

**ISABEL AZEVEDO CARVALHO**

**IDENTIFICATION OF *Mycobacterium avium* subspecies *paratuberculosis* (MAP)  
IN RETAIL MILK AND IN INFLAMMATORY BOWEL DISEASE (IBD) PATIENTS  
IN BRAZIL**

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*.

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**APROVADA: 25 de setembro de 2012.**

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***To my parents, Nélia and Belmiro,  
for their endless love and support that made this dream possible...***

***Aos meus pais, Nélia e Belmiro,  
por seu amor e apoio sem fim, que tornaram esse sonho possível...***

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*“Um menino caminha e caminhando chega no muro  
e ali logo em frente a esperar pela gente o futuro está...”*  
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## BIOGRAPHY

Isabel Azevedo Carvalho, daughter of Belmiro de Carvalho Lage Filho and Maria Nélia Azevedo Carvalho, was born on May 3, 1983, in Ipatinga, MG.

In March 2001, she began to study Veterinary Medicine at the Federal University of Viçosa, MG, finishing in May 2006. She has the title of *Magister Scientiae* in the Postgraduate Program in Veterinary Medicine, Federal University of Viçosa, obtained in July 2008, under the supervision of Professor Maria Aparecida Scatamburlo Moreira.

In 2009, she was a Substitute Professor at the Federal University of Viçosa, in the areas of Veterinary Epidemiology and Sanitation, teaching Veterinary Epidemiology, Epidemiology Applied to Environmental Sanitation, and Sanitation. In July 2012, she has successfully completed an open competition exam for Substitute Professor at the Federal University of Uberlândia, for teaching matters related to Environmental Health and Health Surveillance.

Currently, she is a student of the Postgraduate Program in Veterinary Medicine, at the doctoral level. She had a fellowship of CAPES (Coordination for Improvement of Higher Education Personnel) and continues under the guidance of Professor Maria Aparecida Scatamburlo Moreira. She has experience in the Veterinary Preventive Medicine and Public Health areas, and in the use of molecular biology tools.

## SUMMARY

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## RESUMO

CARVALHO, Isabel Azevedo, D. Sc., Universidade Federal de Viçosa, Setembro de 2012. **Identificação de *Mycobacterium avium* subespécie *paratuberculosis* (MAP) em leite pasteurizado e em pacientes com doença inflamatória intestinal no Brasil.** Orientadora: Maria Aparecida Scatamburlo Moreira. Coorientadores: Maria de Lourdes de Abreu Ferrari e Abelardo Silva Júnior.

*Mycobacterium avium* subespécie *paratuberculosis* (MAP) é o agente etiológico da paratuberculose, uma enterite granulomatosa crônica que acomete todas as espécies de ruminantes, domésticos e silvestres, em todo o mundo. A doença é caracterizada por diarreia persistente e perda de peso progressiva e tem distribuição mundial e prevalência bastante variável, dependendo da metodologia utilizada. MAP tem importância em saúde pública, uma vez que pode ter um papel na etiologia da doença de Crohn (CD). A CD é uma doença inflamatória intestinal de etiologia desconhecida, que se caracteriza por inflamação crônica, focal, assimétrica transmural e ocasionalmente granulomatosa, que pode acometer qualquer segmento do tubo digestivo, da boca ao ânus. Leite e derivados seriam os veículos de transmissão do micro-organismo dos animais para o homem. Uma vez que MAP pode estar relacionado à doença de Crohn, existe uma preocupação com a qualidade e a inocuidade do leite e produtos lácteos, visando prevenir ou minimizar riscos à saúde humana. Considerando que o Brasil é um país grande produtor de leite, a presença de MAP em rebanhos brasileiros, a importância da qualidade do leite e o possível potencial zoonótico de MAP, este estudo objetivou avaliar a eficácia da pasteurização, em laboratório, contra MAP e verificar a presença de MAP em leite pasteurizado comercializado na região de Viçosa - MG, e em fragmentos de intestino humano (biópsias frescas e incluídas em parafina) de pacientes com CD,

colite ulcerativa (UC) e controles (nIBD) atendidos em um centro de referência para tratamento de doenças intestinais, no Brasil, por cultivo e PCR. No estudo de pasteurização em laboratório, verificou-se que, independentemente do binômio tempo-temperatura utilizado, MAP resistiu à pasteurização quando presente em altas concentrações ( $10^6$  e  $10^7$ ) na amostra inicial, sendo esse um fator preocupante considerando o possível papel da bactéria na etiologia da CD. No estudo para verificação da presença de MAP em amostras comerciais de leite pasteurizado, MAP foi identificado, por cultivo, em uma amostra, produzindo o primeiro relato da presença de MAP em leite pasteurizado presente no comércio no Brasil. No estudo para verificação da presença de MAP em fragmentos de intestino humano, MAP foi detectado, por PCR, em todos os grupos de pacientes testados, embora a maior carga bacteriana tenha sido detectada no grupo CD, produzindo também o primeiro relato da presença de MAP em fragmentos de biópsia de intestino humano no Brasil. O papel patogênico de MAP na CD, entretanto, permanece controverso e inconclusivo.

