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Review

Paratuberculosis in Animals and Humans – an Actual Health and Economic Problem

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Abstract

Mycobacterium avium subspecies *paratuberculosis* is the etiological agent of paratuberculosis in animals (Johne's disease) and in humans (Crohn's disease). The review summarized the etiology, epidemiology, pathogenesis and diagnosis of the disease in domestic and wild animals, and humans as well as future challenges to diagnostics, control prebiotics promoting the growth of probiotic compounds public health concern. Special attention is paid to the enormous economic losses worldwide of affected farms associated with decreased production of milk, meat and their products, increased incidence of mastitis and infertility in animals, and higher costs of in-patient care, ambulatory care and drugs for humans. The necessity to develop a National program for rapid diagnosis, monitoring and control of paratuberculosis in Bulgaria, similar to such programs in other affected countries, is explained. In addition, this program will enable the conducting epidemiological analyses and assessment of the risk to animal and human health.
Keywords: paratuberculosis, animals, humans, etiology, pathogenesis, diagnosis, control

Mycobacterium avium subspecies paratuberculosis е етиологичният причинител на паратуберкулозата при животните (болест на Джон) и хората (болест на Крон). Този обзор разглежда етиологията, епидемиологията патогенезата и диагнозата на заболяването при домашните и дивите животни и хората, както и новите предизвикателства пред диагностиката, контрола и общественото здравеопазване. Обръща се внимание на големите икономически загуби в световен мащаб, които паратуберкулозата причинява на засегнатите ферми, поради намаленото производство на мляко, месо и продукти от тях, увеличаването на маститите и безплодието при животните и разходите за лечение на заболелите хора – медикаменти и амбулаторно обслужване. Обосновава се и необходимостта от разработване на Национална програма за бърза диагностика, мониторинг и контрол на паратуберкулозата в България, подобно на прилаганите такива в други засегнати от заболяването страни. Тази програма ще позволи провеждане на епидемиологични анализи и съвременна оценка на риска за здравето на животните и хората.

Paratuberculosis in domestic animals

History and world prevalence of paratuberculosis

Paratuberculosis is one of the oldest diseases in cattle described in 1829 in England by Johne, and Frothingham in 1895. For the first time they discovered an acidalcohol-resistant or the first time they discovered an acidalcohol-resistantbacillus in the intestine of a bovine animal affected with chronic diarrhea (Johne and Frothingham, 1895). In the USA the disease was discovered in cattle in the early 1900s, and in New Zealand was first diagnosed in 1912 (Kopecky, 1961). Infectivity of the disease was proved by Bang in 1906. Later, in 1907, McFadyean described the disease in England, and in 1908 Vucovic reported paratuberculosis in sheep in Bosnia (Chiodini *et al.,* 1984). Twort (1910) and Twort and Inggram (1913) obtained a pure bacterial culture and gave a general description of the disease named "Johne's disease" (Huhn, 1965). The bacterium was named *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) at the suggestion of Thorel *et al*. (1990).

Paratuberculosis is a chronic, progressively

developing intestinal infection mainly affecting domestic large and small ruminants (cattle, buffalo, sheep, goats, deer, camels, lamas, mouflons), some non-ruminant animals (horses, pigs, rabbits, martens, foxes, weasels, monkeys, chimpanzees) and humans (Pacetti *et al*., 1994; Chamberlin *et al*., 2000; Behr and Collins, 2010; Savova *et al*., 2013; Pradenas *et al*., 2014; Koev *et al*., 2015)

Paratuberculosis is a widespread chronic bacterial disease of ruminants in agriculturally developing and developed countries. Most affected are the countries with bigger production of milk and dairy products, in which 20% -80% of herds are infected (USA, Canada, Japan, Australia, Norway, Iceland, the Netherlands, Belgium, Denmark, Chile) (Herman *et al*., 2005; Masalski, 2008; Salgado *et al.,* 2009, 2011a, 2011b; Pradenas *et al*., 2014). Due to the prevalence in the world for 2004 (7-55% in Europe, 40% US, 9-22% Australia), the disease is categorized by the World Organisation for Animal Health (OIE) in sheet B as a disease with serious economic and health significance (OIE, 2014). OIE records show that 44% of member countries reported paratuberculosis during the past decade (Ryan and Campbell, 2006).

In Bulgaria, Kuyumdjiev first reported the presence of paratuberculosis in cattle in 1950, and seven years later, Ivanov described it in sheep. The disease was studied in more details by Belchev, being determined as an autohtonous infection in some areas of the country (Belchev, 1977). Nowadays, more than three decades later, there is no accurate data on the prevalence of paratuberculosis in animals in the official veterinary medical statistics in Bulgaria. The Veterinary Service in the frame of Bulgarian Food Safety Agency does not register the disease because of its rare manifestation and other different reasons.

Indirect evidence of the disease may be obtained by the presence of nonspecific reactions to PPD-tuberculin during mandatory allergy tests for tuberculosis in cattle and buffaloes. These reactions are often due to infection of animals with other non-tuberculous mycobacteria (Bachvarova *et al.,* 1999). The subclinical cases of the diseases are not tested and monitored. The purposive and systematic research of large and small ruminants in the country is not regulated due to absence of a National Surveillance Program. In many European countries (Sweden, Denmark, Holland, Germany, France, Poland, Hungary, Slovenia) and in the USA and Australia as well, National Programs for the control of animals and their products have been developed. In Sweden, the National Program was built on the principle of "stamping out", and all imported animals are tested for paratuberculosis.

In 2014, in Northeastern Bulgaria several cases of paratuberculosis in a dairy farm were registered (Koev *et al.,* 2015). The diseased cows demonstrated diarrheal syndrome, birth of non-viable calves or presence of postnatal complications, severe dehydration and death. In recent years, the importation of cattle from other countries has increased significantly and therefore early and reliable diagnosis and the epidemiological status concerning this zoonotic disease in our country is imperative.

