Detection of *Mycobacterium avium* subsp. *paratuberculosis* in mouflon muscle tissue

[1]Radka Přibylová-Dziedzinská, [2]Jiří Lamka, [1]Iva Slaná

[1]Veterinary Research Institute
Brno, Czech Republic
[2]Faculty of Pharmacy
Charles University in Prague
Hradec Králové, Czech Republic

Abstract

The aim of this study was to examine muscle tissue (back muscle, leg) from mouflon reared in selected game enclosures in the Czech Republic for the presence of the causative agent of paratuberculosis (*Mycobacterium avium* subsp. *paratuberculosis;* MAP). Faecal and muscle tissue samples were collected to determine the current state of health of the animals. Thirty-three of the 48 animals tested were found to be positive in either the muscle or faeces (68.8%). The causative agent of paratuberculosis was found in the muscle tissue of 16 animals (48.5%). Animals affected by paratuberculosis show clinical symptoms resembling symptoms of Crohn's disease in humans. Crohn's disease is a multifactorial disease and MAP is "suspected" to be one of its triggers. The presence of MAP in muscle tissue from almost half of the mouflon tested in this study would, for this reason, seem significant. In view of the increasing popularity of game, certain preliminary recommendations formulated in this article should be taken into consideration in order to reduce the risk of consumption of the meat of game animals contaminated with MAP.

Mouflon, muscle tissue, paratuberculosis, real-time quantitative PCR

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a known causative agent of the chronic disease of the gastrointestinal tract in animals known as paratuberculosis (Johne's disease). This disease is seen most frequently in cattle, though it also occurs commonly in wild ruminants and smaller mammals. A characteristic feature of paratuberculosis is its long incubation period. No clinical symptoms are evident in infected animals during the incubation period, although the agent of paratuberculosis is excreted into the external environment. Clinical symptoms (diarrhoea, emaciation, cachexia, reduced milk yields) appear in cattle a number of years after infection. No clinical symptoms at all are generally seen in wild animals. Clinically and sub-clinically infected animals excrete MAP in faeces which then serve as a source of infection for other healthy animals.

Pathological lesions appear in the mucous membranes of the digestive tract in animals affected by paratuberculosis. These lesions show considerable similarity to intestinal lesions in humans affected by Crohn's disease. Crohn's disease is considered a disease that may be caused by many factors. The most important of these are genetic and immune factors, external environmental factors and infectious agents such as MAP (Economou and Pappas 2008). Milk is the most important source of MAP for man. Generally speaking, the presence of MAP in milk, and similarly in meat, is associated either directly with ongoing illness and the dissemination of the infectious agent in the tissues and organs of the animal or indirectly with secondary environmental contamination.

MAP has been described in animals with a disseminated infection not only in the digestive tract and the mesenteric lymph nodes, but also in other organs such as the liver, spleen, kidneys, lungs, heart and reproductive organs (Pavlik et al. 2000; Antognoli et al. 2008; Mutharia et al. 2010). Some of these organs are widely used for human consumption. Muscle tissue may be contaminated by lymph nodes containing MAP (Antognoli et al. 2008). The agent of paratuberculosis has been described in the

Address for correspondence:
Mgr. Iva Slaná, Ph.D.
Veterinary Research Institute
Hudcova 70, 621 00 Brno
Czech Republic

Phone: +420 777 786 711
E-mail: slana@vri.cz
www.maso-international.cz

past in the diaphragm pillars and the masseter of cattle (Alonso-Hearn et al. 2009; Pribylova et al. 2011), in beef steak (Mutharia et al. 2010), in mutton forequarters and rump steak (Reddacliff et al. 2010) and in the biceps muscle of sheep (Smith et al. 2011). The presence of MAP has not been proven to date in the skeletal muscle of wild ruminants.

