

## Detection of *Mycobacterium avium subsp. paratuberculosis* IS900 in Baby Milk Powder in Egypt

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**Abstract:** Six baby milk powder samples imported from 6 different countries to Egypt were tested to *Mycobacterium avium subsp. paratuberculosis* IS900, the specific fragment for MAP was detected using IS900 PCR and all samples were found positive (100%). These results correspond to the epidemiological results reported and the wide spread of paratuberculosis and Crohn's disease in the last decade. The possible risk of killed MAP cells or bacterial structures in baby milk powder was discussed in respect to the autoimmune Crohn's disease cause. An Egyptian national programme is needed to decrease the risk of exposure for children and people under the highest risk for Crohn's disease. Importers of baby milk powder should import milk powder from countries which use MAP free milk.

**Key words:** Johne's disease • Paratuberculosis • Control • Crohn's disease • IS900 • Baby milk powder

### INTRODUCTION

Paratuberculosis (Johne's disease) is a widely distributed infectious disease of cattle and other domestic and wild ruminants caused by *Mycobacterium avium subsp. paratuberculosis* (MAP) infection [1]. The disease etiological factor is an acid-resistant, Gram-positive, slowly-growing bacillus. The first symptoms of the disease include periodical persistent diarrhea, body mass loss and successive wasting appear 6 months to 15 years after infection. The clinical form of the disease usually occurs in older individuals and is hardly ever observed in those aged under two years. The infected animals excrete MAP both with feces as well as with milk [2]. Up to 70% of dairy herds suffer from this infection in most European countries, the United States and Canada. 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 [3, 4]. 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 [5]. Massive shedding of MAP in faeces contaminates the environment and transmits the infection to other animals. Evidence of

the pathogen has been found not only in the intestine but also in milk, lymph nodes and different parenchymatous organs [6, 7]. Confirmed MAP isolates were cultured from 1.8% of the commercially pasteurized milk samples in the U.K [8]. Similar data were published from the U.S.A. [9]. In the U.K. study, 11.8% of samples of retail milk were MAP-positive by PCR. In Switzerland, 19.7% of bulk-tank milk samples were IS900 PCR positive [10]. Goat and sheep bulk tank milk samples were also PCR-positive for IS900 (23.0 and 23.8%, respectively), providing presumptive evidence for the presence of MAP in Switzerland [11]. MAP has been cultivated from cheese [12, 13] as well.

Pickup *et al.* [14] reported that MAP cultivation needs up to four months for cultivation with some forms not growing *in vitro* at all. The concentration of MAP, quoted in colony forming units (CFU), does not convey the total number of cells present. Molecular techniques offer a more rapid and specific detection of MAP and its quantification in milk, cheese and meat.

Crohn's disease is a chronic autoimmune inflammatory bowel disease with similar pathological changes to paratuberculosis [15]. Crohn's disease was first described in 1932 as a chronic inflammation of the terminal ileum. Crohn's disease affects primarily young adults and the elderly, but can occur at any age. It is now recognized that although involvement of the ileocaecal area is most frequent (50%), the disease can affect any

area of the gastrointestinal tract from mouth to anus, including the nasal cavity. In children and adolescents, there is often upper gastrointestinal tract involvement. Symptoms include fever, diarrhea, cramping pain in the abdomen, nausea, vomiting, anaemia and weight loss [16].

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 [17]. 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 [18].

In all European 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. 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 [19]. An increase of incidence was also reported [20-23].

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. The finding of *M. paratuberculosis* in milk has raised the questions as to whether or not milk could act as a reservoir for exposure of the general public to *M. paratuberculosis*. [24]. Laboratory-scale and commercial-scale studies on pasteurization of milk containing *M. paratuberculosis* indicated that the mycobacterium could survive the heat treatment [25]. 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 [26].

The current investigations were carried out to detect *Mycobacterium avium* subsp. *paratuberculosis* IS900 in baby milk powder in Egypt.

## MATERIALS AND METHODS

**Samples:** Six dried milk baby food products (Infant formula) registered in the Egyptian market, imported from outside Egypt and purchased from the Egyptian market stores were tested. The milk for these formulas is pasteurized before it is dried during preparation of these products.

### DNA Extraction, IS900 PCR and Electrophoresis [27]:

Only one gram of the powdered milk samples were diluted in 9ml 1X TE buffer and centrifuged at 30,000 rpm. The pellet was resuspended in 500ul of 1X TE buffer, extracted by heat block at 100°C/ 10min according to Hruska *et al.* [26]. The extracted DNA samples were applied to IS900 PCR according the following program: 1 cycle at 94°C, 10 min; 50 cycles at 94°C, 59 sec, 60°C, 30 sec and 72°C, 59 sec; followed by a final extension cycle at 72°C, 10 min. The following highly sensitive IS900 primers were used.

Forward: 5'-CCGCTAATTGAGAGATGCGATTGG-3'.

Reverse: 5'-AATCAACTCCAGCAGCGCGGCCTCG-3'.

