluation of the capacity of the methods used to identify the disease.

Materials and Methods

The study was conducted in the herd of Holstein-Friesian dairy cattle of 230 heads. Blood samples for serological tests were collected from cows aged over 18 months. The tests were performed using the Mycobacterium paratuberculosis Antibody ELISA Kit by IDEXX. Test results showed low levels of serop-

revalence in relation to JD at < 2.5%. Eighteen cows were chosen for evaluation of the capacity of the methods used to identify the disease, including four MAP faeces culture positive. Five of the selected animals rendered the positive result, one result was doubtful and eleven rendered negative results in ELISA test for anti-MAP antibodies detection. During the next stage of the study, samples of faeces and milk were collected from the selected cows. The samples were collected three times, a month and 6 months after the first sample collection.

I. Faeces samples were tested by culturing for MAP isolation according to the procedure described by Pavlik et al. (2000).

II. Milk samples were analysed to:

a) detect live MAP cells by culturing method (Szteyn et al. 2008)

b) the presence of the specific IS-900 fragment, characteristic for MAP preceded by DNA isolation from milk. The DNA was obtained using the QIAamp DNA Mini Kit by Qiagen according to the manufacturer's instruction

c) the presence of MAP antibodies using the ELISA test (*Mycobacterium paratuberculosis* Para-Tb AB by SVANOVA) according to the manufacturer's instructions.

III. Confirmation the presence of MAP in cultures, similarly to the material isolated from milk directly, was obtained by detecting the IS-900 fragment in the PCR reaction (Szteyn et al. 2008).

IV. Statistical analysis of the results was conducted by applying the PQStat application (diagnostic reliability) computing sensitivity, specificity, positive predictive value and negative predictive value.

Results

The presence of living MAP cells was detected by culturing method in 10 out of 54 milk samples tested. The method of direct DNA isolation from milk and confirming the presence of the MAP specific IS-900 fragment in the genetic material rendered the positive result in 19 samples. On the other hand, anti-MAP antibodies were detected in 4 milk samples only by applying the ELISA method. Seven of the other milk samples proved doubtful results (+/−). The numbers of positive and doubtful results obtained in the individual test series differed significantly and were as follows: culturing test 2+, DNA IS-900 6+, presence of antibodies 1+, 2 (+/−) in the first series, in the second series 4+, 7+, 2+, 3 (+/−), respectively and in the third series 4+, 6+, 1+ 2 (+/−), respectively. The indicators of sensitivity, specificity of tests as well as positive and negative predictive value computed

based on the statistical analysis are presented in Table 1. The highest specificity was presented by antibodies detection method using the ELISA test while the lowest was obtained in case of direct DNA isolation. ELISA method sensitivity was the lowest while direct DNA isolation from milk samples offered the highest sensitivity. The differences between the negative predictive values were much smaller than the differences in positive predictive values between the three methods applied. In statistical analyses doubtful results of the test were not taken into account in computing the diagnostic indicators for the ELISA method, because they may not be classified either to the positive or to the negative results group.

Table 1. Evaluation of methods for detecting MAP in milk samples.

Values for diagnostic test	Methods for milk sample testing		
	MAP Culture	IS-900 MAP DNA	ELISA
Sensitivity	63.6%	75%	42.8%
Specificity	89.4%	74.3%	97.4%
PPV	70%	47.3%	75%
NPV	91.8%	90.6%	89.1%

Discussion

Different methods have been used for detecting the MAP infected animals and to limit the spreading of paratuberculosis in cattle (Timms et al. 2001). The appearance of subclinical cases of the disease in a herd with confirmed low seroprevalence causes the largest difficulties in selecting the subsequent detection method (Logar et al. 2012). Possible links between paratuberculosis and Crohn's disease as well as inflammatory bowel disease (IBD) in people causes increased interest in MAP detection in milk. Particularly so, because other studies show that mycobacteria are resistant to high temperatures and may survive industrial pasteurisation conditions (Mc Donadl et al. 2005, Cerf et al. 2007). MAP is excreted by infected animals periodically and collecting a number of samples at time intervals from the same animals increases the probability of infection detection (Pinedo et al. 2008). Conventional MAP isolation methods applying classic culturing show low sensitivity as a consequence of the necessity of applying inhibitors of growth of other microflora contained in the sample (Whitlock et al. 2000, OIE 2008). That chemical decontamination may also decrease the count of MAP cells by even as much as 10^3 cfu (Reddacliff et al. 2003). Moreover, given the long culture incubation