## ABSTRACT

CARVALHO, Isabel Azevedo, D. Sc., Universidade Federal de Viçosa, September, 2012. **Identification of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in retail milk and in inflammatory bowel disease (IBD) patients in Brazil.** Adviser: Maria Aparecida Scatamburlo Moreira. Co-advisers: Maria de Lourdes de Abreu Ferrari and Abelardo Silva Júnior.

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, a chronic granulomatous enteritis that affects all ruminant species. This disease is characterized by persistent diarrhea and progressive weight loss. It has a worldwide distribution and its prevalence varies widely depending on the methodology used. MAP has a public health impact, since it has been suggested to play a role in the etiology of Crohn's disease (CD). CD is an inflammatory bowel disease of unknown etiology, characterized by chronic, focal, asymmetric, and transmural and occasionally granulomatous inflammation, which can affect any segment of the digestive tract from mouth to anus. Milk and milk-products may be the transmission vehicles of MAP from animals to man. Since MAP has been associated with CD etiology, there is a concern about the quality and safety of milk and dairy products, to prevent or minimize risks to human health. Considering that Brazil is a great milk producer, the presence of MAP in Brazilian herds, the importance of milk quality and the possible role of MAP in CD, this study aimed: to evaluate the effectiveness of pasteurization in the laboratory against MAP; to evaluate the presence of MAP in retail pasteurized milk in Viçosa city, Minas Gerais State, Brazil; and to verify the presence of MAP in biopsies of the human intestine (fresh and formalin-fixed paraffin-embedded fragments) of patients with CD, ulcerative colitis (UC), and controls (nIBD) attending a referral center for treatment of intestinal

diseases in Brazil, by PCR and culture. In the study of pasteurization in the laboratory, regardless of the time-temperature binomial used, MAP resisted pasteurization when present at high concentrations ( $10^6$  and  $10^7$ ) in the original sample. The findings suggest that retail pasteurized milk may serve as a vector for MAP and human exposure. In the study to verify the presence of MAP in pasteurized milk samples, MAP was identified by cultivation in one sample, producing the first report of the presence of MAP in retail pasteurized milk samples in Brazil. In the study to verify the presence of MAP in fragments of human intestine, MAP was detected by PCR in all groups of patients tested, although the highest bacterial load was detected in the CD group, producing also the first report of the presence of MAP in intestinal tissue biopsies from humans in Brazil. The pathogenic role of MAP in CD, however, remains controversial and inconclusive.

## GENERAL INTRODUCTION

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, a chronic granulomatous enteritis that affects all ruminant species, wild and domestic, worldwide. This disease is characterized by persistent diarrhea and progressive weight loss and its prevalence varies widely depending on the methodology used.

Economic losses due to bovine paratuberculosis are estimated at 200- to 250-million dollars annually in the USA alone, due to decreased milk production, reduction in levels of milk protein, premature culling, increased susceptibility to other diseases, reduced fertility, and increased sanitary costs. In Brazil, studies about this disease are only just beginning and there are no estimates about economic losses caused by the disease.

Besides its great economic impact on agriculture, MAP also has implications for public health, since it may have a role in the etiology of Crohn's disease (CD). CD is an inflammatory bowel disease with unknown etiology, characterized by chronic, focal, asymmetric, and transmural and occasionally granulomatous inflammation, which can affect any segment of the digestive tract, from mouth to anus, although with preference for the distal portion of the small intestine and the proximal portion of large intestine. The pathogenesis of CD, although not fully understood, involves primarily four aspects that interact with each other and environmental factors: i) genetic factors; ii) factors related to intestinal microbiota, its antigens, metabolic products, and food antigens; iii) factors related to the intestinal barrier, including aspects related to innate immunity and intestinal permeability; and iv) factors related to immunoregulation based on adaptive or acquired immunity. Clinical signs depend on the severity and location. The most common clinical manifestations are abdominal pain, diarrhea, and weight loss. The disease progresses with periods of exacerbation and remission, even after surgical resection of the affected areas. There is a high

percentage of complications such as abscess, fistula, stricture, perforation of the free cavity, and anoperineal involvement.

Dairy products are the major vehicle for transmission of MAP from animals to humans, since milk is the second most important elimination route of bacteria. Currently, Brazil is the fifth largest producer of milk with approximately 5% of worldwide production. The Southeast region is the largest producer, accounting for 35.5% of all production and Minas Gerais State contributes 27.3% of milk production in the country. Although MAP is detected in Brazil, the exact prevalence of its occurrence is not known.