Cattle with paratuberculosis are sent to the slaughterhouse where the meat of the given animals is further processed for human consumption after removal of tissue with visible tuberculous lesions. In view of the merely sporadic occurrence of clinical symptoms of paratuberculosis in wild animals, these animals pass through the inspection process "unnoticed". MAP is known to be able to survive for a considerable period of time in various environments and can also survive pasteurisation temperatures. This indicates that meat and meat products that have not been adequately heat treated represent a significant risk to consumers. The aim of this study was to test the muscle tissue (back or leg muscle) of mouflon reared in 9 game enclosures in the Czech Republic by means of real-time quantitative PCR (qPCR). Samples of faeces were also taken from each animal to determine the presence of the agent of paratuberculosis in order to determine the current state of health of the animals.

Materials and Methods

Forty-eight mouflon (*Ovis musimon*) from 9 game breeding enclosures in the Czech Republic were included in the study. Paratuberculosis had been detected by cultivation methods at all these enclosures in the past. The group of tested mouflon included animals that had died of natural causes, animals that had been eliminated due to the occurrence of clinical symptoms of paratuberculosis, and animals shot during the hunting season.

Faeces were taken from the rectum of each animal with the use of disposable gloves and a sample of muscle tissue taken in a sterile manner from the leg or back of each animal. The samples were transported to the laboratory at a temperature of 4 °C and, unless processed directly following transport, frozen at -70 °C. Histopathological examination of each animal (intestine, ileocaecal region) for the presence of granulomatous lesions was performed.

The DNA was isolated from faeces with a commercial QIAamp DNA Stool Kit (Qiagen). The procedure for the isolation of DNA from faeces was modified in accordance with a paper previously published by Kralik et al. (2011). Briefly, 0.5 g of faeces was divided into two test tubes and buffer ASL and zirconia/silica beads (Biospec) added to each. Following incubation, the sample was homogenised in a MagNA Lyser instrument (Roche), centrifuged, and 0.75 ml of the liquid phase taken from each test tube and added to a test tube containing an InhibitEx tablet. Column purification of the supernatant was performed following further homogenisation and centrifuging. The purification method is described in greater detail in the paper by Kralik et al. (2011). The DNA was isolated from tissue with a DNeasy Blood & Tissue Kit (Qiagen); the isolation method has been described in the publication by Slana et al. (2010). Briefly, 0.5 mg of tissue was lysed with buffer ATL (Qiagen) and Proteinase K (Sigma) at 56 °C. This was followed by homogenisation in the presence of zirconia/silica beads in a MagNA Lyser instrument. The DNA was then precipitated with ethanol and purified in a kit column.

The DNA isolated from faeces and tissue was used for amplification of MAP specific insertion sequence IS*900* by the qPCR (Slana et al. 2008). An internal amplification control enabling detection of the presence of possible PCR inhibitors in the sample was added to each qPCR reaction. Absolute quantification of MAP cells in the sample was performed on the basis of a calibration curve derived from a decimal dilution of a plasmid with a known number of IS*900* copies. This calibration curve was part of each qPCR. The absolute number of MAP cells in the sample (per gram of faeces or tissue) was calculated on the basis of previously determined information (Kralik et al. 2011; Pribylova et al. 2011). Duplicate tests were performed on each sample.

Results

Thirty-three of the 48 mouflon (68.8%) returned a positive result for at least one tested sample (back muscle, leg muscle, faeces). Muscle tissue was MAP positive in 16 cases (48.5% of positive results); in 12 of these (36.4% of positive results) the faeces was also found to be positive; the faeces was negative in the remaining 4 animals (12.1% of positive results). The occurrence of MAP in faeces (independent of the results for muscle tissue) was demonstrated in 29 mouflon (87.9% of positive results). The concentration of MAP cells in the faeces ranged from 10^7 to 10^1 (MAP cells per gram of faeces); the number of MAP cells per gram of muscle tissue ranged from 10^4 to 10^1 (Table 1).