period the result can be obtained after 6 weeks at the earliest although usually the process takes longer – 12 to 14 weeks. The conducted experiments applying culturing methods allowed establishing the mycobacteria detection limits. The sensitivity of methods, as reported by authors of tests performed on samples of artificially contaminated milk was diversified ranging from 10-100 cfu/ml (Pila et al. 2002) to 200-300 cfu/ml (Millar et al. 1996). Low detection thresholds of the culturing method might result in not detecting all the animals infected if the MAP cell numbers excreted with milk are low. Milk samples centrifugation applied during preparation for culturing and obtaining three fractions may result in differences in numbers of mycobacteria per individual fraction. As proven, centrifugation at 2500 x g for 15 minutes renders the highest numbers of MAP cells in the sediments at 69.4%, 17.6% in the remaining whey and 13% in the fat (Grant et al. 2000). In our study milk samples were centrifuged and the culturing method sensitivity was computed at 63.6%. The method of direct MAP-DNA isolation from milk samples is not a routine method for detecting infection in cattle and little data concerning that diagnostic method has been published so far (Pinedo et al. 2008). It has been shown, however, that the specificity of the method may reach 100%, while the PCR reaction sensitivity was determined at 0.01 pg DNA/10 genome copies while in case of conventional methods it is 0.1 pg/100 genome copies (Mobius et al. 2008). The IS-900 MAP fragment contains 1451 pairs of alkalis occurring in 14-20 copies in the genome (Bull et al. 2000). The method sensitivity in our studies was determined at 75% and it was higher than that of the other two methods. Although the specificity of the direct MAP-DNA isolation method was the lowest according to. Logar, application of a modified method with high DNA extraction yield and real time PCR may help to increase the sensitivity and specificity of MAP detection by direct DNA isolation from the samples (Logar et al. 2012). Detection of antibodies using the ELISA test is the most frequently used method for serological JD diagnosing. However, the concentration of antibodies in milk is related not only to the level of antibodies in serum but also the milk yield, lactation period and even the calving number (Sweeney et al. 2006). Studies so far conducted milk sample testing with ELISA test has shown 12% more positive results than the tests in the serum from the same animals. Our studies have shown that the ELISA test sensitivity was the lowest compared to the other methods applied at slightly below 43% coupled with the highest specificity of over 97%. The diagnostic test sensitivity and specificity values depend on the results of comparison to the reference test that differentiates clearly between in-

fected and non-infected animals (Leon and Duffy 2006). In case of paratuberculosis, the MAP isolation from animal faeces is the "golden standard". In evaluating the ability of the methods used to identify the disease, the predictive values were computed. The highest probability of the disease occurrence is shown by the positive predictive values (PPV) for the ELISA test while the direct DNA isolation from milk samples method shows the lowest PPV. Absence of the disease is indicated by the negative predictive values (NPV) that was the highest in case of MAP culturing from milk samples and the lowest for the ELISA test. These results may serve as a guide to choose the most appropriate method for diagnosis of MAP infections by milk sample testing.

Conclusions

1. Examination of milk sample for MAP presence does not detect all the cows infected.

2. The highest sensitivity in MAP detection in milk is shown by the direct DNA isolation method with confirmation of the presence of IS-900 fragment while the lowest sensitivity is offered by anti-MAP antibodies detection using the milk ELISA test.

3. The highest MAP detection specificity in milk is shown by the anti-MAP antibodies detection method using the milk ELISA test while the lowest is offered by direct DNA isolation and confirmation of the presence of IS-900 fragment of the MAP in milk.

Acknowledgements

References

Böttcher J, Gangl A (**2004**) Mycobacterium avium ssp. paratuberculosis-combined serological testing and classification of individual animals and herds. J Vet Med B Infect Dis Vet Public Health 51: 443-448.

Bull TJ, Hermon-Taylor J, Pavlik I, El-Zaatari F, Tizard M (**2000**) Characterization of IS-900 loci in Mycobacterium avium subsp. paratuberculosis and development of multiplex PCR typing. Microbiology 146: 2185-2197.

Cerf O, Griffiths M, Aziza F (**2007**)Assessment of the prevalence of Mycobacterium avium subsp. paratuberculosis in commercially pasteurized milk. Foodborne Pathog Dis. 4: 433-447.

Collins MT (**2003**) Paratuberculosis: review of present knowledge. Acta Vet Scand 44: 217-221.

Grant IR, Pope CM, O'Riordan LM, Ball HJ, Rowe MT (**2000**) Improved detection of Mycobacterium avium

subsp. paratuberculosis in milk by immunomagnetic PCR. Vet Microbiol 77: 369-378.

Hermon-Taylor J (**2001**) Mycobacterium avium subspecies paratuberculosis: the nature of the problem. Food Control 12: 331-334.

Hori-Oshima S, Campos-Barba LA, Moreno-Rosales JF, Renteria-Evangelista TB, Lopez-Valencia G, Dela Mora A, Corral-Madriod A (**2007**) Seroprevalence of bovine paratuberculosis in dairy cattle herds in the Mexico-U.S. border area in Baja California, Mexico. In: Proceedings of the nineth ICP, Tsukuba, Japan, 71.

Juste RA, Elguezabal N, Pavon A, Garrido JM, Geijo M, Sevilla I, Cabriada JL, Tejada A, Garcia-Campos F, Casado R, Ochotorena I, Izeta A (**2009**) Association Mycobacterium avium subsp paratuberculosis DNA in blood and cellular and humoral immune response in inflammatory bowel disease patients and controls. Int J Infect Dis 13: 247-254.

