Mycobacterium avium subsp. *paratuberculosis* in powdered infant milk: paratuberculosis in cattle – the public health problem to be solved[1]

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ABSTRACT: Fifty one products of dried milk baby food purchased from 10 producers from seven countries available on the Czech market have been tested. IS*900,* the specific fragments for *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) have been detected using PCR in 25 samples (49.0 %) and fragment f*57* by real time PCR in 18 samples (35.3%). 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 *MAP* dead cells or bacterial structures in food is discussed in respect to autoimmune 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.

Keywords: Johne's disease; paratuberculosis control; Crohn's disease; IS*900*, f*57*

Paratuberculosis (Johne's disease), *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) 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* (Kennedy and Benedictus, 2001). Up to 70% of dairy herds suffer from this disease 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 notifiable disease to the OIE, but it is not yet classed as an emergency disease or zoonosis. An OIE Technical Disease Card on Paratuberculosis is not yet available. Milk and meat from infected herds is not banned if the general rules are fulfilled. Diagnosis of the disease is rather difficult as infected animals don't always shed *MAP* in faeces or milk. The serological methods have low sensitivity and specificity, and cultivation of the agent, although considered as the gold standard, takes a long time of several months with some *MAP* forms not growing *in vitro* at all (Pavlik et al., 1999; Machackova et al., 2004).

If the disease 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

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of *MAP* in faeces is the reason for the contamination of the environment and transmission of the disease to other animals. The most susceptible are calves during the first weeks of their life. Evidence of pathogens was 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 the 10 culture-positive pasteurized milk samples were from just 8 (3.3%) of the 241 dairy processing establishments that participated in the survey (Grant et al., 2002). In the same study 11.8% of samples of retail milk were *MAP*-positive by PCR. In Switzerland, 19.7% of bulk-tank milk samples were IS*900* PCR positive (Stephan et al., 2002). Goat's tank-milk and ewe's tank-milk samples were also PCR-positive for the IS*900* (23.0 and 23.8%, respectively), providing presumptive evidence for the presence of *MAP* in Switzerland (Muehlherr et al., 2003). *MAP* has been cultivated from cheese (Mason et al., 1997; Donaghy et al., 2004; Stabel and Lambertz, 2004; Ikonomopoulos et al., 2005) as well.

MAP is very resistant against higher temperatures and chlorination. The organism remains culturable in lake water microcosms 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 inform about the total number of cells presented, which is estimated to be in orders of 10^5 per millilitre of milk. Molecular biology techniques offer a more rapid and very specific detection of *MAP* and its quantification in milk, cheese, and meat.

Autoimmune diseases are considered as very important. 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% 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) had the fifth highest research budget in 2003 (Anonymous, 2005). The American Academy of Microbiology Colloquium "Microbial Triggers of Chronic Human Illness" presented as an example Crohn's disease which "does not result from infection alone but from the confluence of infection and genetic susceptibility. Susceptible individuals, who carry the NOD 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 the chronic disorder when the onset of disease begins some time after the exposition. 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* spp., *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*) are suspected to be possible triggers of Crohn's disease (Carbone et al., 2005), a chronic autoimmune inflammatory bowel disease with similar pathological changes to paratuberculosis (Chiodini, 1989). *MAP* cells contain peptidoglycans and heat shock proteins, which are able to initiate the inflammatory changes in the intestine (Elzaatari 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 have 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 cost 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 common disease found in 30 to 60% of cattle herds. The prevalence of the disease is unknown in sheep, goats and game ruminants in the most countries. Unfortunately the incidence of paratuberculosis

in cattle is not exactly known because it is hard to diagnose and is not supposed to be a zoonosis or emergency animal disease (Ayele et al., 2001). Some authors have described a parallel increase in paratuberculosis and Crohn's disease prevalence and discuss the possible links (Hermon-Taylor and Elzaatari, 2005). Paratuberculosis in cattle was sporadically diagnosed until 1990 in the Czech Republic. Then the import of heifers and dairy products started and an increase of paratuberculosis has been found in slaughtered cattle (Vecerek et al., 2003).

Dried milk baby food products 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 preclinically infected 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 IS*900* 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. Nevertheless, dairies and food producers are not breaking the present legislation for milk and meat products.

Data on the increase of the incidence of Crohn's disease was published from different countries. Some authors noted an increase in children suffering many times from different autoimmune diseases together with the Crohn's disease. In Scotland incidence of Crohn's disease has increased in children by 30% since 1993 (Armitage et al., 2001). 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 (Askling 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 (Figure 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 linked with the bacterial triggers although the live *MAP* cells were not present in food. Under the higher risk are children and direct relatives of Crohn's disease patients. Although the presence of *MAP* IS*900* in dairy products had to be expected, the aim of our study was to check the contamination in some baby food available on the Czech market.

MATERIAL AND METHODS

Samples. Fifty one products of dried milk baby food products from 10 producers operating in seven European Union countries (included two new member states) have been tested. All were used in