Since MAP can be related to CD, there is a concern about the quality and safety of milk and dairy products, to prevent or minimize risks to human health. Studies show that MAP is able to survive heat treatments in the laboratory that simulate industrial conditions of milk pasteurization. In addition, MAP has been detected by culture and PCR in patients with CD and in tissue fragments obtained from patients with CD.

Interest in the MAP resistance to pasteurization temperatures of milk appeared with the first reports detecting MAP in the gut of patients with CD. Studies have investigated the effect of milk pasteurization containing large amounts of MAP both at 63°C for 30min (slow pasteurization) and at 72°C for 15s (flash pasteurization). Several of these studies reported survival of MAP after pasteurization, although there is no consensus on what quantities of MAP present in raw milk would be inactivated by current pasteurization conditions.

Based on the fact that MAP was detected in milk after pasteurization, it is necessary to verify the presence of MAP in pasteurized milk and fragments of the human intestine in Brazil, and to evaluate whether current pasteurization conditions are able to inactivate MAP present in milk. Around the world, more and more research groups have been seeking a possible association between MAP and CD. In Brazil, there are few published studies on the occurrence of MAP and none related to this subject.

## **AIMS**

Considering that Brazil is a major milk producer, the presence of MAP in Brazilian herds, the importance of milk quality and the possible role of MAP in Crohn's disease (CD), this study aimed to: evaluate the effectiveness of pasteurization, identifying the binomial time-temperature that will inactivate MAP; verify the presence of MAP in retail pasteurized milk in Viçosa city, Minas Gerais State, Brazil; and verify the presence of MAP in fragments of human intestine from patients with CD, ulcerative colitis (UC), and controls (nIBD).

## **OBJECTIVES**

- Compare decontamination protocols for MAP isolation from raw milk samples;
- Compare time-temperature binomials used for pasteurization of milk samples, identifying those that will inactivate MAP;
- Verify the IS900 sequence conservation to be used as a tool for identifying MAP;
- Identify MAP in retail pasteurized milk samples, and in the terminal ileum and ascending colon from patients with Crohn's disease, ulcerative colitis, and controls, using culture and PCR;
- Identify MAP in formalin-fixed paraffin-embedded fragments of the terminal ileum and ascending colon, from patients with Crohn's disease, ulcerative colitis, and controls, using PCR and Ziehl-Neelsen;
- Determine scores (0, 1, 2, and 3) for tissue inflammation by analysis of histological slides stained with Hematoxylin-Eosin;
- Associate amount of MAP detected by qRT-PCR and histological results with groups CD, UC, and nIBD.

## **CHAPTER I. MAP vs Crohn's disease**

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## 1. Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease, with the potential to affect any segment of the gastrointestinal tract. Despite the great advances in recent decades, which provided a better understanding of the pathogenesis of the disease, it is yet to be completely elucidated. Presence of genetic factors, luminal factors such as microflora and factors related to the intestinal barrier and immunoregulation are pieces that interact with each other and with environmental factors. The possibility of an infectious etiology has always been widely discussed. In this context, *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has attracted the interest of many researchers because of the similarity between paratuberculosis and CD. In 1913, two decades before the description of CD in 1932, T. K. Dalziel made associations between chronic cases of enteritis in humans and paratuberculosis in cattle (Behr and Kapur, 2008).

Some genetic studies also support the role of MAP in CD and susceptibility genes have been identified, which encode proteins involved in the innate immunity defense against intracellular bacteria. However, no study is conclusive about a causal relationship. It is not possible to conclude that a single agent is solely responsible for the etiology of CD: a multifactorial cause is much more likely (Grant et al., 2001).

Whereas the causal relationship has not been established, therapeutic implications require further studies. Results of these studies could help answer questions about the role of MAP in the etiology of CD.

## 2. *Mycobacterium avium* subspecies *paratuberculosis*

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a small bacillus, Gram-positive, intracellular, acid-resistant bacterium belonging to the Mycobacteriaceae family. It grows slowly and when observed under an optic microscope, usually appears to form small clusters (Figure.1). Like other mycobacteria, this microorganism has a thick cell wall, composed mainly of lipids, which determines its acid-resistant, hydrophobicity and high resistance to chemical processes, such as chlorination of water, and physical processes, such as pasteurization (Harris and Barletta, 2001).



**Figure. 1.** *Mycobacterium avium* subspecies *paratuberculosis* (MAP) stained by Ziehl-Neelsen. Optical microscopy (1000x).