Paratuberculosis in wild animals

Paratuberculosis is a serious problem in wild and captive animals too. Jarmi first reported cases of paratuberculosis in antelope living in a zoo in 1922. In 1949, Dorofeev and Kalacheva described the disease in deer (by Belchev, 1977). Before 1970, isolated cases of paratuberculosis were described in deer, but later many reports of the disease appeared. In 1973, Strogov described cases of paratuberculosis in reindeer in northern Russia, and in 1979 Riemann and co-authors observed it in California in sika deer (Strogov, 1973; Riemann *et al*., 1979). Temple et al. (1979) described the disease in deer, and Jessup *et al.* (1981) found *Map* in elk and white-tailed deer in the US. Pacetti *et al.* (1994) reported paratuberculosis in wild and zoo animals. In Spain, Marco *et al*. (2002) found paratuberculosis in fallow deer. In the Czech Republic the disease is common for fallow deer, roe deer and red deer (Pavlik *et al*., 2000; Machackova *et al.,* 2004). Infection with *Map* has been described in wild and captive deer populations in many other countries (de Lisle *et al*., 1993; Power *et al*., 1993; Fawcett *et al*., 1995; Manning *et al.,* 1998; Godfroid *et al*., 2000; Paolicchi *et al*., 2001; Alvarez *et al*., 2005; Machackova-Kopecna *et al*., 2005; van Kooten *et al*., 2006; Mackintosh *et al*., 2007). Cases of paratuberculosis in red deer in the region of the Italian Alps and Austria have been described too (Glawischnig *et al*., 2006). Tryland and collaborators (2004) carried out serological studies in Norway and showed evidence of the disease in mice, red deer, roe deer and reindeer.

Since 1980, paratuberculosis has been an emerging problem in deer farming with important underestimated losses, mainly due to outbreaks of the clinical disease (Mackintosh *et al*., 2002). In the southern regions of Chile, paratuberculosis is im-

ported from Argentina, New Zealand and Europe, and is widespread among deer used for hunting and meat production (Mereb *et al*., 1994; Lyle *et al.,* 2003; Pradenas *et al.,* 2013). Paratuberculosis has been reported to occur in wild ruminant species, including deer (Chiodini and Kruiningen, 1983), bison (Buergelt and Ginn, 2000) and elk (Manning et al, 2003), as well as nonruminants, such as wild rabbits (Greig *et al*., 1997), their predators, including foxes and stoats, and primates, such as mandrills and macaques, indicating a wide host range (Beard *et al*., 2001). Due to the close relation between wild and domestic animals in some areas, it is suggested that other wild and captive animal populations could be infected, too (Salgado *et al.,* 2011a). Infections in free-ranging and captive wildlife are also of great concern. Up to one-third of zoos accredited by the American Zoo and Aquarium Association have reported at least one culture-confirmed case of paratuberculosis since 1995 (Manning and Ziccardi, 2000).

In Bulgaria paratuberculosis in wild animals is not well investigated. In the period 2011-2013, 50 samples of shot and dead deer were studied, hinds and mouflons, grown freely in a hunting area in Bulgaria. Histological and microbiological findings typical of paratuberculosis were established in 7 of them and were confirmed by a molecular genetic analysis (Savova *et al*., 2013). These results show the need of a systematic study on the prevalence of this disease in wild animals in their natural habitat. The increase of paratuberculosis in wild animal species is assumed to be caused by the purchase of animals, a strong increase in suckler cow farming (cow-calf herds) with a concentration of pathogens in the environment.

Economic losses and control of paratuberculosis

Paratuberculosis is an important disease with considerable economic consequences. The disease causes significant financial losses through decreased milk production in dairy cattle and meat yield from slaughtered animals (around 59-60 kg less), reduced calf production from both dairy and beef cattle, death of the deseased cows and increased veterinary and related medical costs. During the course of the disease, the absorption of nutrients in normal food intake decreases and animals progressively lose weight (Pavlik, 2006). Besides losses from exhaustion and death, other reasons for financial costs are a significant increase in mastitis, infertility, susceptibility to other infections and disposal of the infected animals (Merkel *et al.,* 1975). In clinically affected dairy cows a 16% decrease was observed in milk production between the current lactation and the lactation two seasons previously. In 1987, the net cost to EU farmers with subclinical Map infection in their animals was estimated at £209 per infected dairy cow (Benedictus *et al*., 1987). Other economic losses come from the cost of programs undertaken by government agencies to reduce the value of the loss of breeding animals (Pavlik, 2006). In the USA, the economic losses due to this infection in livestock reach $ 1.5 billion annually. The cost of reduced productivity reaches $ 200-250 million per year (Harris *et al.*, 1998). Losses from dairy farms are about $ 100 per cow in moderately infected herds and increase to over $ 200 per cow in heavily infected herds, mainly due to the decrease in milk production and increased cow replacement costs (Ott *et al*., 1999).

The control of paratuberculosis can improve animal health and welfare, increasing productivity, reducing potential problems in the market and improving the overall business profitability. The benefits that can be derived from the control of paratuberculosis should be communicated to all stakeholders in the industry to encourage the development and implementation of control programs.

Etiology and pathogenesis

The causative agent of paratuberculosis, *Mycobacterium avium* ssp. *paratuberculosis* (*Map*), is one of the four members of the *Mycobacterium avium* complex (MAC), known also as *M. avium intracellulare* complex (MAIC) - *M. avium* ssp. *avium*; *M. avium* ssp. *silvaticum*, *M. avium* ssp. *paratuberculosis* and *M. avium* ssp. *hominissuis* (Inderlied, 1993). On the basis of growth characteristics and different molecular typing, *Map* strains are classified into three types (I, II and III). Strains of type I and III share similar phenotypic traits and have been isolated mainly from sheep and goats, and rarely from wildlife (S' type). Type II strains were primarily isolated from cattle but also from small ruminants, different wildlife species (predominantly in wild cervids) and humans (C' type). They are the most common in Europe.

The bacteria are acid-fast, aerobic, immobile, non-spore or capsule forming bacilli (1-2 μm by 0,5 μm). In Ziel-Neelsen (ZN) stained smears short, pink or red rods in clumps or single are observed (Fig. 1a). *Map* is a very slow-growing organism, demanding the presence of iron (mycobactin) in culture media for *in vitro* growth and cultivation (Collins, 1996). The appearance of colonies (Fig.

