Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from milk of commercial consumption in Argentine

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ABSTRACT: The objective of this study was to investigate if viable *Mycobacterium avium* subsp *paratuberculosios (Map)* is present in commercial

pasteurized milk in Argentina. Seventy commercial milk (18 pasteurized, 30 ultra-pasteurized and 22 ultra-pasteurized high temperature were collected over seven months. Milk samples (50 ml) were centrifuged and the pellet was suspended in 0.75% of hexadecylpyridinium over night. and the pellet inoculated on Herrold's egg yolk medium with mycobactin and sodium pyruvate. *Map* was isolated from 2 (2.86 %) of 70 pasteurized milk, one from pasteurized and other from ultra-pasteurized milk. Both positive culture samples were also positive with *IS*900-PCR. The isolates were analyzed by PCR and the RFLP of these isolates were pattern A, one of the most prevalent *Map* type in Argentina. This study provides evidence that viable *Map* is present in comercially pasteurized milk. This result has become very important since human exposure to *Map* is a potential risk for Crohn´s disease.

Keywords: Johnes disease, *Mycobacterium avium* subsp *paratuberculosis,* milk, IS*900*

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the causative agent of paratuberculosis (PTBC) or Johne's disease. It affects domestic and wild animals and causes chronic enteritis in cows, producing symptoms such as diarrhea, weight loss, and progressive emaciation that can eventually lead to death (Collins *et al.*, 1996). *Map* has been also linked to human Crohn's disease, a systemic disorder that causes mainly a chronic inflammation of the intestine. During the development of this pathology, *Map* can parasitize immunoregulatory cells in the intestine of people with acquired or inherited susceptibility, thus resulting in an immunodepression of the mucosal lining of the intestine. This causes symptoms such as profuse diarrhea, inflammation of lymphatic ganglions and intestine, general immunological depression and weakening (Hermon-Taylor, 2002).

In the last ten years, there has been important progress in the research of the relationship between this microorganism and Crohn's disease. This research has mainly focused on food products as the transmission pathway. The dogma that *Map* is not a zoonotic microorganism is changing due to recent reports of two cases. The first case is that of a 36-year-old patient with haemophilia, AIDS and low amount of lymphocytes in blood who developed a profuse diarrhea; acid alcohol-resistant bacilli were visualized in the biopsy of his colon, liver and bone marrow, and both the culture analyses and PCR were positive (Richter *et al*., 2002). The second case was that of a six-year-old boy from whom *Map* was isolated by means of a culture obtained from his lymph nodes (Greenstein, 2003).

Although the etiologic agent of the PTBC grows slowly in culture media, this method continues to be the best for its detection. There are other fast, sensitive and specific methods for the detection of the disease such as the polymerase chain reaction (PCR) and the search for restriction fragment length polymorphism (RFLP). These analyses are based on the identification of IS*900*, an insertion sequence that appears to belong only to *Map* (Green *et al.*, 1989).

The way in which *Map* is transmitted in not fully understood yet, but some lines of evidence suggest that humans can get infected through contaminated milk, although relatively little is known about the survival of *Map* during the production of milk. Some authors suggest that pasteurization is capable of

destroying mycobacteria. Stabel *et al.* (2001) have shown that when milk is inoculated with *Map* and subjected to a treatment of 65°C for 30 minutes, such mycobacteria are destroyed since no viable microorganisms were found after 28 days of incubation. Likewise, Pearce *et al.* (2002) have stated that the pasteurization under commercial conditions provides an effective inactivation of *Map*, regardless of the type of milk or the recovery medium. In contrast, other authors support the theory that when *Map* is present in milk, it is able to resist the pasteurization conditions (Grant 1998, Grant *et al.,* 1996, Grant *et al,* 1997, Grant *et al,* 1999, Grant *et al,* 2000, Grant *et al,* 2002a, Grant *et al*, 2002b, Grant *et al*, 2003; Grant 2006, Millar *et al.*, 1996).