The first observation of the microorganism was made in 1895 by Johne and Frothingham, who isolated an acid-resistant bacterium from the ileum of animals with chronic granulomatous inflammation. The disease was called paratuberculosis, according to its similarity with intestinal tuberculosis. Although the disease had been reported in 1895, identification of the agent was assigned by Twort in 1910 who could grow the microorganism for the first time in laboratory (Cocito et al., 1994).

Morphology of MAP colonies depends on the medium used for growth. In Herrold Egg Yolk Medium (HEYM), colonies are small, measuring about 1 to 2 mm, generally white, convex and smooth, while in Middlebrook Agar they become more wrinkled. Even under optimal conditions, colonies may take 3 to 4 months or longer to become visible (Harris and Barletta, 2001). Other features of MAP, which serve to differentiate it from other bacteria, are its dependence on mycobactin *J* for in vitro growth, a compound extracted from mycobacterial cells that helps in iron uptake, and the presence of insertion element IS900, which appears as 14 to 18 copies within the genome (Green et al., 1989).

The complete genome of MAP K-10 was sequenced in 2004 by researchers at the University of Minnesota, USA. Analysis showed that MAP K-10 has a circular sequence of 4,829,781 base pairs with 69.3% G+C. When comparing the genome of MAP and other mycobacteria, researchers have suggested two hypotheses to explain the extremely slow growth of MAP. Firstly, the presence of an insertion sequence, MAP0028c/IS1311, much closer to oriC in MAP compared to *M. tuberculosis*, would be detrimental to chromosomal replication, leading to a wider range of generation. The second theory is the presence of the gene map0638, with a

higher replacement rate compared to *M. tuberculosis*. This gene is responsible for regulating synthesis of purine and therefore has a role in rate of protein synthesis and cell growth (Li et al., 2005).

### **3. Paratuberculosis**

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of paratuberculosis or Johne's disease, chronic infectious enteritis, characterized by the presence of persistent diarrhea and progressive weight loss, which mainly affects domestic and wild ruminants worldwide and may also affect several other species of mammals, including primates.

In the USA, bovine paratuberculosis is well documented and it is estimated that economic losses for the dairy industry are of the order of millions of dollars annually. These losses are due to decreased production, reduced protein content of milk, premature culling, increased susceptibility to other diseases, reduced fertility and increased health costs (Hendrick et al., 2005).

Under natural conditions, the transmission of the microorganism is usually horizontal, by ingesting food or water contaminated with MAP. Transmission can also occur vertically by intrauterine infection, colostrum from females infected or contaminated semen.

Paratuberculosis usually manifests in young adult cattle. After an incubation period of about 2 to 5 years clinical symptoms begin to appear. However, infected animals eliminate the microorganism in their stools and in minor amounts in milk even before the onset of clinical signs, which contributes to the spread of the agent. Confinement contributes to the infection of animals and can be one of the reasons for higher prevalence of the disease in dairy cattle herds compared to meat cattle. The long incubation period has great importance in the economic impact of disease because infected animals are responsible for the unnoticed spread of the agent and, therefore, of the disease in herds.

In vivo, the primary target of MAP infection is the M cells from Peyer's patches and the primary lesions occur on the walls of the small intestine and mesenteric lymph nodes. The multiplication of the microorganism leads to the extent of injury to the ileum, jejunum, cecum and colon, interfering with the intestinal metabolism. The main gross lesions are characterized by thickening of the intestinal mucosa, which

presents an aspect grid with transverse folds, well-exposed and enlarged mesenteric lymph nodes, and the main histopathological findings consist of enteritis and granulomatous lymphangitis and lymphadenitis associated with the presence of acid-fast bacilli resistant into macrophages.

Typical clinical signs of the disease are rapid and progressive weight loss (Figure 2) and intermittent diarrhea, which becomes progressively more severe. Animals continue with a normal appetite but cannot effectively absorb nutrients. Lower body condition scores are generally found. In the final stage, chronic diarrhea of animals becomes untreatable and then, they will die in cachectic state. During the early stages of paratuberculosis, the immunity is characterized by a strong cell-mediated immune response and in the later stages there is a humoral immune response. Antibody concentrations become higher with the progression of the disease when the lesions become more extensive, reflecting the amount of antigen present.



**Figure. 2.** Animal with paratuberculosis showing low body score

Diagnosis can be based on the detection of the etiologic agent or detection of immune response to this agent. Several methods have been used to diagnose the disease such as fecal culture, immunological tests, histopathological tests and molecular tests.

MAP isolation by fecal culture is considered the "gold standard" for diagnosis of paratuberculosis, despite having low sensitivity and requiring up to 16 weeks until the first colonies can be seen. To detect infected animals in the early stages of the disease by this method is very difficult due to the slow growth of MAP. The agent is grown on specific medium and confirmation of the identity of the colonies is done by

its dependence of micobactin J or targeting the insertion sequence IS900 on molecular tests, an element pattern that enables the genetic identification of MAP.