León EA, Duffy SJ (**2006**) Pruebas diagnósticas: principios y métodos para su evaluación e interpretación. In: Cacchione R, Durlach R, Martino P eds. Temas de Zoonosis III – 1st edition. Buenos Aires, Asociación Argentina de Zoonosis. 416-422.

Logar K, Kopinč R, Bandelj P, Starič J, Lapanje A, Ocepek M (**2012**) Evaluation of combined high-efficiency DNA extraction and real-time PCR for detection of Mycobacterium avium subsp. paratuberculosis in subclinically infected dairy cattle: comparison with faecal culture, milk real-time PCR and milk ELISA. BMC Vet Res 8:49.

McDonald WL, O'Riley KJ, Schroen CJ, Condron RJ (**2005**) Heat inactivation of Mycobacterium avium subsp. paratuberculosis in milk. Appl Environ Microbiol. 71: 1785-1789.

Manning EJ, Collins MT (**2001**) Mycobacterium avium subsp. paratuberculosis: pathogen, pathogenesis and diagnosis. Rev Sci Tech 20:133-150.

Millar D, Ford J, Sanderson J, Withey S, Tizard M, Doran T, Hermon-Taylor J (**1996**) IS900 PCR to detect Mycobacterium paratuberculosis in retail supplies of whole pasteurized cow's milk in England and Wales. Appl Environ Microbiol 62: 3446-3452.

Mobius P, Hotzel H, Rassbach A, Kohler H (**2008**) Comparison of 13-single – round and nested PCR assays targeting IS900, ISMav2, f57 and locus 255 for detection of Mycobacterium avium subsp. paratuberculosis. Vet Microbiol 126: 324-333.

Nielsen SS, Toft N (**2009**) A review of prevalences of paratuberculosis in farmed animals in Europe. Prev Vet Med 88: 1-14.

Office International Des Epizooties (**2004**) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Part II(mammals, birds and bees). 347.

Office International Des Epizooties (**2008**) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Part II (mammals, birds and bees). 276.

Ott SL, Wells SJ, Wagner BA (**1999**) Herd-level economic losses associated with Johne's disease on US dairy operations. Prev Vet Med 40: 179-192.

Pavlik I, Matlova L, Bartl J, Svastova P, Dvorska L, Whitlock R (**2000**) Parallel faecal and organ Mycobacterium avium subsp. paratuberculosis culture of different productivity types of cattle. Vet Microbiol 77: 309-324.

Pillai SR, Jayarao BM (**2002**) Application of IS900 PCR for

detection of Mycobacterium avium subspecies paratuberculosis directly from raw milk. J Dairy Sci 85: 1052-1057.

Pinedo PJ, Williams JE, Monif GRG, Rae DO, Buergelt CD (**2008**) Mycobacterium paratuberculosis shedding into milk: association of ELISA seroreactivity with DNA detection on milk. Intern J Appl Res Vet Med 6: 137-144.

Reddacliff LA, Vadali A, Whittington RJ (**2003**) The effect of decontamination protocols on the numbers of sheep strain Mycobacterium avium subsp paratuberculosis isolated from tissues and faeces. Vet Microbiol 95: 271-282.

Ridge SE, Heuer C, Cogger N, Heck A, Moor S, Baker IM, Vaughan S (**2010**) Herd management practices and transmission of Johne's disease within infected dairy herds in Victoria, Australia. Prev Vet Med 95: 186-197.

Singh SV, Singh AV, Singh R, Sharma S, Shukla N, Misra S, Singh PK, Sohal JS, Kumar H, Patil P K, Misra P, Sandhu K S (**2008**). Sero-prevalence of bovine Johne's disease in buffaloes and cattle population of North India using indigenous ELISA kit based on native Mycobacterium avium subspecies paratuberculosis "Bison type" genotype of goat origin. Comp Immunl Microbiol Infect Dis 31: 419-433.

Sweeney RW, Whitlock RH , McAdams S, Fyock T (**2006**) Longitudinal study of ELISA seroreactivity to Mycobacterium avium subsp. paratuberculosis in infected cattle and culture-negative herd mates. J Vet Diagn Invest 18: 2-6.

Szteyn J, Wiszniewska-Łaszczych A, Ruszczyńska A (**2008**) Effectiveness of Mycobacterium paratuberculosis isolation from raw milk by means of direct isolation of DNA and classic culture. Pol J Vet Sci 11: 25-28.

Timms VJ, Gehringer MM, Mitchell HM, Daskalopoulos G, Neilan BA (**2001**) How accurately can we detect Mycobacterium avium subsp. paratuberculosis infection? J Microbiol Meth 85: 1-8.

Whitlock RH, Wells SJ, Sweeney RW, Van Tiem J (**2000**) ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. Vet Microbiol 77: 387-398.

Wiszniewska-Łaszczych A, Szteyn J, Ruszczyńska A, Hebel T, Lipiec M (**2009**) Paratuberculosis infection in a dairy herd in the Żuławy region. Med Weter 65: 484-486.