Mycobacterium avium subsp. paratuberculosis in powdered infant formula

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ABSTRACT

Fifty one powdered infant formula products produced by 10 companies from seven countries available on the Czech market were tested. Milk used for these products is pasteurized prior to drying. IS900, the specific fragments for Mycobacterium avium subsp. paratuberculosis (MAP) were detected using PCR in 25 samples (49.0 %) and fragment f57 by real time PCR in 18 samples (35.3 %). One sample was positive by culture, but the finding was not successfully repeated. These results correspond to the epidemiological situation in Europe and are not unexpected. Paratuberculosis in cattle was almost unknown in the Czech Republic until 1990. An increase in the number of cows with paratuberculosis found in slaughterhouses and the incidence of Crohn’s disease in the last decade is evident. The possible risk of killed MAP cells or bacterial structures in food is discussed in respect to an autoimmune cause Crohn’s disease. The national programmes of paratuberculosis control and certification of paratuberculosis–free herds should be strongly supported to decrease the risk for children and other people under higher risk. Producers should use MAP-free milk for baby food production on a voluntary basis.

Key words: Johne’s disease, paratuberculosis, control, Crohn’s disease, IS900, f57, infant formula

INTRODUCTION

Paratuberculosis (Johne’s disease), Mycobacterium avium subsp. paratuberculosis (MAP) infection and Crohn’s disease are the focus of growing interest, with the number of research projects and published results doubling between 1994 and 2003 (Hruska, 2004). Paratuberculosis is the widely distributed infectious disease of cattle and other domestic and wild ruminants caused by MAP infection (Kennedy and Benedictus, 2001). Up to 70% of dairy herds suffer from this infection in most European countries, the United States and Canada. The financial losses were already estimated at about $1.5 billion per year in the USA in 1998 (Stabel, 1998). Paratuberculosis is a notifiable disease for the OIE, but it is not classed as zoonotic or an emergency disease or. An OIE Technical Disease Card on paratuberculosis is not yet available. Milk and meat from infected herds is not banned if the general food-handling rules are fulfilled. Diagnosis of the disease is rather difficult as infected animals don’t always shed MAP in faeces or milk. Serologic methods have low sensitivity and specificity, and cultivation of the agent, although considered “the gold standard”, takes several months with some MAP forms not growing in vitro at all (Pavlik et al., 1999; Machackova et al., 2004).

If the infection is not efficiently controlled it is guaranteed to spread MAP to most animals in the herd, although the genetic influences in the susceptibility of cattle to paratuberculosis have been reported (Koets et al., 2000). Subsequently, as a result of different stress factors e.g. parturition, malnutrition, transportation etc., some animals suffer from the clinical form of the disease. Massive shedding of MAP in faeces contaminates the environment and transmits the infection to other animals. The most susceptible are calves during the first weeks of their life. Evidence of the pathogen has been found not only in the intestine but also in milk, lymph nodes, and different parenchymatous organs (Pavlik et al., 2000; Ayele et al., 2004). Confirmed MAP isolates were cultured from 1.8% of the commercially pasteurized milk samples in the U.K (Grant et al., 2002). Similar data were published from the U.S.A. (Ellingson et al., 2005). In the U.K. study

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The risk associated with the presence of cultivable MAP in retail dairy products has been noted by an increase of paratuberculosis was found in slaughtered cattle (Vecerek et al., 2003). MAP has been cultivated from cheese (Donaghy et al., 2004; Ikonomopoulos et al., 2005) as well.

MAP is very resistant to high temperatures and chlorination. The organism remains cultivable in lake water for 632 days and persisted for up to 841 days (Pickup et al., 2005). MAP cultivation needs up to four months for cultivation with some forms not growing in vitro at all. However, the concentration of MAP, quoted in colony forming units (CFU), does not convey the total number of cells present. Molecular biology techniques offer a more rapid and specific detection of MAP and its quantification in milk, cheese, and meat.

Autoimmune diseases are an important health problem for humans. The Autoimmune Diseases Coordinating Committee of the U.S. National Institute of Health reported to Congress that the prevalence of all autoimmune diseases ranged from 5 to 8 percent of the U.S. population (14.7 to 23.5 million people) in 2003. The expenditures for autoimmune diseases research reached nearly $600 million. The inflammatory bowel disease Crohn’s disease, a chronic autoimmune inflammatory bowel disease with similar pathological changes to paratuberculosis (Chiodini, 1989), had the fifth highest research budget in 2003 (Anon, 2005). The American Academy of Microbiology Colloquium “Microbial Triggers of Chronic Human Illness” used Crohn’s disease to illustrate a condition that does not result from infection alone but from the confluence of infection and genetic susceptibility. Susceptible individuals, who carry the NOD2 or TNFR polymorphisms, may respond to certain commensal intestinal flora, stimulating acute inflammation that leads to chronic inflammation and colitis. The Colloquium also stated that it can be extremely difficult to prove that a pathogen is the cause of a chronic disorder when the onset of disease begins some time after the exposure. Often times it is not practical or even possible to use Koch’s postulates to prove the infectious nature of chronic illnesses (Carbone et al., 2005).

