Mycobacterium avium subsp. *paratuberculosis* in powdered infant milk: *F57* competitive real time PCR

K. Hruska, I. Slana, P. Kralik, I. Pavlik

Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in concentrations from 48 to 32 500 cells per gram of powdered infant milk were found in 18 out of 51 investigated samples (35%) in this study. More than 10 000 cells per gram were present in four samples (7.8%). Such concentrations mean that one package of milk contains 5 million MAP cells, which are ingested by a bottle-fed baby over the course of several days. Premature babies and bottle-fed newborns can be affected by pro-inflammatory triggers from a huge number of mycobacteria despite not suffering from infection with bacteria or viruses often linked with the etiology of Crohn's disease.

Keywords: Crohn's disease; public health; food safety; environmental risk

Paratuberculosis in ruminants (Johne's disease) is not yet considered a zoonosis and contamination of milk with MAP is not subject to regulatory standards. Countless authors have reported that milk from cows suffering from paratuberculosis is contaminated with MAP (Collins, 1997; Anon, 2000, 2010; Grant et al., 2001; Corti and Stephan, 2002; Nacy and Buckley, 2008; Slana et al., 2008b; Eltholth et al., 2009; Botsaris et al., 2010; Donaghy et al., 2011). Herd prevalence of paratuberculosis is more than 50% in many countries (Nielsen and Toft, 2009). Cultivable MAP are present in around 2% of retail pasteurized milk and cheese (Grant et al., 2002; Slana et al., 2009; Gill et al., 2011). The presence of IS*900* and *F57* from MAP in powdered infant milk is not surprising as milk from paratuberculosis-contaminated herds is used for the production of milk products. We have found IS*900* in 48.9% of 51 retail powdered infant milk samples produced by 10 companies from seven European countries (Hruska et al., 2005). Potable or bottled water is another possible source of mycobacteria in baby formula (Papapetropoulou et al., 1997; Falkinham, 2003; Pedley et al., 2004).

Muramyldipeptides, released from peptidoglycans constituting the mycobacterial cell walls, are potent immunomodulators and are known triggers of inflammation (Ellouz et al., 1974; Carbone et al., 2005; Maeda et al., 2005; Coulombe et al., 2009). Heat shock protein, present in the mycobacteria, can also participate in this process. Hence, even dead mycobacteria in milk or water used for baby formula pose a risk for newborn babies, as their immunomodulatory effects are beyond doubt (Pettis et al., 2000; Maeda et al., 2005; Coulombe et al., 2009).

Genetic factors linked with Crohn's disease, such as NOD2, are well documented (Brant et al., 2007). The hypothesis that MAP and perhaps other mycobacteria in baby formula play the role of the missing environmental factor in the etiology of Crohn's disease (Hruska, 2009; Hruska and Pavlik, 2010) is supported by many papers which describe breast feeding as a protective factor against Crohn's disease (Bergstrand and Hellers, 1983; Davis, 2001; Klement et al., 2004; Mikhailov and Furner, 2009; Barclay et al., 2009), type I diabetes mellitus (Hanson, 1998; Davis, 2001; Virtanen and Knip, 2003; Peng and

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Hagopian, 2006; Dow, 2006; Banwell et al., 2008), asthma (Oddy, 2004), multiple sclerosis (Banwell et al., 2008), junevile idiopathic arthritis (Ellis et al., 2010), allergic diseases (Hanson et al., 2002) and other autoimmune diseases. Some authors have published data on direct links between MAP or cow's milk and the mentioned diseases (Hanson, 1998; Davis, 2001; Virtanen and Knip, 2003; Dow, 2006). Comprehensive studies and reviews are available on the links of Crohn's disease with social and ethnic factors (Economou and Pappas, 2008; Hou et al., 2009), urban dwelling and communal water availability (Falkinham et al., 2008; Kaevska and Hruska, 2010; van Ingen et al., 2010), showers and bathrooms with hot water supplies (Gent et al., 1994; Duggan et al., 1998), the use of home refrigerators (Hugot et al., 2003) and higher hygienic standards (Guarner, 2007; Klement et al., 2008). Some important findings have been published as far back as 20 years ago (Thomson, 1993; Colombel and Gowerrousseau, 1994; Wurzelmann et al., 1994). All these hypotheses can be linked with bottle feeding and MAP or mycobacterial triggers of proinflammatory cytokine cascades. Gut mucose permeability and formation of immunity and exposure to mycobacterial triggers of inflammation in the first days or weeks after birth could have a delayed manifestation of inflammation in the target tissues many years later (Colombel and Gowerrousseau, 1994; Kawabata et al., 1994; Wurzelmann et al., 1994; Ponsonby et al., 2009).