Several serological tests have been used for rapid detection of infected animals including complement fixation, agar gel immunodiffusion and ELISA. However, because disease immunity is mediated by cells in the early stages and humoral in the later stages, these tests generally have high sensitivity in infected animals showing clinical signs, and low sensitivity in animals that do not. Therefore they are most useful in the clinical stages of disease. Among the serological tests, ELISA is the most commonly used for its high sensitivity and acceptable specificity. Despite the low sensitivity and specificity, intradermal tests have also been used. Infected animals show a cellular response when in contact with purified MAP proteins. These tests are not recommended because it may cause crossreactivity of these proteins with tuberculin during the tuberculin test, leading to false positive results. This is a test performed by control programs for diagnosis of tuberculosis, a disease caused by *M. bovis* in countries where this disease is present.

Rapid detection of microorganisms of slow growth has become possible through use of molecular biology techniques. Discovery of insertion sequence IS900 in the MAP genome and the development of the polymerase chain reaction (PCR) revolutionized this field.

PCR is used successfully to detect MAP DNA in samples of pasteurized milk and fresh milk and is more sensitive than fecal culture. Using PCR it is possible to detect concentrations as low as 10UFC/mL milk. It is a highly sensitive and specific technique for the detection of MAP, as well as fast, and can reduce diagnosis time from months to just two days. PCR is also very versatile, considering that it can be used in stool, tissues, milk, blood and semen. There is currently no satisfactory treatment for animals affected by MAP. Vaccines currently available against MAP do not protect animals completely and there is also the aforementioned problem of possible cross-reactions. Thus, vaccination is not recommended.

General management measures such as general hygiene facilities, separating animals by age, and identification and disposal of infected animals can be cited as preventative measures.

### **3.1 Paratuberculosis in Brazil**

In Brazil, the first notification of paratuberculosis occurred in Rio de Janeiro, in an imported animal (Dupont, 1915). Afterwards, the disease was reported in the Southeastern (Santos and Silva, 1956; Dacorso Filho et al., 1960; Silva, 1961; Nakajima et al., 1991; Ristow et al., 2007; Costa et al., 2010), Southern (Portugal et al., 1979; Ramos et al., 1986; Driemeier et al., 1999), Mid-western (Brautingam et al., 1996; Acypreste et al., 2005), and Northeastern (Mota et al., 2010; Oliveira et al., 2010) regions of the country in animals born and raised in Brazil. The first report in raw milk samples is recent (Carvalho et al., 2009).

The first report of the presence of MAP in milk samples in the country was fairly recent. Although researches on paratuberculosis in Brazil have increased considerably, the studies published in the area are still few and the economic impact of the disease has not been measured in the country. In Brazil, the estimated prevalence of paratuberculosis is higher than in other countries. Further studies are needed to subsidize control measures in the national herd, since, in Brazil, there is no health program for this disease.

### **4. Crohn's disease**

Crohn's disease (CD) is an inflammatory bowel disease of unknown etiology, which is characterized by chronic, focal, asymmetric, transmural and granulomatous inflammation, and can affect any segment of the digestive tract, from mouth to anus, although with preference for the distal small intestine and proximal large intestine. The disease has three phenotypes which are: stenosing, penetrating, and not stenotic and non-penetrating.

Incidence and prevalence of CD varies greatly with geographic location. USA, Britain, Scandinavia (especially Norway and Sweden), Italy and countries of northern Europe are considered areas with greater impact. Intermediate incidence areas are represented by the countries of southern Europe, South Africa, Australia and New Zealand. Low incidence is reported in Asia and South America.