MAP and other agents (Clostridium, Campylobacter jejuni, Campylobacter feacalis, Listeria monocytogenes, Brucella abortus, Yersinia pseudotuberculosis, Yersinia enterocolica, Klebsiella spp., Chlamydia spp., Eubacterium spp., Peptostreptococcus spp., Bacteroides fragilis, Enterococcus feacalis, and Escherichia coli) have been considered possible triggers of Crohn’s disease (Carbone et al., 2005). MAP cells contain peptidoglycans and heat shock proteins that are able to initiate the inflammatory changes in the intestine (El-Zaatari et al., 1995; Chamaillard et al., 2003). The highest reported prevalence of Crohn’s disease to date is in north-eastern Scotland, where almost 0.15% of the population has the disease. It is not far from the truth that Crohn’s disease affects hundreds of thousands of people around the world. Based on the latest epidemiology research from the United States, the most likely conclusion is that there are 400,000 people in the United States who suffer from Crohn’s disease. Since the population of the United States is 270 million people, this means that the current prevalence of Crohn’s disease in the United States is 148 cases per 100,000 people. In the United States, in 1990, Crohn’s disease costs between $1.0 and 1.2 billion. Other countries with a high prevalence of Crohn’s disease are Canada, Sweden, Norway, Germany, United Kingdom, Netherlands, Belgium, France, Switzerland, Austria, Spain, Portugal, Greece, Italy, Ireland, Australia, New Zealand, and many countries of Eastern and Central Europe. In all these countries bovine paratuberculosis is a commonly found infection in cattle herds but the incidence of paratuberculosis in cattle is not known with precision because it is hard to diagnose and under-reported (Ayele et al., 2001). The prevalence of the disease is also unknown in sheep, goats and game ruminants. Some authors have described a parallel increase in paratuberculosis and Crohn’s disease prevalence and discuss the possible links between them (Hermon-Taylor and El-Zaatar, 2005). Paratuberculosis in cattle was sporadically diagnosed until 1990 in the Czech Republic. When the import of heifers and dairy products started an increase of paratuberculosis was found in slaughtered cattle (Vecerek et al., 2003).

Dried milk baby food products (infant formula) originating from nine European countries including two new EU member states were all available on the Czech market in 2004. In all these countries paratuberculosis is present in dairy herds where milk and beef from pre-clinically affected animals can be sold on the market. The risk associated with the presence of cultivable MAP in retail dairy products has been noted by a
number of authors. The presence of the specific IS900 was also confirmed. These findings are unsurprising as the prevalence of paratuberculosis in dairy cattle herds is high and MAP can be present in milk even in cows without the clinical form of the disease. Current milk and meat products regulations are being met by dairies and food producers.

Data on the increase of the incidence of Crohn’s disease has been published from different countries. Some authors noted an increase in children with different autoimmune diseases, including Crohn’s disease. In Scotland the incidence of Crohn’s disease has increased in children by 30% since 1993 (Armitage et al., 2001). An increase of incidence was also reported in Denmark (Fonager et al., 1997), Israel (Shapira and Tamir, 1994), Minnesota, U.S.A. (Loftus et al., 1998) and in the region of Northern Stockholm (Asking et al., 1999; Hildebrand et al., 2003). The index of patients treated for Crohn’s disease in the Czech Republic between 1995 and 2004 increased to 2.9; in the age category up to 19 years to 4.6 and in patients older than 65 to 6.6 (Fig. 1).

Milk and dairy products are important components of human nutrition. However, the autoimmune character of the Crohn’s disease does not exclude a risk for genetically susceptible people when linked with bacterial triggers. This may occur even though live MAP cells are not present in food. At higher risk are children and direct relatives of Crohn’s disease patients. Given the presence of MAP IS900 in dairy products has been reported, the aim of this study was to evaluate MAP contamination in dried milk baby food (infant formula) available in the Czech Republic.

Fig. 1: Crohn’s diseases in the Czech Republic in 1995 to 2004. Age in years 0-19, 20-65, 65 and older and all ages are depicted in full, hatched, open and horizontally hatched bars, respectively (Institute of Health Information and Statistics of the Czech Republic)

MATERIALS AND METHODS

Samples
Fifty one dried milk baby food products (infant formula) from 10 producers operating in seven European Union countries were tested. The milk for these formulas is pasteurized before it is dried during preparation of these products.