A tentative interpretation of ongoing or previous infections with Crohn's disease could be provided by data regarding the duration of exposure to triggers, which is strongly linked with chronic human diseases (Carbone et al., 2005). The fact that the human body can be affected by a huge number of non-cultivable mycobacteria even if the host does not suffer from mycobacteriosis as a disease with clinical symptoms is not yet regarded as a possible missing piece of the etiological puzzle of many inflammatory diseases. Data on the number of MAP cells in powdered infant milk can be important for further understanding the etiology and pathogenesis of Crohn's disease and other lifestyle diseases.

MATERIAL AND METHODS

Fifty one dried milk baby food products from 10 producers operating in seven European Union countries and available on the Czech market were tested by competitive real time quantitative PCR for *F57* (Slana et al., 2008a). The results of testing the same samples by PCR for IS*900* have been already published (Hruska et al. 2005).

RESULTS AND DISCUSSION

MAP cells were found in 18 samples (35%) and concentrations ranged between 48 and 32 500 per gram of dried milk (Table 1). More than 10 000 cells per gram of dried milk were estimated to be present in four samples (7.8%). Eighteen samples were found to be *F57*-positive, while 13 samples were also IS*900*-positive. *F57* was not detected in 12 samples positive for IS*900*. The differences between both methods are the subject of ongoing experiments; however, we have sufficiently reliable controls for the elimination of false positive results. We are confident in the reliability of the estimated numbers of cells and believe that the numbers may even be higher.

The concentration of 10 000 cells per gram of dried milk represents 5 million MAP cells in one package of 500 g. Usually two packages of the same batch are purchased together; therefore, the exposure of one baby to immunomodulators from 10 million MAP cells is accomplished within several days according to the age of the baby and daily amount of ingested milk. However, exposure can be higher and longer if the batch is not changed or if the new one is not MAP-free or is negligibly contaminated. Moreover, mycobacteria with proinflammatory triggers can be ingested also from potable or bottled water, used for formula preparation or can be ingested or inhaled during bathing or swimming. Thus, the total amount of triggers, having a possible impact on the baby in a critical time of immune maturation, can be very significant.

Muramyldipeptide and other components of the mycobacterial cell wall can have an impact on immunity and very likely participate in the development of some human chronic inflammatory diseases. Powdered or liquid milk as a substitute for breast feeding of premature babies and newborns should be produced from MAP-free milk or some limit of contamination should be established. The contamination of water should be regulated in a similar manner. The presence of mycobacteria in milk and water represents a public health problem which needs to be addressed urgently.

Table 1. The quantity of *Mycobacterium avium* subsp. *paratuberculosis* cells in powdered infant milk*

	F57	IS*900***
Number of samples examined	51	51
Number of samples positive	18 (35.3%)	25 (49%)
Number of MAP per 1 g	4.84×10^1	positive
	5.39×10^1	negative
	6.48×10^1	positive
	1.05×10^2	negative
	1.21×10^2	positive
	1.24×10^2	negative
	1.37×10^2	inhibition
	1.58×10^2	positive
	1.87×10^2	positive
	2.29×10^2	positive
	2.50×10^2	positive
	2.55×10^2	positive
	2.59×10^2	positive
	4.92×10^2	positive
	1.05×10^4	positive
	1.61×10^4	positive
	2.68×10^4	inhibition
	3.25×10^4	positive

*Only three *F57*-positive samples were IS*900*-negative, in two samples the PCR was inhibited. In twelve IS*900*-positive samples *F57* was not detected
**Hruska et al. (2005)

REFERENCES

Anon (2000): Possible links between Crohn's disease and paratuberculosis. Report of the Scientific Committee on Animal Health and Animal Welfare, Adopted 21 March 2000 (SANCO/B3/R16/2000), 1–76.