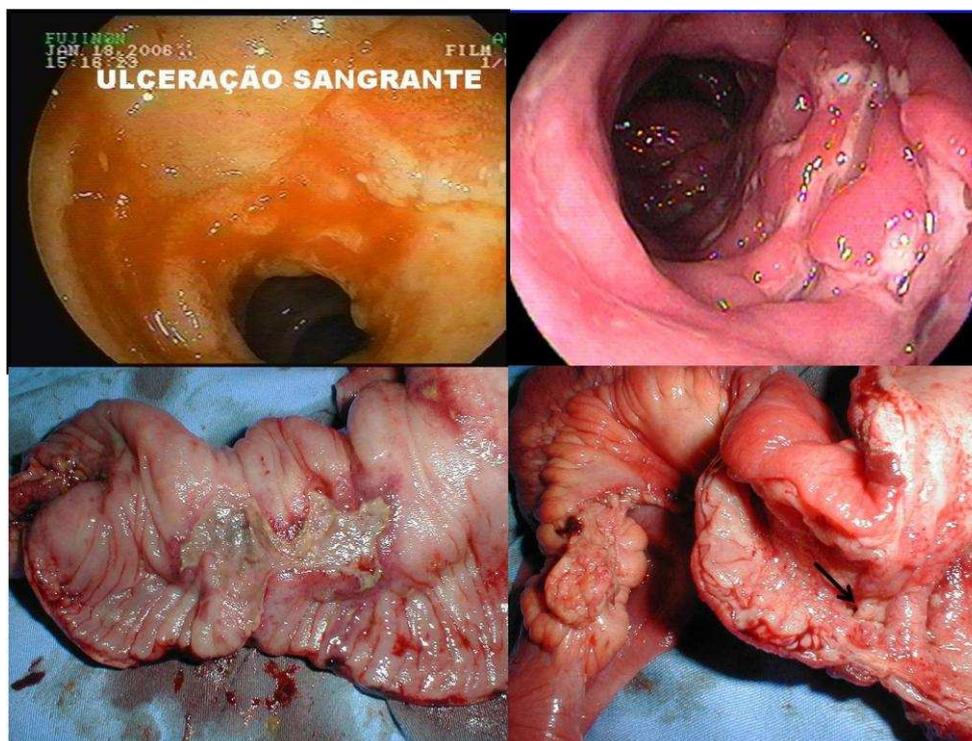
The disease can affect individuals of any age, but occurs with greater frequency in patients between 20 and 40 years old. It affects people in their most productive period, with an enormous impact on quality of life of patients. A second

peak in incidence, less obvious, is described in patients between 60 and 80 years old, setting a bimodal presentation. The disease has no predilection for sex.

Pathogenesis of CD, although not fully understood, fundamentally involves four aspects that interact with each other and with environmental factors: a) genetic factors; b) luminal factors related to the intestinal microbiota, its antigens, metabolic products and food antigens; c) factors related to the intestinal barrier, including aspects related to innate immunity and intestinal permeability; and d) factors related to immunoregulation, based on the adaptive or acquired immunity.

CD is characterized by periods when the disease is active and others where it is in remission. Symptoms depend on the severity and location of intestinal involvement. In approximately one third of patients, CD involves the small intestine, especially ileum, and in some cases the jejunum. About 20% to 25% of cases present only colonic lesions. The isolated involvement of the jejunum is rare, as well as cases involving the esophagus, stomach and duodenum. The most common clinical manifestations are abdominal pain, diarrhea and weight loss. CD progresses with periods of remission and exacerbation, even after surgical resection of the affected areas. It has a high percentage of complications with formation of abscesses, fistulas, stenosis, cavity free perforation and anoperineal involvement (Figure 3). About 70% of individuals affected by the CD undergo surgery and 30% suffer from repeated bowel resections. This disease may be associated with extraintestinal manifestations, and rheumatological, dermatological, ophthalmological, and hepatobiliary nephrology manifestations are the most frequent.

Diagnosis of the disease represents a major challenge, especially when it comes to early diagnosis. It is based on a combination of clinical, laboratory, radiological, endoscopic and histopathological findings. There is no pathognomonic test for the diagnosis of CD. To date there is no medical or surgical treatment that provides healing of CD. As inflammation is the maximum expression of the disease pathophysiology, clinical therapy, which involves different groups of medications such as aminosalicylates, corticosteroids, immunomodulators and biologic therapy are aimed at blocking the inflammatory cascade and ending the inflammation and scarring of the intestinal mucosa.



**Figure. 3.** Endoscopic appearance of Crohn's disease (CD). A: bleeding ulcerated lesion; B: serpiginous ulcerations in the mucosa showing cobblestones appearance. Surgical specimens. C: ileum with extensive ulcerated lesion, D: intestinal wall thickening and opening of the fistulous orifice (arrow).

Although it is a benign disease with low mortality rate, CD is accompanied by high morbidity, with an unpredictable course and the phases of activity, exteriorized through uncomfortable manifestations and/or complications, even when the best care and treatments are implemented. Patients have to live for a long time with limitations that affect directly their daily lives, impacting greatly on the quality of life (Sands, 2006; Brand, 2009).

#### **4.1 Crohn's disease in Brazil**

Brazil is located in South America, a region considered to have a low incidence of CD. Despite the paucity of epidemiological studies that allow us to know the real incidence and prevalence of the disease, reports of cases, predominantly from universities, have shown that this disease is not rare, and that its incidence had increased in recent decades. This epidemiological profile, now recognized in Brazil, has been previously observed in different countries with a high incidence. Nowadays, these countries have been evolving for stability in the frequency of the disease. For the different areas of Brazil, CD is most frequently observed in states located in the

south and southeast, areas with higher socioeconomic development. Currently, there is a joint effort of hospitals for inflammatory bowel diseases to collect reliable statistical data that would serve as an international database.