IS900 determination
A total 20 mg of dry milk samples were diluted in 200 µl of MAP-free water. DNA was isolated by QIAamp DNA Blood Kit (QIAGEN, Germany) according to manufacturers’ instructions. From the resulting volume 200 µl a total of 4 µl of DNA was used for PCR (Ayele et al., 2005). The highly sensitive PCR (sensitivity in
tenths of specific loci per reaction) was performed with Taq PCR Master Mix Kit (QIAGEN, Germany) using primers IS900-P3N: 5’-GGG TGT GGC GTT TTC CTT CG-3’ and IS900-P4N: 5’-TCC TGG GCG CTG AGT TCC TC-3’ in a concentration of 10 µmol per reaction. The expected length of amplification product was 257 bp. An internal standard with a length of 591 bp was used to control false negatives.

Real time PCR for the specific fragment f57 was based on partial sequence described by (Poupart et al., 1993) (GenBank Acc. No. X70277; http://www.ncbi.nlm.nih.gov). BLAST analysis revealed that this fragment is located in a coding region of the hypothetical protein MAP0865 predicted according to the complete MAP genome sequence (GenBank Acc.No. AE017229). Primers and specific TaqMan probes were designed according to the above mentioned f57 sequence and synthesized in TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany. A full description of the method and results will be published separately.

MAP cultivation
Culture of the infant formula products was performed according to Ayele et al. (2004). The milk powder was reconstituted (1 g in 5 ml of distilled water) and the mixture was centrifuged at 2,500 rpm (800 x G) for 15 minutes. The supernatant was decanted and the sediment resuspended in 5 ml 0.75% HPC (hexa-decyl-pyridinium chloride: cetylpyridinium chloride, No. 102340 Merck, Darmstadt, Germany). The tubes were incubated for 4 hours at room temperature with intermittent agitation and centrifuged again for 15 minutes under the same conditions. The supernatant was decanted and the sediment resuspended in 1 ml sterile distilled water. A 200 µl aliquot of the suspension was inoculated onto three Herrold’s egg yolk media with mycobactin J.

RESULTS

The IS900 insertion sequence was found in 25 samples (49%). Products from three manufacturers with the highest numbers of samples tested, i.e. 24, 11, and 7 were PCR-positive 47.7, 45.5, and 85.7% of cases, respectively (Table 1).

Fragment f57 was found in 18 of 51 samples tested (35.3%). An isolate of MAP was made from one sample. Repeated testing of this sample was did not isolate MAP.

<table>
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<th>Producer</th>
<th>No. of products</th>
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<th>Negative</th>
<th>Inhibition*</th>
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<td>25</td>
<td>23</td>
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*If the internal standard with a length of 591 bp was negative, the result was evaluated as inhibition or "false negative"

DISCUSSION

The results are not unexpected. Dairy products from herds not warranted as MAP-free are likely to contain IS900 from the dead cells in varying amounts unless action is taken to control the infection and thereby reduce MAP contamination of milk.
A single isolate of MAP from one of 51 samples was obtained, but upon repeat culture no additional isolation was made. The non-uniform distribution of the organism and the small number of cells surviving milk treatment protocols used to produce infant formula could explain this finding.

Crohn’s disease is a chronic inflammatory bowel disease similar to paratuberculosis in ruminants. It is classified as an autoimmune disease but its trigger mechanisms are not fully understood. The primary impulse may be the sensitisation of the innate immune system at an early age (Hermon-Taylor and Bull, 2002). The innate immune system is the most ancient and ubiquitous system of defence against microbial infection. The microbial sensing proteins involved in innate immunity recognise conserved and often structural components of microorganisms. Published data has strengthened the association of MAP with Crohn’s disease (Chiodini et al., 1984; Chiodini et al., 1986; Chiodini, 1989; Hermon-Taylor et al., 2000; Autschbach et al., 2005; Sechi et al., 2005). Crohn’s disease affects hundreds of thousands of people around the world. The current prevalence of Crohn’s disease is 50 to 150 cases per 100,000. Paratuberculosis is a common disease in dairy and beef cattle herds in countries with a high prevalence of Crohn’s disease.

Many papers describe the presence of mycobacteria-specific DNA sequences in Crohn’s disease patients. Specific probes based on the IS900 sequence (Green et al., 1989) are usually used to detect MAP although some different specific loci were described (Poupart et al., 1993; Eriks et al., 1996; Bannantine et al., 2002; Vansnick et al., 2004). The presence of of MAP antigen and the antibody array from Crohn’s disease patients indicate a unique immune response to MAP and suggest that this organism may play some role in the pathogenesis of Crohn’s disease. The insertion sequence IS900 reveals a unique protein product, p43. The anti-p43 antibody identifies p43 as a 28 kDa processed product in Western blots of protein extracts from MAP (Tizard et al., 1992). Mycobacterial 65KDa heat shock proteins (Hsp65) are among the most extensively studied mycobacterial proteins, and their immunogenic characteristics have been suggested to be the basis for autoimmunization in chronic inflammatory diseases (Elsaghier et al., 1992; Stevens et al., 1992; Szewczuk and Depew, 1992).