Anon (2010): Assessment of food as a source of exposure to Mycobacterium avium subspecies paratuberculosis (MAP). Journal of Food Protection 73, 1357–1397.

Banwell B, Bar-Or A, Cheung R, Kennedy J, Krupp LB, Becker DJ, Dosch HM (2008): Abnormal T-cell reactivities in childhood inflammatory demyelinating disease and type 1 diabetes. Annals of Neurology 63, 98–111.

Barclay AR, Russell RK, Wilson ML, Gilmour WH, Satsangi J, Wilson DC (2009): Systematic review: The role of breastfeeding in the development of pediatric inflammatory bowel disease. Journal of Pediatrics 155, 421–426.

Bergstrand O, Hellers G (1983): Breast-feeding during infancy in patients who later develop Crohns-disease. Scandinavian Journal of Gastroenterology 18, 903–906.

Botsaris G, Slana I, Liapi M, Dodd C, Economides C, Rees C, Pavlik I (2010): Rapid detection methods for viable Mycobacterium avium subspecies paratuberculosis in milk and cheese. International Journal of Food Microbiology 141 (Suppl.1), S87–S90.

Brant SR, Wang MH, Rawsthorne P, Sargent M, Datta LW, Nouvet F, Shugart YY, Bernstein CN (2007): A population-based case-control study of CARD15 and other risk factors in Crohn's disease and ulcerative colitis. American Journal of Gastroenterology 102, 313–323.

Carbone KM, Luftig RB, Buckley MR (2005): Microbial triggers of chronic human ilness. American Academy of Microbiology Colloquium, 1–14.

Collins MT (1997): Mycobacterium paratuberculosis: A potential food-borne pathogen? Journal of Dairy Science 80, 3445–3448.

Colombel JF, Gowerrousseau C (1994): Etiology of Crohns-disease – current data. Presse Medicale 23, 558–560.

Corti S, Stephan R (2002): Detection of Mycobacterium avium subspecies paratuberculosis specific IS*900* insertion sequences in bulk-tank milk samples obtained from different regions throughout Switzerland. BMC Microbiology 2, 15.

Coulombe F, Divangahi M, Veyrier F, de Leseleuc L, Gleason JL, Yang Y, Kelliher MA, Pandey AK, Sassetti CM, Reed MB, Behr MA (2009): Increased NOD2-mediated recognition of N-glycolyl muramyl dipeptide. Journal of Experimental Medicine 206, 1709–1716.

Davis MK (2001): Breastfeeding and chronic disease in childhood and adolescence. Pediatric Clinics of North America 48, 125–141.

Donaghy JA, Johnston J, Rowe MT (2011): Detection of Mycobacterium avium ssp. paratuberculosis in cheese, milk powder and milk using IS900 and f57-based qPCR assays. Journal of Applied Microbiology 110, 479–489.

Dow CT (2006): Paratuberculosis and type I diabetes is this the trigger? Medical Hypotheses 67, 782–785.

Duggan AE, Usmani I, Neal KR, Logan RFA (1998): Appendicectomy, childhood hygiene, Helicobacter pylori status, and risk of inflammatory bowel disease: a case control study. Gut 43, 494–498.

Economou M, Pappas G (2008): New global map of Crohn's disease: Genetic environmental and socioeconomic correlations. Inflammatory Bowel Diseases 14, 709–720.

Ellis JA, Munro JE, Ponsonby AL (2010): Possible environmental determinants of juvenile idiopathic arthritis. Rheumatology 49, 411–425.

Ellouz F, Adam A, Ciorbaru R, Lederer E (1974): Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. Biochemical and Biophysical Research Communications 59, 1317–1325.