In United Kingdom, viable *Map* has been detected in 1.7 % of samples of commercial pasteurized milk (Grant *et al.*, 2002a) and in 6.7 % of samples of naturally infected raw milk subjected to commercial scale pasteurization at 72°C for 15 or 25 s (Grant *et al.*, 2002b). In addition, viable organisms have been found in a low number of bottles of commercial pasteurized milk in California, Minnesota and Wisconsin (USA). Two point eight percent of the 702 samples analyzed contained viable *Map* (Ellingson, 2005). These results indicate that this pathogen is occasionally capable of surviving commercial pasteurization.

Recently, in Czech Republic were tested powered infant milk from seven countries and IS900 were detected in 49% of samples and using real time PCR were detected the fragment f57 in 35% of them. Additionally, one sample were positive by culture showed viable *Map* is present (Hruska et al, 2005).

Goat's milk, which is frequently consumed without pasteurization, can contain cells of *Map* and could constitute a potential source for human infection. This mycobacterium could also be present in cheese and other products that are produced with non-pasteurized milk (Cirone, 2004).

The objective of the present work was to identify the presence of *Map* in commercial pasteurized milk to different thermal treatments.

MATERIALS AND METHODS

Samples collected

One-liter bottles of fluid milk commercial scale in supermarkets were purchased at random over seven months (once a month). Eighteen pasteurized milk (*past*) and thirty ultra pasteurized (*upast)* were kept at 4-6ºC, and twenty-two bottles of ultra high temperature (*uat*), were kept at room temperature.

Treatment of milk samples

Fifty ml of milk was taken from each of 70 samples, transferred to sterile plastic tubes and centrifuged at 2500 *g* for 30 min; the supernatant was withdrawn to obtain the pellet. The *upast* and *uat* milk were not subjected to decontamination processes with hexadecylpyridinium (HPC) at 0.75%. The *past* milk was decontaminated over night (ON) since it may contain contaminant microorganisms.

Laboratory examination for maycobacteria

Culture examination. The pellet from the non-decontaminated samples was re-suspended in 25 ml of saline buffer pH 7 (PBS), while that from the decontaminated ones was re-suspended in 25ml of HPC at 0.75%. The samples were agitated until complete dissolution, and kept at room temperature ON. They were centrifuged at 2000 g for 30 minutes, 1 ml broth brain-heart (with vancomycin, amphotericyn and nalidixic acid) was added to each pellet, and they were kept at 37°C ON. Six drops (120 μl) were taken from each sample, and cultured in triplicate in Herrold´s medium (H), H medium plus mycobactin and piruvate (HMP), and H medium plus mycobactin and antibiotics (HMA) (Paolicchi, 2003). The cultures were observed every 15 days for four months, and the development of suspicious colonies of Map was identified. The colonies were analyzed microscopically by means of Ziehl Neelsen staining. The counts were expressed in cfu/ml of milk.

Identification of mycobacterial isolates. Two cfu of the growth of each of the strains was taken from an HMP culture medium kept at 37°C with colony development and an aliquot of the reaction mixture was added. The extracted DNA was amplified in a reaction mixture containing: 2.5 μl of each primer IS*900* (50 ng/μl), P90 5’ (GAAGGGTGTTCGGGGCCGTC) and P91

(GAGGTCGATCGCCCACGTGAC) (Khare *et al*., 2004), 5 μl of the buffer 10 x (200mM Tris HCl pH 8.4, 500 mM KCl and 50 mM $MgCl_2$), 4μl dNTP mix (2.5 mM of each dNTP), and 0.25 μl of *Taq* polymerase (5 units/μl) per reaction. Each sample was homogenized and transferred to a tube of PCR in aliquots of 20 μl. Each mixture was covered with mineral oil and submitted to amplification. The samples were subjected to 25 cycles at 94°C for 3 minutes, to 94°C for 1 minute, to 65°C for 1 minute and to 72°C for 2 minutes; and to one cycle at 72°C for 4 minutes. For each set of PCRs both a positive (*Map* DNA) and a negative control (sterile distilled water) were used. An aliquot of each mixture of amplification was subjected to electrophoresis in agarose gel at 2% with Tris-borate buffer EDTA (TBE; 89 mM Tris, 1 mM boric acid, 2 mM EDTA). The DNA bands were observed under UV light after ethidium bromide staining. The samples were classified as positive only if the correct band size (217 bp) was identified in the gel.