In one follow up study, researchers evaluated 100 patients with CD in university referral center for inflammatory bowel disease. The mean follow-up was 47.3 months, with variations from one month to 9.5 years. As for gender distribution, 59% were female and 41% male. Age of patients ranged from 16 to 69 years old, with an average of 29.9 and a median of 27 years old. 5% of patients had a family history of ulcerative colitis or CD.

Among the clinical manifestations, abdominal pain was the most common symptom observed in 98% of cases, followed by chronic diarrhea in 83% and weight loss in 82% of patients. Regarding the behavior of the disease, stenosing form was observed in 35% of cases, followed by non-penetrating and not stenosing form in 34% of cases and fistulizing form was described in 31% of cases. Of the extraintestinal manifestations, rheumatological manifestation was the most frequently observed, followed by skin and eyes manifestations.

50% of patients underwent surgical procedures and 63% were hospitalized at least once. The authors conclude that the profile of CD in evaluated patients was similar to that described in the literature, and these data were corroborated by other Brazilian authors (Faria et al., 2004; Santana et al., 2008; Torres et al., 2010).

##### **5. *Mycobacterium avium* subspecies *paratuberculosis* vs Crohn's disease**

Paratuberculosis and Crohn's disease (CD) are two diseases that share many clinical and histopathological similarities. Both diseases are characterized by chronic inflammation, weight loss and there is no cure.

While there have been, in recent years, various hypotheses about the etiology of CD and the mechanisms that trigger it may be cited as diet, environmental factors, genetic predisposition and autoimmune responses, the two main causal theories are infection and the autoimmune response. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has been cited as the leading candidate from the point of view of infection.

Considering the high prevalence of MAP in dairy herds and the resistance of microorganisms to disinfectants and pasteurization, several countries have created programs to control the disease considering its possible zoonotic potential.

### **5.1 Facts that support MAP in Crohn's disease**

Despite extensive research and large and important advances in the past few decades, the etiology of Crohn's disease (CD) remains unknown, hindering the development of a specific therapy. Due to the similarity of clinical signs and histopathological findings between the two diseases, associations between paratuberculosis in cattle and chronic ileocolitis in humans have been made. Interest in participation in the infectious etiology of CD has increased with the isolation and detection of DNA of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in samples from patients with the disease. MAP is not classified as a zoonotic agent, but it represents a major concern in the field of public health because of the associations that have been made.

The controversial zoonotic potential of CD grows as more research has been seeking an association between MAP and CD. In recent studies, MAP strains were isolated from human intestinal tissues and blood from patients with CD. It is known that patients with CD are substantially more positive for MAP, regardless of method used, when compared with individuals with ulcerative colitis or individuals without inflammatory bowel disease. The isolation of MAP and detection of MAP DNA from breast milk of nursing mothers with a diagnosis of CD has also been reported and a possible maternal-fetal transmission of MAP may be suggested. These results, however, were not replicated by other researchers and there is no evidence of increased frequency of CD in children of mothers with CD (Naser et al., 2000; Schwartz et al., 2000; Bull et al., 2003; Naser et al., 2004).

In several published reports, presence of antibodies against MAP has also been demonstrated in serum samples from patients with CD by serological tests. Using these tests, there are also significant differences when compared with sera from patients with CD, ulcerative colitis and control patients. This is another fact that supports the association of MAP with CD (Olsen et al., 2009; Rosenfeld and Bressler, 2010).

Molecular techniques have been used for providing faster results than conventional diagnostic techniques. Using PCR-based sequence IS900, MAP DNA has also been found in a significantly higher proportion of patients with CD than in patients with ulcerative colitis or patients without inflammatory bowel disease (Abubakar et al., 2008).

Besides all this evidence, CD's patients have been treated successfully using antimycobacterial drugs. If MAP is involved in the etiology of CD, it is expected that antimycobacterial drugs should improve the clinical status of affected patients (Hermon-Taylor, 2002).

Contaminated milk would be the main, but not the only, vehicle of transmission of MAP from animals to human beings. Infected animals can eliminate the organism in this way and furthermore can occur a milk contamination with via fecal material, a route through which the organism is eliminated at higher concentrations. It is known that occurrence of MAP in milk is well documented and several studies have shown that the microorganism can remain viable after being subjected to standard conditions of pasteurization and processes used to produce cheese if it is present in large concentrations. In addition, although not recommended, it is known that raw milk is consumed fresh in many parts of the world, besides being used for the manufacture of several dairy products. A causal association of MAP with CD would have important implications for the processing of milk and other dairy products (Grant et al., 2001).