Table 1. Findings of *Mycobacterium avium* subsp. *paratuberculosis* in mouflon muscle and faeces with IS*900* real-time quantitative PCR

Animal number	Cells per gram	
	Muscle tissue (back / leg)	Faeces
1	3.62×10^4	5.39×10^7
2	1.49×10^4	3.68×10^7
3	2.74×10^4	2.60×10^2
4	1.32×10^4	-
5	1.16×10^3	3.69×10^6
6	2.20×10^3	3.35×10^2
7	2.89×10^3	5.54×10^2
8	2.20×10^3	3.52×10^1
9	3.10×10^3	4.85×10^1
10	1.14×10^3	-
11	1.06×10^3	-
12	2.25×10^3	-
13	6.11×10^2	1.37×10^2
14	3.97×10^2	4.19×10^2
15	7.30×10^2	1.32×10^2
16	7.16×10^1	4.57×10^6
17	-	3.26×10^4
18	-	2.11×10^3
19	-	1.04×10^3
20	-	1.57×10^3
21	-	5.15×10^3
22	-	2.11×10^3
23	-	1.95×10^2
24	-	4.46×10^2
25	-	4.70×10^2
26	-	5.77×10^2
27	-	4.12×10^2
28	-	1.00×10^2
29	-	3.16×10^2
30	-	3.66×10^2
31	-	2.34×10^2
32	-	5.88×10^2
33	-	2.50×10^2

Discussion

Although slaughter animals are subjected to veterinary inspection at the slaughterhouse, only tissues and organs with clearly visible pathological changes are condemned. Such pathological changes are, however, only visible in animals in an advanced stage of infection in the case of paratuberculosis (Manning and Collins 2001). This means that animals with clinical paratuberculosis may find their way directly into the human food chain along with animals with sub-clinical symptoms of paratuberculosis (Grant 2005).

In this study, MAP was determined in the muscle tissue of both clinically ill animals (animals eliminated due to clinical symptoms) and animals that did not show clinical symptoms (natural death, commercial shooting). The presence of MAP in the muscle was, with the exception of 4 individuals, always accompanied by MAP in the faeces

(Table 1). The fact that MAP was not detected in the faeces of the given 4 individuals may have been caused by intermittent shedding of the agent of paratuberculosis in the faeces. Although animals infected with paratuberculosis excrete MAP bacteria in the faeces, there are certain periods of time when they are not excreted or when the number of bacteria excreted is beneath the level of detectability. When cultivation methods are used to detect the presence of MAP, the absence of bacteria in the faeces may be accredited (in addition to intermittent shedding) to the inadequate sensitivity of cultivation techniques or the use of aggressive methods of decontamination (Pavlik et al. 2000). In view of the fact that the extremely sensitive qPCR method was used for detection in this study, the likeliest explanation for the absence of MAP in the 4 mouflon above is the effect of intermittent shedding of MAP.

The presence of MAP was proven in the skeletal muscle of 48.5% (16 of 33 positive mouflon) of the group of mouflon tested. Alonso-Hearn et al. (2009) described the occurrence of MAP in 13% of samples of diaphragm muscle in cattle. The proportion of positive results in diaphragm muscle from cattle was significantly higher in another study, amounting to as much as 68% with the use of IS*900* qPCR (Pribylova et al. 2011). Pribylova et al. (2011) also demonstrated the agent of paratuberculosis in the masticatory muscles of cattle (38.9% positive by IS*900* qPCR). The presence of MAP in the muscle tissue of mouflon is evidence not merely of infection of the digestive tract, but also of the general dissemination of the agent of paratuberculosis throughout the body. The presence of MAP can also be expected to be seen in other organs in these animals, as well as their muscle tissue. Although the animals in question showed disseminated infection, not all the individuals showed even mild clinical symptoms of paratuberculosis. The fact that wild game may not necessarily manifest clinical symptoms of paratuberculosis represents a significant problem in terms of the spreading of the disease. Animals that look healthy at first glance, though which are in fact infected, may be a source of infection for individuals that actually are healthy.