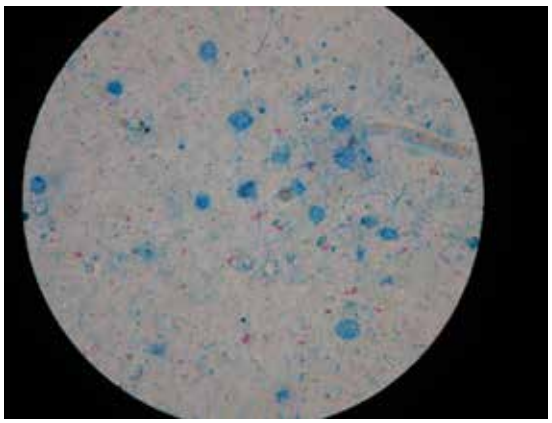

Fig. 1a. Light microscopy of ZN-stained smear (1000X)

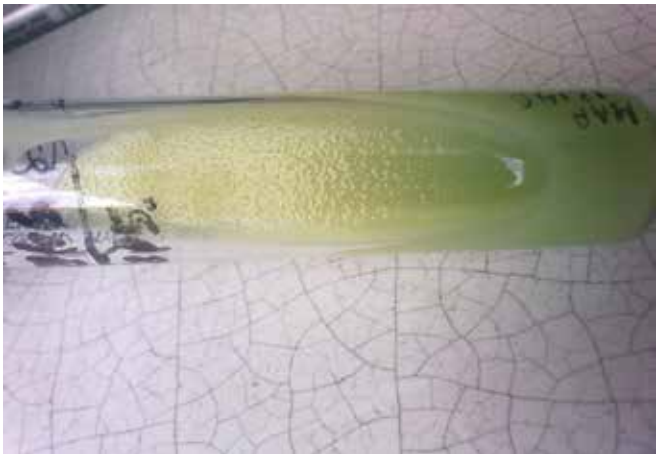

Fig.1b. Bacterial growth of *M. paratuberculosis* on solid Herold's medium with Mycobactin J

1b) usually occurs 12–16 to 24 weeks when *Map* is isolated from cattle, and up to 9 months when isolated from sheep (Belchev, 1977; Masalski, 2008). The slow growth rate may be due to the presence of a thick, waxy and complex cell wall, primarily composed of mycolic acids and arabinose derivatives.

Like other mycobacteria, *Map* possesses a lipid-rich cell wall, thus resulting in acid fastness, hydrophobicity and increased resistance to chemicals and chlorine. Paratuberculous mycobacteria are also resistant to drying, low temperature and decay. In water they survive 3-7 months, and in the soil from 3 months to 5 years (Watando, 2001). Bacteria are isolated from infected intestine, stored for 18 months in a refrigerator at 4°C and after one year storage at a freezing temperature (-14ºC). UV rays kill bacteria for 100 hours, but in boiling water they die after 2 min (Belchev, 1977; Hruska and Kaevska, 2012). In naturally contaminated with *Map* bovine faeces exposed to a variety of natural conditions as freezing, drying, sunlight, high and low temperatures and rain, bacteria have been isolated between 152 and 246 days (Manning and Collins, 2001). Live bacteria could still be isolated after pasteurization of milk (Herman *et al.,* 2005; Mundo *et al.,* 2013). Experiments with *Map* contaminated milk products (yoghurt and fermented milk products containing probiotic cultures) stored for 6 weeks at 6°C showed that *Map* counts in yoghurt did not change substantially, while in fermented milk products containing probiotic cultures, *Map* numbers decreased but live cultures were still isolated (Van Brandt *et al.,* 2011).

After ingestion, *Map* enters the intestinal tissue through the M-cells in the Peyers patches of the small intestine. Regional enterocytes may also be involved in the uptake of the bacterium. The bacterial cells are then phagocytosed by resident subepithelial macrophages or dendritic cells, in which they multiply. *Map* is able to survive within the macrophages by inhibiting phagosome acidification and phagolysosomal fusion. This is a crucial process in the establishment and evolution of the disease. With disease progression, infected macrophages migrate into local lymphatics, resulting in bacterial spread to mesenterial lymph nodes. The infection may be disseminated, with mycobacteria detectable in extra-intestinal lymph nodes (mammary, pulmonary, hepatic and head) and associated organs.

Paratuberculosis refers to "open" infections with large excretion of the causative agent in the environment (Kruze et al., 2006). The animals secrete mycobacteria with faeces, semen and breast milk without showing clinical symptoms (Sweeney, 1996). The bacteria can also enter the bloodflow after intestinal disorders (Harris, 1998). Drinking water, where the paratuberculous bacteria can survive for 7-9 months, has an important role in the transmission of the infection. Healthy herds are infected by sharing pastures and water sources with sick animals (Belchev, 1977). The disease has a zonal character. Predisposing factors are acidic soils and soils deprived of micronutrients and macronutrients, which provide a suitable environment for paratuberculous mycobacteria.

Subsequent to infection, *Map* is initially controlled by a T-helper (Thl) response with IFN-y production. The progression of the disease from a subclinical to a clinical state is associated with a switch from a Thl to a Th2 immune response, leading to antibody production that is less effective in controlling infection.

Young animals (cattle, buffaloes, sheep and goats) are thought to be most susceptible to natural infection with *Map* by close contact with con-

taminated faeces, feed, water, colostrum and milk or indirect contact with infected animals (Pavlik *et al.,* 2010). Carnivores may be infected by eating an infected prey species. Contaminated colostrum and milk produced by infected dams is a major route of transmuission for neonates. Transmission occurs mainly by the horizontal faecal-oral route, or vertically through the placenta to the fetus or through infected semen (Larson and Kopecky, 1970; Chiodini *et al*., 1984; OIE, 2014). The intrauterine transmission, although less prevalent, is more common in farmer deer than in cattle or sheep.

Judge *et al*. (2006) investigated routes of intra-species transmission of *Map* in rabbits through random selection of the rabbit population. Four hundred and eighty-seven rabbits were sampled from two sites in Scotland, where *Map* had previously been isolated from the livestock and rabbit populations (Greig *et al.,* 1997; Greig *et al.,* 1999). No pathology was noted in any animal, but *Map* was isolated from the testes, uterus, placenta, fetuses, and milk in rabbits at both sites. The obtained results provide evidence for the potential transmission of *Map* in rabbit populations via vertical (transplacental), pseudovertical (ingestion of contaminated milk or feces) and horizontal routes. Vertical and pseudo-vertical transmission occurred in 14% of offspring entering the population at 1 month of age (Judge *et al*., 2006).

The presence of these routes of transmission within natural rabbit populations will contribute to the maintenance of *Map* infections within such populations and, therefore, the environment. These observations support the assumption that rabbits are a significant true reservoir and source of the *Map* livestock. Therefore, it is essential that rabbits should be included in the management and control strategies of paratuberculosis in farmed ruminants.