Eltholth MM, Marsh VR, Van Winden S, Guitian FJ (2009): Contamination of food products with Mycobacterium avium paratuberculosis: a systematic review. Journal of Applied Microbiology 107, 1061–1071.

Falkinham JO (2003): The changing pattern of nontuberculous mycobacterial disease. Canadian Journal of Infectious Diseases 14, 281–286.

Falkinham JO, Iseman MD, de Haas P, van Soolingen D (2008): Mycobacterium avium in a shower linked to pulmonary disease. Journal of Water and Health 6, 209–213.

Gent AE, Hellier MD, Grace RH, Swarbrick ET, Coggon D (1994): Inflammatory bowel-disease and domestic hygiene in infancy. Lancet 343, 766–767.

Gill CO, Saucier L, Meadus WJ (2011): Mycobacterium avium subsp. paratuberculosis in dairy products, meat, and drinking water. Journal of Food Protection 74, 480–499.

Grant IR, Rowe MT, Dundee L, Hitchings E (2001): Mycobacterium avium ssp. paratuberculosis: its incidence, heat resistance and detection in milk and dairy products. International Journal of Dairy Technology 54, 2–13.

Grant IR, Ball HJ, Rowe MT (2002): Incidence of Mycobacterium paratuberculosis in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. Applied and Environmental Microbiology 68, 2428–2435.

Guarner F (2007): Hygiene, microbial diversity and immune regulation. Current Opinion in Gastroenterology 23, 667–672.

Hanson LA (1998): Breastfeeding provides passive and likely longlasting active immunity. Annals of Allergy Asthma and Immunology 81, 523–537.

Hanson LA, Korotkova M, Haversen L, Mattsby-Baltzer I, Hahn-Zoric M, Silfverdal SA, Strandvik B, Telemo E (2002): Breast-feeding, a complex support system for the offspring. Pediatrics International 44, 347–352.

Hou JK, El-Serag H, Thirumurthi S (2009): Distribution and manifestations of inflammatory bowel disease in Asians, Hispanics, and African Americans: A systematic review. American Journal of Gastroenterology 104, 2100–2109.

Hruska K (2009): Possible risk of autoimmune or autoinflammatory diseases triggered by mycobacteria. 333–338. In: Kazda J, Pavlik I, Falkinham III JO, Hruska K (eds): The Ecology of Mycobacteria: Impact on Human and Animal's Health. 1st ed. Springer. 565 pp.

Hruska K, Pavlik I (2010): Frequently asked questions on the links between mycobacteria and Crohn's disease. In: Keynote lecture delivered at the International Conference on Gastro-Intestinal Microbial Ecology, 8–11 November 2010, Kosice, Slovak Republic. http://centaur.vri.cz/docs/files/FAQ_2010_Kosice.pdf

Hruska K, Bartos M, Kralik P, Pavlik I (2005): Mycobacterium avium subsp. paratuberculosis in powdered infant milk: paratuberculosis in cattle – the public health problem to be solved. Veterinarni Medicina 50, 327–335. http://www.vri.cz/docs/vetmed/50-8-327.pdf

Hugot JP, Alberti C, Berrebi D, Bingen E, Cezard JP (2003): Crohn's disease: the cold chain hypothesis. Lancet 362, 2012–2015.

Kaevska M, Hruska K (2010): Mycobacteria in water, feedstocks and food: analysis of publications. Veterinarni Medicina 55, 571–580. http://vri.cz/docs/vetmed/55-12-571.pdf

Kawabata Y, Nagao S, Asano T, Nishikawa T, Takada H (1994): Guinea-pigs prepared with various bacteria and their components to induce a necrotic reaction provoked with muramyldipeptide. FEMS Immunology and Medical Microbiology 9, 287–297.

Klement E, Cohen RV, Boxman J, Joseph A, Reif S (2004): Breastfeeding and risk of inflammatory bowel disease: a systematic review with meta-analysis. American Journal of Clinical Nutrition 80, 1342–1352.