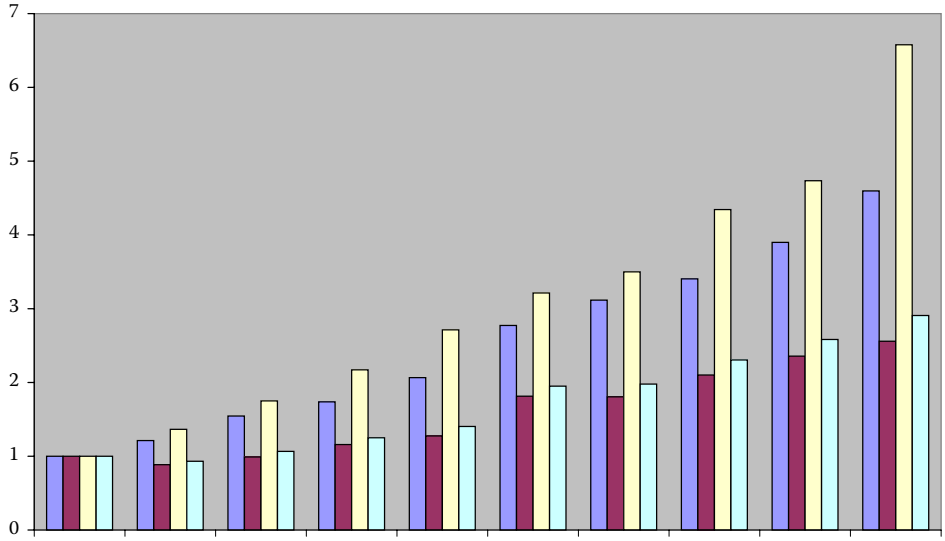

Figure 1. Crohn's diseases in the Czech Republic in 1995 to 2004: age in years 0–19, 20–65, 65 and older and all ages are depicted in bars of blue, red, yellow, and light blue, respectively (Institute of Health Information and Statistics of the Czech Republic)

Table 1. *IS 900* tests results

Producer	No. of products	Positive	Negative	Inhibition	Positive (%)
1	24	10	13	1	**41.7**
2	11	5	6		**45.5**
3	7	6	1		**85.7**
4	3	1	1	1	33.3
6	1	1			100.0
9	1		1		0
10	1	1			100.0
7	1			1	0
5	1	1			100.0
8	1		1		0
Total	51	25	23	3	
Percent		**49.0**	45.1	5.9	

the University Hospital, Brno, and were available on the Czech market.

IS*900* 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 added to PCR (Ayele et al., 2005). High sensitive PCR (sensitivity in tenths of specific loci per reaction) was performed with Taq PCR Master Mix Kit (QIAGEN, Germany) using primers IS*900*-P3N: 5´-GGG TGT GGC GTT TTC CTT CG-3´ and IS*900*-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 f*57* 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 coding region of hypothetical protein *MAP*0865 predicted according to the complete *MAP* genome sequence (GenBank Acc. No. AE017229). Primers and specific TaqMan probe 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.

RESULTS

Insertion sequence IS*900* was found in 25 samples (49 %). Products from three producers with the highest numbers of samples tested, e.g. 24, 11, and 7 were positive 47.7, 45.5, and 85.7% of cases, respectively (Table 1).

Fragment f*57* was found in 18 of 51 samples tested (35.3%).

DISCUSSION

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 already for two decades (Chiodini et al., 1984, 1986, 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 and in sheep, goats and game ruminants in countries with a high prevalence of Crohn's disease.

A lot of papers describe the presence of mycobacterial specific DNA sequences in Crohn's disease patients. Specific probes based on the sequence of IS*900* (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 the antigen of *MAP* and the antibody activities from Crohn's disease patients were described indicating the unique immune response to *MAP* and suggesting that this organism may play some role in the pathogenesis of Crohn's disease. Insertion sequence IS*900* reveals a unique protein product, p43. The anti-p43 antibody also identified p43, as a 28 kDa processed product in Western blots of protein extracts from *MAP* (Tizard et al., 1992). Mycobacterial 65 kDa 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 high intensity of antibody reactions of some sera from Crohn's disease patients compared with that from noninflammatory bowel disease patients showed a positive correlation with mycobacterial diseases (Elzaatari et al., 1995). Serum antibodies (IgG, IgA, and IgM) to the 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). Antibody activities from Crohn's disease patients were tested by immunoblotting against recombinant antigens that were 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 and therefore 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 very important for a further research (Bannantine et al., 2004).

Multiple genetic variants of NOD2/CARD15 have been associated with 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). The comprehensive reviews on experimental data supporting the genetic disposition to Crohn's disease and immunity, inflammation and allergy in the gut were published recently (Kobayashi et al., 2005; MacDonald and Monteleone, 2005; Maeda et al., 2005).

Paratuberculosis in cattle poses an important problem for farmers causing remarkable economical loses (Mason et al., 1997; Stabel, 1998; Kennedy and Benedictus, 2001). Crohn's disease is also very important for both the people suffering from the severe chronical illness with all its painful and difficult attributes and for the huge expenditure for treatment and additional costs (Juan et al., 2003; Bassi et al., 2004; Ebinger et al., 2004). Information already available is sufficient to accept the possibility of a risk for consumers resulting not only from the 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 people under the highest risk, e.g. in newborns, children and genetically susceptible persons, namely patients suffering from Crohn's disease and their direct relatives.

We consider the following topics to be the most important:

– to consider the hypothesis of a possible link between the *MAP* structural compound and Crohn's disease
– to decrease the risk of *MAP* for consumers by means of introduction of dairy and beef products *MAP* free and to encourage the producers to start this on a voluntary basis
– to support the 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 assessed as a herd disease and the certification must be based on periodical 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 suspected clinical cases of paratuberculosis and postmortal 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 free of *MAP* or with minimal contamination. Thus a reliable quantitative or semiquantitative method for identifying MAP or its specific components is a necessary prerequisite.

To avoid panic and misinterpretation a serious information dissemination should be immediately started. Beef and dairy products are an important component of human nutrition and cannot be omitted. The dairy industry is a great sector of agriculture and food production and should be supported in a rapid and efficient solution of the problem.

CONCLUSION

The possible risk of the *Mycobacterium avium* subsp. *paratuberculosis* dead cells or bacterial structures in food in respect with 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 for children and people under the highest risk. Producers should use *MAP* free milk for baby food production on a voluntary basis.

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