Conclusions

Game has been increasing in popularity in recent years, and this meat is appearing on the consumer's menu ever more frequently. The role of MAP in the etiology of Crone's disease has never been either entirely confirmed or ruled out. The finding of MAP in the skeletal muscle of mouflon is a serious concern from the viewpoint of food safety considering the fact that there is no outbreak of clinical symptoms of paratuberculosis in the majority of wild game, and paratuberculous animals in a clinical and sub-clinical condition are regularly sent to slaughter. Meat is considered a marginal source of infection for man in comparison with cow's milk, though the conclusions of previously published studies in cattle and this study in mouflon indicate that the presence of MAP bacteria in meat is higher than might be expected. For this reason, provisional measures to minimise the risk to the consumer should incorporate the following recommendations:

1. Do not use the meat of game animals with clinical symptoms of paratuberculosis for human consumption.
2. Perform a thorough inspection of the intestines and the intestinal lymph nodes when inspecting wild game not showing clinical symptoms of paratuberculosis.
3. Do not consume raw or undercooked game meat.

Acknowledgements

The work was supported by project LO1218 of Ministry of Education, Youth and Sports of the Czech Republic under the NPU I program and by Grant No. QJ1210113 of the Ministry of Agriculture of the Czech Republic.

References

Alonso-Hearn M, Molina E, Geijo M, Vazquez P, Sevilla I, Garrido JM, Juste RA 2009: Isolation of *Mycobacterium avium* subsp *paratuberculosis* from muscle tissue of naturally infected cattle. Foodborne Pathog Dis **6**: 513-518

Antognoli MC, Garry FB, Hirst HL, Lombard JE, Dennis MM, Gould DH, Salman MD 2008: Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection in dairy cattle and its association with ante mortem test results. Vet Microbiol **127**: 300-308

Economou M, Pappas G 2008: New global map of Crohn's disease: Genetic environmental and socioeconomic correlations. Inflamm Bowel Dis **14**: 709-720

Grant IR 2005: Zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*: the current position. J App Microbiol **98**: 1282-1293

Kralik P, Slana I, Kralova A, Babak V, Whitlock RH, Pavlik I 2011: Development of a predictive model for detection of *Mycobacterium avium* subsp. *paratuberculosis* in faeces by quantitative real time PCR. Vet Microbiol **149**: 133-138

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

Mutharia LM, Klassen MD, Fairies J, Barbut S, Gill CO 2010: *Mycobacterium avium* subsp. *paratuberculosis* in muscle, lymphatic and organ tissues from cows with advanced Johne's disease. Int J Food Microbiol **136**: 340-344

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

Pribylova R, Slana I, Kralik P, Kralova A, Babak V, Pavlik I 2011: Correlation of *Mycobacterium avium* subsp. *paratuberculosis* counts in gastrointestinal tract, muscles of the diaphragm and the masseter of dairy cattle and potential risk for consumers. Int J Food Microbiol **151**: 314-318

Reddacliff LA, Marsh IB, Fell SA, Austin SL, Whittington RJ 2010: Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from muscle and peripheral lymph nodes using acid-pepsin digest prior to BACTEC culture. Vet Microbiol **145**: 122-128

Slana I, Kralik P, Kralova A, Pavlik I 2008: On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS*900* and *F57* competitive real time quantitative PCR and culture examination. Int J Food Microbiol **128**: 250-257

Slana I, Kaevska M, Kralik P, Horvathova A, Pavlik I 2010: Distribution of *Mycobacterium avium* subsp. *avium* and *M. a. hominissuis* in artificially infected pigs studied by culture and IS*901* and IS*1245* quantitative real time PCR. Vet Microbiol **144**: 437-443

Smith SI, West DM, Wilson PR, De Lisle GW, Collett MG, Heuer C, Chambers JP 2011: Detection of *Mycobacterium avium* subsp. *paratuberculosis* in skeletal muscle and blood of ewes from a sheep farm in New Zealand. N Z Vet J **59**: 240-243