Analyzed strains. The two strains isolated from commercial milk were analyzed by using the method described by Paolicchi *et al* (2002). After digestion, the restriction endonuclease *Bst*EII was used and the type of RLFP-IS*900* of *Map* isolated was designed arbitrarily by using the letters “A”, “B”, “C” and “E” to design the diferente patterns of RFLP (Moreira *et al*., 1999; Pavlík *et al*., 1995; Pavlík *et al*., 1999).

RESULTS

Colonies with the typical morphology of *Map* were identified in one *past* and in one *uat* sample of milk after 8 weeks on Herrold’s media (Table 1). Both strains isolated from commercial milk were positive for the PCR IS*900*.(photo 1) The results of the RFLP analysis revealed that both strains of *Map* belong to a pattern called “A” or C17 European strains type (photo 2).

DISCUSSION

Map is a microorganism that can be found in different productive systems and in the agricultural-food chain, and thus represents a risk for Public Health. In the present work, we demonstrate that it is possible to identify viable *Map* from the commercial pasteurized milk that was obtained from dairy herds

and animals presumably infected with PTBC. This indicates *Map*'s capacity to resist inactivation by heath treatments to which milk is subjected before commercialization and consumption. Although the number of milk bottles examined in this work was low, the positive samples found represent 2.86% of all samples tested. This finding is the first of its kind in Argentina as regards commercial milk and indicates the risk of infection to which the population that usually consumes this product is exposed.

The genetic identification in the *Map* strains isolated from commercial milk revealed that they belong to pattern "A", which is the most important one in our country. On the other hand, the two strains analyzed by PCR from a colony of *Map* culture (PCR colony) were positive, thus confirming the identity of this mycobacterium found in commercial milk.

Studies performed in United Kingdom revealed that *Map* is present in 1.7% of samples of commercial pasteurized milk (Grant *et al.*, 2002a). In addition, viable organisms have been found in low numbers (2.8% of a total of 702 samples) in commercial pasteurized milk in California, Minnesota and Wisconsin in the USA (Ellingson, 2005). These results indicate that is pathogen is capable of surviving commercial pasteurization. Due to these results, certain European governments introduce measures to reduce *Map* in the food chain as way of preventing of the disease (Greenstein, 2003).

An important clinical finding that reinforces the concept of zoonosis is that *Map* has also been cultured from maternal milk from two women with Crohn's disease. This was confirmed by means of the detection of the insertion sequence IS*900* typical of *Map* (Naser *et al.*, 2000). Our previous works in cheese made with cows' and sheep's milk artificially inoculated with *Map,* indicated that this pathogen would resist the condition of maduration in cheese (Cirone, 2004).

The results obtained in this work, which are important as regards Public Health, demonstrate that the controls in the sanitary systems in the dairy farm and milk industry should be tightened with the aim of protecting consumers from risk of infection with *Map* and any other potentially pathogenic bacteria.

Further qualitative and quantitative research is still necessary in order to clarify the rol *Map*'s resistance to heath treatments performed in the milk

industry, as well as to evaluate its survival to the different elaboration processes used for dairy products. Also, fast, reliable and inexpensive detection methods should be developed so that efficient control measures can be later applied both in primary production and in the manufacture or processing of food.