Klement E, Lysy J, Hoshen M, Avitan M, Goldin E, Israeli E (2008): Childhood hygiene is associated with the risk for inflammatory bowel disease: A population-based study. American Journal of Gastroenterology 103, 1775–1782.

Maeda S, Hsu LC, Liu H, Bankston LA, Iimura M, Kagnoff MF, Eckmann L, Karin M (2005): NOD2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. Science 307, 734–738.

Mikhailov TA, Furner SE (2009): Breastfeeding and genetic factors in the etiology of inflammatory bowel disease in children. World Journal of Gastroenterology 15, 270–279.

Nacy C, Buckley M (2008): Mycobacterium avium paratuberculosis: Infrequent human pathogen or public health threat? A report from the American Academy of Microbiology, 37 pp.

Nielsen SS, Toft N (2009): A review of prevalences of paratuberculosis in farmed animals in Europe. Preventive Veterinary Medicine 88, 1–14.

Oddy WH (2004): A review of the effects of breastfeeding on respiratory infections, atopy, and childhood asthma. Journal of Asthma 41, 605–621.

Papapetropoulou M, Tsintzou A, Vantarakis A (1997): Environmental mycobacteria in bottled table waters in Greece. Canadian Journal of Microbiology 43, 499–502.

Pedley S, Bartram J, Rees G, Dufour A, Cotruvo JAE (2004): Pathogenic mycobacteria in water – A guide to public health consequences, monitoring and management. Emerging Issues in Water and Infectious Disease Series, World Health Organization titles with IWA Publishing. 222 pp.

Peng H, Hagopian W (2006): Environmental factors in the development of Type 1 diabetes. Reviews in Endocrine and Metabolic Disorders 7, 149–162.

Pettis RJ, Hall I, Costa D, Hickey AJ (2000): Aerosol delivery of muramyl dipeptide to rodent lungs. AAPS Pharmsci 2, article 25, 1–9.

Ponsonby AL, Catto-Smith AG, Pezic A, Dupuis S, Halliday J, Cameron D, Morley R, Carlin J, Dwyer T (2009): Association between early-life factors and risk of child-onset Crohn's disease among Victorian children born 1983–1998: A birth cohort study. Inflammatory Bowel Diseases 15, 858–866.

Slana I, Kralik P, Kralova A, Pavlik I (2008a): On-farm spread of Mycobacterium avium subsp. paratuberculosis in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. International Journal of Food Microbiology 128, 250–257.

Slana I, Paolicchi F, Janstova B, Navratilova P, Pavlik I (2008b): Detection methods for Mycobacterium avium subsp. paratuberculosis in milk and milk products: a review. Veterinarni Medicina 53, 283–306. http://www.vri.cz/docs/vetmed/53-6-283.pdf

Slana I, Bartos M, Roubal P, Babak V, Pavlik I (2009): Mycobacterium avium subsp. paratuberculosis and M. avium detected by culture, IS900 and IS901 highly sensitive PCR in bulk tank milk from dairy herds in the Czech Republic between 2002 and 2004. Czech Journal of Food Sciences 27, 372–378.

Thomson ABR (1993): IBD epidemiology – ongoing issues and new ideas – the Canadian perspective. Canadian Journal of Gastroenterology 7, 142–148.

van Ingen J, Blaak H, de Beer J, Husman AMD, van Soolingen D (2010): Rapidly growing nontuberculous mycobacteria cultured from home tap and shower water. Applied and Environmental Microbiology 76, 6017–6019.

Virtanen SM, Knip M (2003): Nutritional risk predictors of beta cell autoimmunity and type 1 diabetes at a young age. American Journal of Clinical Nutrition 78, 1053–1067.

Wurzelmann JI, Lyles CM, Sandler RS (1994): Childhood infections and the risk of inflammatory bowel-disease. Digestive Diseases and Sciences 39, 555–560.

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Corresponding Author:

Prof. MVDr. Karel Hruska, CSc., Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic
Tel. +420 533 332 014, E-mail: hruska@vri.cz