In the epidemiology of *Map* infection, recently the possible role of synanthropic rodents has been reported. Ruminants could be infected by sick rodents through contaminated faeces, food or water (Beard *et al.,* 2001). *M. paratuberculosis* has been isolated from internal organs and feces of asymptomatic rodents, e.g. from the brown rat and common vole (Durnez *et al.*, 2008; Kopecna *et al.,* 2008)

Studies of authors reporting the relationship of the disease between wild and domestic animals have aroused the interest of veterinarians, foresters, ecologists, etc. In their view, wild animals can become infected from domestic ruminants in jointly inhabited regions (Riemann *et al*., 1979; Marco *et al.,* 2002). Chiodini and Van Kruiningen (1983) have proved the transmission of the infectious agent from deer to cattle. In wildlife, paratuberculosis is running subclinically being characterized by low mortality, due to the relatively low virulence of mycobacteria (Jessup and Williams, 1999).

Infection is not always accompanied by a disease. The organism of the infected animals may develop cell mediated immunity and remain healthy. The animal can get rid of the infection or remain a carrier for long period of time. This means that the greater the amount of mycobacteria , the more likely it is for infection to occur.

Clinical symptoms and phatomorphological chances

Johne's disease is a chronic infectious inflammation of the intestinal tract of ruminants (paratuberculous enteritis). The subclinical form of infection is most commonly found in the affected populations. The disease remains hidden, but animals are carriers and emitters of the infectious agent. This makes them a potentially infectious threat to the rest of the herd. In cows with subclinical mastitis, a reduction in mammary secretion and infertility can be observed (Iliev, 1972; Belchev, 1977; Jungersen, 2002). These animals show reduced effectiveness in the utilization of the feed.

The typical clinical manifestations in sick animals are progressive afebrile weight loss and reduced food intake with preserved appetite, chronic persistent greenish diarrhea, diffuse intermandibular oedemas and eventual death (Machackova-Kopecna *et al*., 2005). Death occurs as a result of cachexia and dehydration of the organism (Suárez, 2006). In contrast to cattle, diarrhoea is not a feature in small ruminants. This is probably due to their greater ability to reabsorb water in the large intestine, though in advanced cases the faeces may become soft and unformed (Machackova-Kopecna *et al.,* 2005). Chiodini *et al.,* (1984) reported that animals with clinical form of infection excreted up to 10^{10} CFU/g in the faeces. Actually, animals contribute infectious material to a common environment and this environment serves as an indirect source of transmission The dominant transmission strategy for *Map* is not well known, and the distinction between the two transmission pathways, direct or indirect, remains poorly elucidated (Heuer *et al*., 2012). This year, Slater *et al*. used the term "super-shedders" for infectious individuals that contribute a disproportionate amount of infectious pathogen load to the environment. A super-shedder host may produce up to 10 000 times more pathogens than other

infectious hosts. Moreover, the authors showed that the effect of super-shedders for *Map* is limited and that the effect of the individual bacterial load is limited and the relationship between bacterial load and the infectiousness is highly concave. A 1000-fold increase in the bacterial contribution is equivalent to up to a 2–3 fold increase in infectiousness (Slater *et al*., 2016). Paratuberculosis is characterized by an extended incubation period. The disease symptoms do not occur in animals before 3-5 years, and even at a more mature age (Stabel, 2004).

The main pathological changes affect the intestine, lymph and lymphoid tissues. In advanced clinical cases a gelatinous atrophy of fat stores and serous effusions in body cavities are observed. In sheep and goats with paratuberculosis major changes are often lacking or difficult to detect (Clarke, 1997). In cows, postmortem, mostly affected are the small intestine and mainly the ileum (Figure 3), but changes may be detected in the jejunum and colon. They are thickened, soft to the touch, hyperemic, and in advanced disease the surface of the bowel is folded like cerebral cortex (Figure 4).

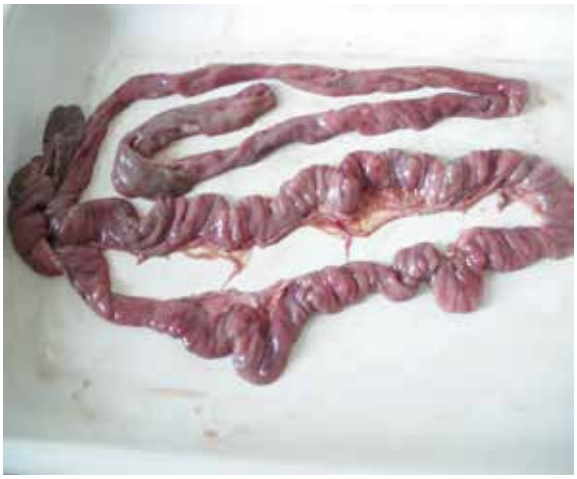

Fig. 3. Highly pleated small intestine of mouflon

Fig.4. Intestinal tract of deer pleated as „cerebral cortex“

In sick animals enlarged mesenteric lymph nodes and dilation of mesenteric lymphatic vessels were observed (Iliev, 1977; Savova *et al*., 2013; Pradenas *et al*., 2014; Koev *et al*., 2015). In the mucosa of the small intestine, including the intestinal villi and propria, a proliferative inflammation is established by histological examination. Granulomas without caseosation and presence of macrophages with foamy cytoplasm and large number of epithelioid cells were observed. The giant cells of Langhans-type are absent or single cells could be found (Pradenas *et al*., 2014).

Diagnosis of paratuberculosis

Diagnosis of paratuberculosis, especially subclinical cases, continues to be a challenge in animals and humans. The diagnosis in sick ruminants is based on *Map* detection in faeces and milk as well as patho-anatomical and histopathological examination of tissues originating from the lower part of the jejunum, ileum, ileocecal valve and the associated lymph nodes (Clarke, 1997; Kruze *et al.,* 2013). Diagnosis of the disease is a difficult, long and labour-consuming process that depends on the stage of development of the disease and the extent of its spread in the herd. It is hampered by the lack of single diagnostic methods that can be used to detect both forms of the disease - subclinical and clinical. The histopathological observation of lesions is insufficient to confirm the disease, especially due to the similarity of lesions caused by *Map* and *M. avium* (*Maa*). Co-infection with *Map* and *Maa*, which causes clinical disease and possibly death, complicates bacteriological diagnosis of mycobacterial infections (Godfroid *et al.,* 2005). Furthermore, lesions detected during the slaughter of animals subclinically infected with paratuberculosis, may lead to problems because their lesions were similar to those of tuberculous animals (Campbell, 1995).