The electrophoresis grade Agarose was prepared in 1x electrophoresis buffer to reach the required 1.5% concentration. The Agarose was cooked in a microwave with agitation till being clear. The Agarose was allowed to cool, then 0.5 µg/ml ethidium bromide was added. The Agarose was poured in the electrophoresis mould to make 4 mm depth. The comb was inserted and left to solidify. The comb was removed gently. The TAE buffer was poured until covering the gel. The sample was injected with loading dye and sunk in the well. The cathode and anode were matched with power supply at 100 volt. The current was stopped when the loading dye reached 2:3 to the gel. The transilluminator was used to detect the desired 229 bp band.

## RESULTS

All six powdered milk samples were found to be positive to IS900 PCR and gave a band of 229 bp molecular size confirming the presence of IS900 sequence specific for *Mycobacterium avium* subsp. *paratuberculosis* as shown in Fig. 1.

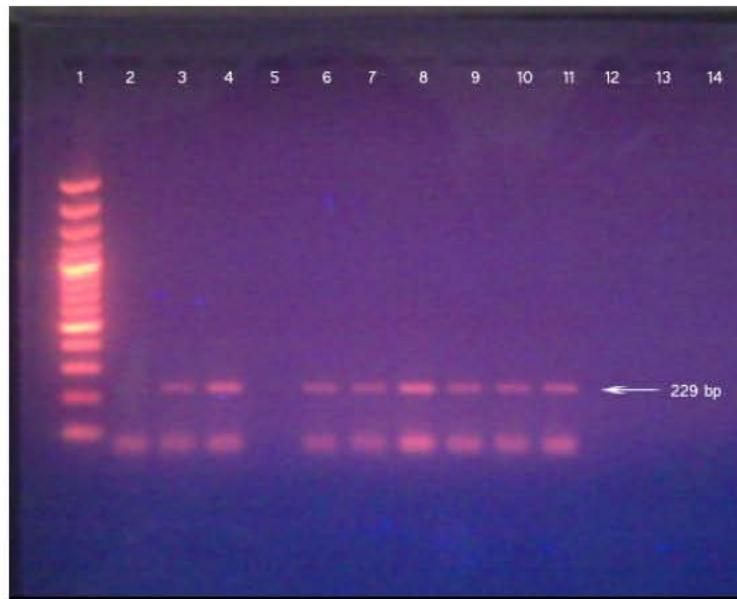


Fig. 1: Showing IS900 PCR application on six powdered milk samples which all were positive to IS900 PCR. Lane(1) represents the 100bp marker ladder. Lane (2) represents the negative control. Lanes(3 and 4) represent the positive controls. Lanes (6 to 11) represent the positive milk powder samples showing a band of 229 bp molecular size confirming the presence of IS900 sequence specific for *Mycobacterium avium subsp. paratuberculosis* while lane (5) was left without sample as a separator between samples and controls

## 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 [28]. The microbial sensing proteins involved in innate immunity recognize conserved and often structural components of microorganisms. Published data has strengthened the association of MAP with Crohn's disease [15, 29, 30-33]. 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 [34] are usually used to detect MAP although some different specific loci were described [35-38]. The presence 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 revealed 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 [39]. 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 [40-42].

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 [43]. Crohn's disease patients' antibodies were tested by immunoblotting against recombinant antigens identified from MAP genomic library [44]. 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 [45]. Peptidoglycan-polysaccharide complexes were detected intracellularly in the mucosa and submucosa of the bowel wall of Crohn's disease patients. The results showed 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 [46].

Comprehensive reviews of experimental data supporting a genetic disposition to Crohn's disease and immunity, inflammation and allergy in the gut were published [47- 49].

Paratuberculosis in cattle causes considerable economic losses for farmers [1,50, 51]. Crohn's disease is also important, both for the pain and difficulties it causes and for the huge expenditure for treatment [52-54]. 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 and even DNA.

Up to 70% of dairy herds suffer from the paratuberculosis infection in most European countries, the united states and Canada as Stable [51] declared. Map is very resistant to high temperature and chlorination. The organism remains life in lake water for 632 days and persisted for up to 841 days as stated by Pickup *et al.* [14]. Hruska *et al.* [26] used IS900 PCR for detection of *Mycobacterium avium subsp. paratuberculosis* in powdered milk infant formula and found that more than 70% were positive to IS900 PCR and 2% were positive to culture which cause infection of infants who were highly susceptible to infection and less developed immune system. The methods of production of powdered milk (spray and tray methods) could be able to kill all organisms in milk, due to Map-like all mycobacterium gathered in clumps and coating of organism with concentration of milk proteins and milk fat make it more resistant to heat which may make escape of at least one live bacillus beside ovens efficacy. 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.

Regarding to the obtained results, IS900 PCR assay was applied on six purchased fortified powder milk infant formula imported from 6 different foreign countries to Egypt and found that all samples were positive to the presence of DNA material of *Mycobacterium avium subsp. paratuberculosis* (100%) as shown in Figure 1. These results revealed that the milk which was used in powdered milk production was obtained from Map shedder cattle and these results agreed with the data supported by Hruska *et al.* [26].

The possible risk of *Mycobacterium avium subsp. paratuberculosis* dead cells or bacterial structures in milk and food in respect to autoimmune Crohn's disease should be carefully monitored to decrease the risk of

exposure for children and people under the highest risk for Crohn's disease and a national programme should be developed for controlling the disease in Egypt. Importers of baby milk powder should import milk powder from countries which use MAP free milk on a voluntary basis.

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