In humans, the strong antibody reactions of some sera from Crohn’s disease patients compared with non-inflammatory bowel disease patients showed a positive correlation with mycobacterial diseases (El-Zaatari et al., 1995). Serum antibodies (IgG, IgA, and IgM) to protoplasmic antigen of MAP were quantified in patients with Crohn’s disease and in control subjects using an enzyme-linked immunosorbent assay (Suenaga et al., 1999). Crohn’s disease patients’ antibodies were tested by immunoblotting against recombinant antigens identified from MAP genomic library (Naser et al., 2000). Immunoglobulin M (IgM), IgA-, and IgG1- and IgG2-isotype-specific enzyme-linked immunosorbent assays for MAP-derived antigens (heat shock proteins of 70 kDa (Hsp70) and 65 kDa (Hsp65), lipoarabinomannan, and MAP purified protein derivative (PPD) was measured by Koets (Koets et al., 2001). Peptidoglycan-polysaccharide complexes were detected intracellularly in the mucosa and submucosa of the bowel wall of Crohn’s disease patients. The results show the presence of bacterial peptidoglycan in the bowel wall and the immune responsiveness, especially at the site of inflammation, to these antigens in active Crohn’s disease. These results present suggestive evidence for the role of peptidoglycan in the etiology and/or pathogenesis of Crohn’s disease (Klasen et al., 1994). The results of mycobacterial genomics are important for a further research (Bannantine et al., 2004).

Multiple genetic variants of NOD2/CARD15 have been associated with a susceptibility to Crohn’s disease. NOD2/CARD15 recognizes muramyl dipeptide (MDP) derived from bacterial peptidoglycan (PGN), but the molecular basis of recognition remains elusive (Tanabe et al., 2004). Comprehensive reviews of experimental data supporting a genetic disposition to Crohn’s disease and immunity, inflammation and allergy in the gut were published recently (MacDonald and Monteleone, 2005; Kobayashi et al., 2005; Maeda et al., 2005).

Paratuberculosis in cattle causes considerable economic losses for farmers (Mason et al., 1997; Stabel, 1998; Kennedy and Benedictus, 2001). Crohn’s disease is also important, both for the pain and difficulties it causes and for the huge expenditure for treatment (Juan et al., 2003; Bassi et al., 2004; Ebinger et al., 2004). The information already available is sufficient to support the possibility of a health risk for consumers resulting not only from viable MAP, but also from inactive or dead cells and even from their structural components. The number of MAP cells present in food is very important. Intake should be
minimised in highest risk people e.g. in newborns, children and genetically susceptible persons, namely patients suffering from Crohn’s disease and their direct relatives.

It is most important
- to consider the hypothesis of a possible link between MAP structural components and Crohn’s disease
- to decrease the risk of MAP for consumers by introducing MAP-free dairy and beef products and to encourage producers to start this on a voluntary basis
- to support national programmes for certification of dairy and beef cattle herds free of paratuberculosis
- to support the national control programmes for paratuberculosis.

Certification and control programmes have already started in some countries. Paratuberculosis should be considered a herd disease and certification must be based on periodic culture of pooled faeces samples and PCR confirmation of specific DNA sequences from milk four times a year. Culling of shedding animals, careful evaluation of suspect clinical cases of paratuberculosis and post-mortem inspection of all culled cows is recommended to reach the status of a paratuberculosis-free herd. Closing the herd until it is possible to purchase animals from guaranteed paratuberculosis-free herds is absolutely essential. Finally, producers of baby food formulas should require milk either free of MAP or with minimal MAP contamination. Thus a reliable quantitative or semi-quantitative method for identifying MAP or its specific components in milk is necessary.

To avoid panic and misinterpretation, thorough education on this topic should be provided immediately. Beef and dairy products are an important component of human nutrition and cannot be omitted. The dairy industry is a valuable sector of agriculture and food production and should be supported in order to rapidly reach a solution of the problem.

CONCLUSION

The possible risk of Mycobacterium avium subsp. paratuberculosis dead cells or bacterial structures in food in respect to autoimmune Crohn’s disease should be carefully monitored. National programmes of paratuberculosis control and certification of paratuberculosis-free herds should be strongly supported to decrease the risk of exposure for children and people under the highest risk for Crohn’s disease. Producers of infant formula should use MAP free milk on a voluntary basis.

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