The degree of reliability of the methods used largely depends on the stage of infection in infected herds. Diagnostic techniques of paratuberculosis aimed, on the one hand, to carry out screening and monitoring of herds for the presence of disease and the degree of infection, and on the other, to determine the individual status of the infected animals. The diagnosis of paratuberculosis in the wild is hampered by the low virulence of *Map*, chronic or subclinical course of the disease and the lack of effective diagnostic tests (Jessup and Williams, 1999). Therefore, the authors focus on prevention of the disease**,** the aim being to reduce animal den-

sity and to limit their transportation (Marco *et al,* 2002). For the diagnosis of *Map* infection in live animals two approaches are used:

- direct isolation of the bacterial causative agent from fecal and milk samples cultivated on a differentiating nutrient media, and in recent years accomplished by PCR;

- indirect diagnosis by determining the immunological status of the animals through tuberculinization and serological testing with ELISA and gamma interferon test.

Detection of the causative agent

Direct microscopy of ZN-stained smears from faeces, intestinal mucosa or lymph nodes is a rapid, economical way for obtaining a diagnosis. The diagnosis depends on the presence of short, red-staining rods clustered in clumps bacteria, showing intracellular growth, which is typical of *Map*. The presence of predominantly single acid-fast bacilli is unconvincing as they can be environmental mycobacteria. The test has a low sensitivity in the early stages of the disease, but when the animal reaches the clinical stage, the test reaches virtually 100% sensitivity.

The isolation of a *Map* culture from faeces during the progress of disease is the most sensitive test with 100% specificity and is therefore regarded as the "gold standard". However, the test is very labour-intensive, time consuming and expensive. It is not standardized and varies considerably in different qualified laboratories. The decontamination procedure for the tested samples, especially faeces, is an essential step in all used methods. Cultivation of bacteria may take several months. In order to complete successfully the elimination of the fast growing bacteria, a crucial step is the effectiveness of decontamination. At present there are two basic methods in use for culturing *Map* from clinical specimens: the method using oxalic acid and NaOH for decontamination and Löwenstein-Jenssen medium for growth, and the method using hexadecylpyridinium (HPC) for decontamination in combination with Herrold's Egg Yolk Medium (HEYM). Both media contain mycobactin. Colonies can be observed after 4 to 24 weeks or more.

A faster method for isolation of *Map* is by using the radiometric system BACTEC 460. The growth of mycobacteria is measured by the release of $^{14}CO_2$ from palmitate in bacterial metabolism (Collins *et al.,* 1990). The authors also introduced three other rapid methods based on fluorescence - BACTEC 9000, and both systems MGIT (Becton Dickinson) and Bact (Organon Technique). The introduction of these rapid techniques for isolation of *Map* (radiometric and fluorescence based methods) from fecal samples, create serious problems due to overgrowth of other bacteria (spore forms and fungi) and are currently unenforceable (Collins *et al*., 1990)

Molecular techniques have been very valuable for diagnosis and epidemiological investigations and have overturned many epidemiological assumptions. The technique of the polymerase chain reaction (PCR) may be used to identify specific mycobacteria in faeces or tissues and also to confirm identification of colonies. The PCR test offers an attractive alternative for the traditional culture methods. It has higher sensitivity and yields faster results from the culture. PCR is not only a more rapid test (1-2 days as opposed to six months), but it can also offer high levels of specificity. Today, various *Map*-specific insertion sequences (IS900, IS901, IS1245, f57, IS*Map*2, IS*Map*04) linked to avian virulence and applicable for detection and identification of *Map* by PCR have been revealed. Of these, IS900 is widely used as target for PCR detection of *Map*. This insertion sequence is specific for the bacterium and is present in 14 to 18 copies in the genome. The presence of IS900-like sequences in non-*Map* mycobacteria shows the need to verify the result of DNA sequencing. In order to improve the specificity and to make differentiation between single and mixed mycobacterial infections, a successful multiplex PCR has been developed (Moss *et al*., 1991; Ritacco *et al*., 1998; Stabel *et al.,* 2004).

Detection of an immune response to M. paratuberculosis

The diagnosis of subclinical paratuberculosis presents an important point in the control of this disease. Immunologic assays such as complement-fixation test (CFT), enzyme-linked immunosorbent assay (EL1SA) and agar gel immunodiffusion assay (AGID) are used to diagnose paratuberculosis in a herd. All these methods rely on the presence of antibodies to *M. paratuberculosis* in the blood serum. Because subclinical infected animals usually do not produce measurable antibody titers to the final stages of the disease, these tests are ineffective in detecting subclinical infection in a herd.

The complement fixation test (CFT) is the most widely used standard test for paratuberculosis in cattle. The test has sufficiently high sensitivity for detecting the advanced clinical cases, but is not

a completely reliable tool for the identification of less infected animals. Despite the fact that the outcome of the test gives no guarantee about the status of the individual animal, the CFT test is often the only valuable test requested for import and export of cattle within the EU (Ridge *et al*., 1991; Sockett *et al*., 1992; Yokomizo *et al.,* 1991).

ELISA is the preferred test for herd analysis because of the convenience of sample collection, rapid laboratory turnaround time and low cost of the test. The serum antibodies against *Map* are detected using different crude fractions of the bacterium; lipoarabinomannan (LAM) or PPD's. False positive results can occur as a result of cross reactions with related bacteria as *Corynebacterium*, *Mycobacterium* and *Nocardia*, or in countries where bovine tuberculosis is endemic. The occurrence of false positives is therefore also geographically dependant. A problem in accounting of the ELISA test may occur in the use of vaccination against paratuberculosis in cattle. The specificity of the test is increased in the so-called absorbed ELISA by absorption with sonicates from environmental mycobacteria, like *M. phlei* or *M. vaccae*. Since the commercial absorbed ELISA kits became available, it has become the most widely used serological test for diagnosis of paratuberculosis in cattle. To avoid receiving ELISA false positive results, it *Map* specific recombinant antigens can be used.

Reports on the sensitivity of the absorbed ELISA are contradictory (Collins *et al.,* 1991; Ridge *et al*., 1991; Sweeney *et al*., 1995). More reliable is the sensitivity of the test when ELISA is used in a herd or group of animals, where the disease has existed for a longer period of time because the serum antibodies are increased in the later stages of the disease. The test showed very low sensitivity in young, recently infected animals and therefore ELISA can be used to detect specific antibodies in farms where the disease has started spreading recently. Therefore, the test will be useful in programs for certification of flocks where a sufficient number or only older animals are tested.