Other similar works should be performed in different regions of Argentina in order to evaluate the presence of *Map* in milk and derived dairy products, and to relate them both to the prevalence of PTBC in cattle and other animal species or to the incidence of Crohn's disease in humans. On the other hand, it would be important to develop and adjust the fast and reliable identification methods to detect the presence of *Map* in food products before they are placed in the market for human consumption.

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REFERENCES

Cirone K. (2004): Characterization and viability of *Mycobacterium avium* subsp. *paratuberculosis* in milk, cheese and water. (Caracterización y viabilidad de *Mycobacterium avium* subsp. *paratuberculosis* en leche, quesos y agua.) Thesis of Graduate at Universidad Nacional de Mar del Plata, Faculty of Agrarian Sciences. Argentina. 65 p.

Collins M.T., Goodger W.J., Nordlund K.V., Pelletier J. (1996): Associations between subclinical paratuberculosis and milk production, milk components, and somatic cell counts in dairy herds. J Am Vet Med Assoc. 208(11):1872-1876.

Ellingson J., Anderson J., Koziczkowski J., Radcliff., R., Sloan S., Allen S., Sulllivan N. (2005): Detection of Viable *Mycobacterium avium* subsp. *paratuberculosis* in Retail Pasteurized Whole Milk by Two Culture Methods and PCR; J of Food Prot. 68 (5): 966-972;

Grant I.R., Ball H.J., Neill S.D., Rowe M.T. (1996): Inactivation of *Mycobacterium avium* subsp*. paratuberculosis* in cows' milk at pasteurization temperatures. Appl Environ Microbiol. 62 (2): 631-636.

Grant I.R., Ball H.J., Rowe M.T. (1997): A novel staining technique for assessing clumping and viability of *Mycobacterium avium* subsp. *paratuberculosis* cells during pasteurization; Proceedings of the 5th International Colloquium on Paratuberculosis. International Association of Paratuberculosis Inc, Rehoboth, MA, USA. p. 456-458.

Grant I.R. (1998): Letter to the editor: Does Mycobacterium avium subsp. paratuberculosis survive current pasteurization conditions? Appl Environ Microbiol. (7): 2760-2761.

Grant I.R., Ball H.J., Rowe M.T. (1999): Effect of higher pasteurization temperatures, and longer holding times at 72 degrees C, on the inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk. Lett Appl Microbiol. 28 (6):461-465.

Grant I.R., Pope C.M., O'Riordan L.M., Ball H.J., Rowe M.T. (2000): Improved detection of *Mycobacterium avium* subsp*. paratuberculosis* in milk by immunomagnetic PCR. J. Vet. Microbiol. 77(3-4):369-378.

Grant I.R., Hitchings E.I, Ball H.J., Rowe M.T. (2002a): Impact of commercial HTST pasteurization on *Mycobacterium avium* subsp. *paratuberculosis* in naturally infected cow's milk. Appl Environ Microbiol. 68(2): 602-607.

Grant I.R., Ball H.J., Rowe M.T. (2002b): Incidence of *Mycobacterium avium* subsp. *paratuberculosis* in bulk raw and commercial pasteurized cow's milk

from approved dairy processing establishments in the United Kingdom; Appl Envirom Microbiol; 68: 2428-2435;

Grant I.R., Kirk E., Hitchings E.I., Rowe M.T. (2003): Comparative evaluation of MGIT and BACTEC culture systems for the recovery of *Mycobacterium avium* subsp. *paratuberculosis* from milk. J Appl Microbiol. 95: 169-201.

Grant I.R. (2006): Occurrence of *Mycobacterium avium* subsp *paratuberculosis* in foods and the impact of milk processing on its survival. Proceeding of 8°ICP, Copenhague, Denmark.

Green G., Tizard M., Thompson J., Winterbourme D., Mc Fadden J., Hermon-Taylor J. (1989):. Sequence and characteristic of IS*900*, and insertion sequence identified in human Crohn's disease isolate of *Mycobacterium avium* subsp*. paratuberculosis*. Nucleic acid Research. 17: 9063-9073.