Diagnosis of paratuberculosis is usually based on the combined results of faecal culture and ELISA (Sweeney *et al*., 1995). Both tests have their drawbacks: bacterial culturing is a slow, laborious and expensive process, and the sensitivity of ELISA is not sufficiently clarified, particularly for the final stage of eradication.

The agar gel immuno-diffusion test (AGID) can be used for the detection of paratuberculosis in different animals (cattle, sheep, goats and deer). However, the used antigens are not specific for *Map* (Shermann *et al.,* 1984). The relatively low cost of the AGID make this test very attractive for use in small ruminants (sheep and goats). Its sensitivity is lower than the ELISA test, being also closely related to the immunopathological forms of the infection. Sheep and goats with focal and tuberculoid forms (non-clinical infections) are usually negative (Garcia Marin *et al*., 1993). Clinical cases of sheep and goats with numerous bacterial forms are positive for AGID as it must be borne in mind that those which show lymphocyte forms are typically negative (Pérez *et al.,* 1997).

In general, the sensitivity of the AGID test is variable, depending on the group of investigated animals (Nyange, 1991). Like the ELISA, the AGID is based on the detection of antibodies against *Map*. Therefore, it has a relatively low sensitivity in the early stages of the disease, but becomes more useful in later stages.

The optimal diagnosis of paratuberculosis required a combination of T-cells (for early or subclinical cases of *Map* infection) and antibody tests (for chronic clinical cases) in order to identify both early and late cases. Cellular immunity tests can be performed *in vivo* or *in vitro*, using the intradermal test with PPD or johnin (the paratuberculosis equivalent) or the gamma-interferon assay, respectively. The cellular response is less pronounced than in the case of bovine tuberculosis, where the intradermal test is still the most reliable test. In the intradermal test, skin thickness is measured before and 72 hours after injection with PPD. An increase in thickness of more than 2 mm is regarded as a positive reaction.

The gamma-interferon assay is performed by stimulating aliquots of heparinised blood with PPD as the subsequent release of gama-interferon is measured by ELISA (Wood *et al.*, 1991). Sensitisation to avian-PPD or johnin is very common in animals because they contain many cross-reacting antigens. This interferes with the specificity of both tests and makes interpretation of the results more difficult (Billman *et al*., 1992). Moreover, there is a correlation between the positivity to cellular tests and animals showing focal-tuberculoid pathological forms. These are non-clinical and non-shedding animals (Perez *et al.,* 1994). This results in a decreased sensitivity of the tests and reduces their value compared to the much cheaper ELISA.

Treatment and vaccination against paratuberculosis

Drug treatment of paratuberculosis in animals is not recommended. Vaccination in cattle was first introduced in 1926 by Vallee and Rinjard. The vaccination was performed by subcutaneous injection of living, unattenuated paratuberculosis bacilli and was administered at 1 to 30 days of age, (subcutaneously) in the brisket, where the vaccinal nodule is less obtrusive. The vaccines were used in other species, mainly sheep involving subcutaneous administration in both young (15 days to several months old) and adult animals (Sigurdsson, 1960; Benedictus *et al*., 1987; Perez *et al*., 1995; Garcia Marin *et al.*, 1997). Revaccination is not recommended.

According to the guidelines of the specifications of the OIE, currently the World Organisation for Animal Health (WOAH), vaccines may contain live attenuated or killed bacteria incorporated with an adjuvant or freeze dried and adjuvanted on reconstitution bacteria (OIE Paris, 1996). The use of vaccination is still under debate. Following the opinion of some authors, vaccination is the best way to control the infection, especially in sheep and goats, though others conclude that vaccination alone is not sufficient for control of paratuberculosis (Sigurdsson, 1960; Marly *et al.,* 1988; OIE Paris, 1996; Garcia Marin *et al.,* 1997). The sensitisation caused by vaccination not only affects the diagnosis of paratuberculosis, but vaccinated animals become also sensitised to bovine PPD used in the intradermal test for control and eradication of bovine tuberculosis (Stuart, 1965)

Prevention and control of paratuberculosis

The control of the disease is related to diagnostic tests, combined with sanitation programs, and elimination of infected animals. The treatment of animals is unprofitable and is not recommended. In Bulgaria and in other countries, the main approach is the eradication of the disease, reducing *Map* positive herds and preventing the spread of infection to healthy flocks.

The best prevention is achieved by reducing the overpopulation of animals, providing good ventilation in barns, regular disinfection of premises, proper nutrition providing the necessary minerals and vitamins, and timely elimination and eradication of infected animals from the herd (Belchev, 1977; Suárez, 2007).

Paratuberculosis in humans

History

Crohn's disease (CD) is an inflammation of the gastrointestinal tract (from the mouth to the anus) in humans, causing a wide range of symptoms such as general malaise, abdominal pain, diarrhea, vomiting, chronic weight loss, skin rashes (Chiodini, 1989). OIE and WHO reported the prevalence of the disease not only among domestic and wild animals in various countries of the world including Europe but also increasing cases of human disease (Suárez, 2006; OIE, 2014).

A possible relationship between *Map* and Crohn's disease was reported in 1913 by Dalziel in Glasgow who first noticed similarities between general pathology and symptoms of John's disease in cattle and Crohn's disease in humans. In 1904 the disease was described by the Polish surgeon Antoni Leśniowski. Kron, Gainsbourg and Oppenheimer described in 1932 in Mount Sinai Hospital in New York eight cases of regional ileitis, and gave the name Crohn's disease (Crohn *et al*., 1932). Later on, the name of regional ileitis was replaced by regional enteritis when Lockhart-Mummery and Morson, in 1960, described the bowel disease and accepted that the disease is not restricted to the ileum. Subsequently, that entity was called by the USA clinician granulomatous colitis. Together with ulcerative colitis and unclassified chronic colitis, Crohn's disease belongs to a spectrum of diseases more generally designated as "Chronic inflammatory bowel diseases".

Theories of Crohn's disease

After isolation of *Map* from blood culture of a patient, it was assumed that *Map* (the causative agent of paratuberculosis in animals) is also one of the etiological factors in the human disease (Crohn's disease) (Golan *et al*., 2009). Although epidemiological studies are absent, the data of several authors show the relationship between these two diseases (Thompson, 1994; Suárez, 2006; Behr and Collins, 2010; Hermon-Taylor, 2009). This determines the zoonotic nature of the disease (Shafran, 2008; Golan *et al.*, 2009).

The cause of Crohn's disease remains still unknown, although some authors claim that in humans it is an autoimmune disease (Loftus, 2002; Segal, 2006). Now it is generally believed that there is a complex multifactorial etiology, including genetic predisposition, environmental factors (infectious agent, diet or smoking), and an abnormal inflammatory response.

Irrespective of the etiologic agent, several theories of disease mechanisms in Crohn's disease are currently under consideration: 1) a persistent infection, possibly involving mycobacteria (specifically *Map*), the measles virus, *Listeria* spp., *Escherichia coli*; 2) a defective mucosal barrier (leaky gut) which allows uptake of bacterial, dietary and other immunogenic macromolecules; 3) dysregulation of the host immune response with loss of tolerance, aggressive cellular activations and disorders of apoptosis; 4) genetic susceptibility factors; and 5) a combination of some or all of these reasons (Sartor, 1997, 2003; Shanahan, 2002).

Epidemiology of Crohn's disease

The disease is more prevalent in Western populations with northern European and Anglo-Saxon ethnic derivation than in populations of Southern Europe, Asia and Africa (Jayanthi *et al.,* 1992).

The incidence of Crohn's disease is around 13 people per 100,000 of the population in the UK. Similar incidence rates are reported for other countries in Europe (Shivananda *et al*., 1996). All this evidence suggests that the incidence of CD is increasing worldwide. Every year around 3,000 new cases of Crohn's disease are diagnosed in the UK. Crohn's disease typically affects young people between the ages of 16 and 25, although it can also occur in early childhood or later. Most patients with Crohn's disease have a chronic, intermittent disease course and treatment is multifaceted, including anti-diarrhoeals and anti-inflammatory agents to treat symptoms, immunosuppressive drugs aimed at disease remission, and surgery to remove diseased bowel or alleviate complications such as obstructions, stricture or fistula formation (Podolsky, 2002).

The World Congress of Gastroenterology in Vienna in 1998 categorized patients with Crohn's disease into 24 possible subgroups according to the disease location in the gastrointestinal tract, behaviour of disease and age at diagnosis (Gasche *et al.,* 2000). Patients with Crohn's disease are a heterogeneous group and the aetiology may not be the same for all patients.

Aetiology of disease

Crohn's disease is most commonly seen in the distal ileum, but can affect any part of the gastrointestinal tract. *Map* has been sporadically isolated from humans with Crohn's disease of resected tissue specimens after incubation for very long periods (>1 year). Chiodini *et al.* (1984) first isolated initially unclassified *Mycobacterium* sp., identified later as *Map*, by culturing specimens from resected ileum of three young patients with Crohn's disease (McFadden *et al.,* 1987). The obtained three strains were cultured 18 months for primary isolation and identified as Linda, Ben and Dominic. Later the authors reported that all the three strains were isolated initially as nonacid-fast coccobacillary forms that had ultrastructual appearance of spheroplast (cell wall deficient forms), which, after several months of incubation, were transformed into characteristic *Map*-like organisms (Chiodini *et al.,* 1986). This would explain why acid-fast cells were not readily observed in sections of Crohn's tissue by histological examination. Since the reported initial isolations by Chiodini *et al.* (1986), *Map* has been successfully cultured from resected tissue of Crohn's diseased patients in various parts of the world (USA, UK, The Netherlands, Australia, France, the Czech Republic, etc.), and also from breast milk of two lactating Crohn's patients in Florida (Naser *et al.,* 2000). With the MGIT culture system (Becton Dickinson) in the late 1990s, isolation rates of *Map* from resected tissue or biopsies from Crohn's patients have improved significantly: 86% of resected tissues and between 20 and 42% of biopsies from Crohn's patients, compared with 5-6 and 9% of biopsies from control patients positive for *Map*, but tested by culture (Schwartz *et al*., 2000; Bull *et al*., 2003). Naser *et al*. (2004) cultured for the first time a viable *M*ap from peripheral blood in 14 out of 28 (50%) patients with Crohn's disease, in 2 out of 9 patients (22%) with ulcerative colitis, and in 9 out of 15 individuals without inflammatory bowel disease. In a previous study the same research group reported the first isolation of viable *Map* from suckling mother (Nasser *et al*., 2000).

Based on these data, some researchers conclude that *M*ap may be the causative agent of Crohn's disease. Various professional groups, such as farmers, veterinarians and slaughterhouse workers may be affected, although high incidence of Crohn's disease was not reported among these professional groups. The question of whether *Map* is important for public health as a cause of disease in humans has been reviewed by a number of expert groups in recent years, regardless of detected cases of Crohn's disease. The opinion of many experts is that conclusive data proving a causal relationship between *Ma*p and Crohn's disease are not available at present.

In case that *Ma*p takes part in the pathogenesis of Crohn's disease, it could be assumed that

the infection is related with consumption of food or water. The most likely means of transmission of *Map* from cattle to humans are milk (and potentially other dairy products), beef and water. *Map* is cultivated from the milk of cows with clinical and subclinical John's disease (Taylor *et al.,* 1981; Sweeney *et al.,* 1992; Streeter *et al.,* 1995). It can also occur in the milk of other ruminants such as sheep and goats affected by John's disease. Most people consume raw cow's milk from a young age and in this way cow's milk has been recognized as a means of transmission of *Map* from cattle to humans. As most cow's milk is pasteurized prior to consumption, there is considerable interest in determining the effectiveness of milk pasteurization with regard to *Map*. Studies report that *Map* is more heat-resistant than other mycobacteria (including *M. bovis*) and that occasionally low numbers of viable *Map* may survive after milk pasteurization. The ability of *Map* to survive pasteurization, alows the microorganism to be viable in certain dairy products. For example, many cheeses are made from raw or minimally heat-treated cows', sheep's and goats' milk. A recent study found that the number of *Map* cells increased 10-fold during production of cheddar cheese and *Map* remained culturable for 27 weeks of the period of cheese ripening (Donaghy *et al.,* 2004).