Greenstein R. (2003): Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne"s disease. The Lancet infectious disease. 3: 507-513.

Hermon-Taylor J. (2002): *Mycobacterium avium* subsp. *paratuberculosis* and its relation to Crohn's disease. Proceedings of the 7th International Colloquium on Paratuberculosis. Bilbao, Spain. p. 96

Hruska K., Bartos M., Kralik P., Pavlik I. (2005): *Mycobacterium avium* subsp. *paratuberculosis* in powdered infant formula. Veterinarni Medicine (Vet Med Czech) 50:8.

Khare S., Fitch T., Santos R., Romano J., Fitch A., Zhang S., Grant I., Libal M., Hunter D., Adams G. (2004): Rapid and sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk and feces by combination of inmunomagnetic bead separation-conventional PCR and real-time PCR. J. Clinical Microbiol. 42(3): 1075-1081.

Millar D., Ford J., Withey S., Tizard M., Doran T., Hermon-Taylor J. (1996): IS*900* PCR to detect *Mycobacterium avium* subsp*. paratuberculosis* in retail supplies of whole pasteurized cow's milk in England en Wales. Appl Environ Microbiol. 5(11): 3446-2452.

Moreira A., Paolicchi F., Morsella C., Zumarraga M., Cataldi A., Bigi F., Alito A., Overduin P., Van Soolingen D., Romano M. (1999): Distribution of IS*900* restriction length polymorphism types among animal *Mycobacterium avium* subsp. *paratuberculosis* isolates from Argentina and Europe. J Vet Microbiol. 70: 251-259.

Naser, S., Schwarzt D., Shafran I., (2000): Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from breast milk of Crohn's disease patients. Am J Gastroenterol. 95 (4):1094-1095.

Paolicchi F.A., Zumarraga M.J., Gioffre A., Zamorano P., Morsella C:, Verna A., Cataldi A., Alito A., Romano M. (2003): Application of different methods for the diagnosis of paratuberculosis in dairy cattle herds in Argentina. J Vet Med. 950: 20-26.

Pavlik I., Horvathova A., Bartl J., Rychlik I. (1995): Study of epidemilogy and pathogenesis of paratuberculosis using RFLP (Restriction Fragment Length Polymorphism); Proceedings of the Fifth International Colloquium of Paratuberculosis; p. 202-211.

Pavlik I., Horvathova A., Dvoska L., Svastova P., du Maine R., Fixa B., Rychlik I. (1999): Homogeneity / Heterogeneity of *Mycobacterium avium* subsp. *paratuberculosis* strains. Correlation between RFLP-Type and source (Animal, Enviromental, Human). Proceedings of sixth International Colloquium of PTBC. 321-329.

Pearce L.E., Crawford R.A., Troung H.T., Yates G.F., Lisle G.W. (2002): The recovery of *Mycobacterium avium* subsp. *paratuberculosis* following heat treatment of inoculated milk in a turbulent-flow pasteuriser is not adversely affected by decontamination and antibiotic selection. Proceedings of the 7th International Colloquium on Paratuberculosis. Bilbao, Portugal; p. 102.

Richter E., Wessling J., Lugering N., Domschke W., Gerdes-Rush S. (2002): *Mycobacterium avium* subsp. *paratuberculosis* Infection in a patient with HIV, Germany. Emerging Infectious Disease. 8 (7): 729-730;

Stabel J.R. (2001): On-farm batch pasteurization destroys *Mycobacterium avium* subsp. *paratuberculosis* in waste milk. J Dairy Sci. 84 (2):524-527.

Table 1:
Results of cultivated milk samples .

Type of milk	**Milk samples (%) with *Map***
past[a]	1 (5.56%)
upast[b]	0 (0 %)
uat[c]	1 (4.55 %)
total number	**2 (2.86 %)**

a.pasteurized

1b. ultra-pasteurized

2c. ultra- high temperature

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