Johne's disease affects dairy and beef cattle, and theoretically, meat could also be a potential food transmitting *Map* to humans. It is supposed that meat from old dairy cows, which are used for ground meat or beef for human consumption, may represent a source of *Map* infection for consumers (Manning and Collins, 2001). It is hypothesized that when ground beef is obtained from animals with localized infections, for example in a lymph node, *Map* can spread over the entire batch of minced beef. Water may also represent a potential means for transmitting *Map* to humans. Everyone drinks water in its natural state or as an ingredient in other drinks. Water is used in the production and processing of food, so it can reach consumers in this way. Surface water contaminated with effluent from the pasture grazed by animals with Johne's disease, can theoretically enter water supplies that are used for the supply of drinking water. The efficiency with which water treatments, such as sand filtration and chlorination, remove or inactivate *Map* present in water destined for human consumption has not been thoroughly investigated. The levels of chlorination that is typically used for water treatment only results in around a two log10 reduction in numbers of viable *Map* spiked into water (Whan *et al*., 2001).

Pathology

The earliest macroscopic lesions of Crohn's disease appear to be tiny focal "aphthoid" ulcerations of the mucosa, usually with underlying nodules of lymphoid tissue. Sometimes these lesions regress; in other cases, the inflammatory process progresses and involves all layers of the intestinal wall, which becomes greatly thickened. Histologically, changes are most marked in the submucosa, with lymphoedema and lymphocytic infiltration occurring first, and extensive fibrosis later. Patchy ulcerations develop on the mucosa. The combination of longitudinal and transverse ulcers with intervening mucosal oedema frequently creates a characteristic "cobblestone" appearance.

Segments of the diseased bowel are characteristically sharply demarcated from adjacent normal bowel – hence the name "regional" enteritis. Segmental lesions could be separated by normal areas (skip lesions). With reference to disease distribution in the gut, a recent European collaborative study on 125 cases has shown that the ileum alone is involved in about 10% of cases (ileitis); both the ileum and colon are affected in about 60% (ileocolitis); and the colon alone is involved in about 30% (granulomatous colitis). Occasionally, the entire small bowel (jejunoileitis) is involved, and rarely also the stomach, duodenum, or oesophagus.

While granulomas when present are helpful in distinguishing Crohn's disease from other forms of inflammatory bowel diseases, it is the chronic inflammation involving all layers of the intestinal wall that is most characteristic of Crohn's disease. Both lymphocytes and macrophages are present in the granulomas and the cellular infiltrate.

The attached mesentery is thickened and lymphoedematous; mesenteric fat typically extends onto the serosal surface of the bowel. Mesenteric lymph nodes are often enlarged. The transmural inflammation, deep ulceration, oedema, and fibrosis are responsible for obstruction, and deep sinus tracts and fistulas, as well as mesenteric abscesses are the major local complications.

Control and public health concern

Althogh not currently classified as zoonotic agent, *Map* is suspected to be involved in the pathogenesis of Crohn's disease in humans. This recognizes that CD involves the interaction of at least 3 components: a) genetic predisposition, b) an enviromental trigger, and c) unregulated immune

response. The involvment in *Map* is supported by several factors. They include: 1) CD and *Map* share clinical and histopathological similarities; 2) viable *Map* has been cultured from intestinal tissues, blood and milk of patients with CD; and 3) antibodies to *Map* antigen have been identified in the blood of Crohn's patients.

Recent meta-analysis studies indicate that there is an association between the presence of *Map* and CD, but whether this association is causal or coincidental remains a controversial issue. Arguments against the role of *Map* in CD are TNF-gamma inhibitors, which would not be effective if *Map* were the cause of CD. They are effective in the treatment of CD, whereas anti-mycobacterial treatment has limited efficacy. In some countries, precautionary control measures are taken in food sectors, as there is a probability that dairy and meat products are contaminated by mycobacteria. The major concerns are studies demonstrating that *Map* has been cultured from pasteurized retail milk and may be present in drinking water.

Diagnosis and treatment of Crohn's disease

The diagnostic techniques that can be used to prove the disease are influenced by the clinical stage as it has been developed in the affected host and the prevalence in the human society. Detection of the infection of the host during this sub-clinical stage is complicated by the fact that the bacterium does not multiply rapidly and is therefore difficult to detect in faeces (Ridge *et al.*, 1991; Sockett *et al.,* 1992; Sweeney *et al.,* 1995). Traditionally, the treatment of Crohn's disease has involved suppressing the host's immune response with corticosteroids, surgical resection of severely diseased bowel, nutritional supplementation, or administration of antibiotics. The efficacy of these therapies is evaluated by monitoring disease signs, symptoms and quality of life indicators, so called Crohn's disease activity index (CDAI). Early efforts at antibiotic therapy for the treatment of Crohn's disease involve standard drugs used in the treatment of tuberculosis (isoniazid, ethambutol and pyrazinamide) (Hermon-Taylor, 1998). This antibiotic therapy gives different results. Unlike the microorganism that causes tuberculosis, *Map* is probably located intracellularly in macrophages in the form of spheroplasts and therefore is extremely slow-growing (Hermon-Taylor, 2002). These factors must be taken into consideration when choosing drugs for treatment of paratuberculosis in humans.

Health and economic losses

According to the latest data, Crohn's disease affects between 400,000 and 600,000 people in North America. In Northern Europe, there are between 27 and 48 diseased in every 100,000 people (Shivananda *et al.,* 1996; Loftus *et al.,* 2002). A major multicentre European study has calculated an incidence rate for Crohn's disease of 5,6 per 100,000 per year. The disease is life-long, though considerable periods of remission may occur. It is estimated that over 200,000 people are affected by this disease in the European Union. Data of the cost of Crohn's disease to society in Europe are not available. However, a Swedish study has shown that in 1994 Sweden spent over 40 million SK on patients with this condition, 29% of which were direct costs, i.e. costs for in-patient care, ambulatory care and drugs. Indirect costs, i.e. sickness leave and early retirement constituted 71% (Ekbom, 1997).

Crohn's disease results in substantial morbidity and high use of health services. Mortality is not usually a feature of the disease though patients, particularly the young, have double risk mainly because of complications associated with the disease (Prior *et al.,* 1981). An US study found that for a representative patient, projected lifetime costs were $125,404 using mean charges (Silverstein *et al.,* 1999). Total annual medical costs in the US were estimated at $1-1.2 billion for 1992 (Hay J.W. and Hay A.R., 1992). If disability payments and the cost of early retirement are taken into account, the total cost of this disease is much higher.

In conclusion, paratubrculosis remains a subject of public concern in many countries worldwide, because diagnosis and control in animals and humans are difficult and expensive, as a result of possible zoonotic links with Crohn's disease.

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