There is no doubt therefore that there is a potential source of zoonotic infection, considering (1) the widespread dissemination of MAP in dairy herds in Europe, America and Australia, (2) the elimination of MAP in milk of infected animals, (3) the relative strength of MAP to pasteurization process used currently and (4) the recovery of viable MAP in milk samples, water and beef, other potential sources of transmission of MAP. So, considering the association between CD and MAP infection to be correct, the fact that MAP has been detected in foods could be a public health problem.

It is known that the vast majority of studies using many different techniques have detected MAP DNA or cultured the microorganism most frequently in tissue from patients with CD, rather than in those with ulcerative colitis and other disorders. These results are consistent with two possibilities: either MAP infection could cause

CD in a subgroup of patients that are selectively exposed to this microorganism or are genetically susceptible to infection, or alternatively, this microorganism, relatively common in the diet, can colonize selectively to ulcerated mucosa of patients with CD, but not initiate or perpetuate intestinal inflammation (Behr and Kapur, 2008; Hermon-Taylor, 2009; Rosenfeld and Bressler, 2010).

The most plausible theory that would explain a role for MAP in the etiology of CD is related to the recipient NOD2/CARD15. NOD2/CARD15 is an intracellular receptor for muramyl dipeptide (MDP), the smallest immunologically active component of bacterial peptidoglycan. The binding of MDP to the receiver NOD2/CARD15 activates nuclear factor  $\kappa$ B. This may contribute to the elimination of intracellular bacterial infection and secretion of  $\alpha$  defensins by Paneth cells, which constitutively express NOD2/CARD15. The three most common polymorphisms of this gene lead to a defective activation of nuclear factor  $\kappa$ B by the MDP and they are found in 35% of Caucasian patients with CD. NOD2/CARD15 mutations in CD are associated with a decreased expression of  $\alpha$  defensins in the mucosa.

Thus, a plausible explanation linking NOD2/CARD15 to CD is that a defect in this gene could not result in elimination of intracellular infection by MAP and decreased secretion of luminal  $\alpha$  defensins in the mucosa, allowing a greater adhesion and epithelial invasion by the microorganisms ingested (Sartor, 2005).

Despite all the evidence implying an association of MAP with CD, it is not possible to conclude that a single agent is solely responsible for the cause of CD - a multifactorial cause is more likely. The role of MAP in the etiology of CD cannot yet be confirmed or refuted with certainty. The organism can act as a causative agent, it may have a role in the context of infection, it can exacerbate the disease, or it may be non-pathogenic. Clearly, more studies are needed to determine whether MAP infection causes the disease or whether this environmental contaminant innocently lodges in ulcerated mucosa. Well-designed studies are needed to definitively resolve this debate.

## **5.2 Facts that do not support MAP in Crohn's disease**

It has been suggested that a relationship between MAP infection and Crohn's exists due to the clinical and pathological similarities between Crohn's disease (CD) in human patients and paratuberculosis in animals. However, there are also many

arguments against MAP being the causative agent of CD. Despite the similarities between clinical signs and histopathological features of both diseases, there may be differences in clinical and pathological responses between both diseases, which are not expected if both diseases are caused by the same microorganism.

Another important factor to be considered is the lack of epidemiological support considering the infection's transmission. If animals eliminate MAP in large quantities in feces and milk, it would be expected that the prevalence of CD in people in direct contact with animals infected with MAP was great if the association between the CD and paratuberculosis is true. These facts have not yet been reported (Jones et al., 2006).

Not all patients with CD respond well to treatment with anti-MAP. The failure of treatment in such cases can be attributed to the fact that CD can exist in two forms: one form caused or triggered by infection with MAP and otherwise, induced by some other unknown cause. If this is true, the treatment may be ineffective due to inability to identify patients infected with MAP before the start of treatment. There is not enough evidence to assert the effects of antimycobacterial therapy in patients with CD, but it is suggested that this therapy can help maintain remission of clinical signs of disease (Selby et al., 2007).

Genetic profiles of different strains already isolated have been outlined and possible epidemiological associations between the species from which they were isolated have been studied. Studies have shown that human isolates have profiles more similar to those isolated from sheep and goats than to those isolated from cattle. This fact also contrasts with the idea that cow's milk would be the main source of transmission of MAP from cattle to human beings. In contrast to this fact, there are studies reporting that both animals and humans are susceptible to infection by MAP isolates with similar genotypes (Jones et al., 2006).

Just as there are several research groups associating MAP to CD using detection of MAP DNA in samples of intestinal tissues and blood of patients with CD, other groups have shown the opposite: the same levels of detection of MAP in patients with CD compared with patients with ulcerative colitis or patients without inflammatory bowel diseases.

Moreover, the absence of MAP DNA in patients with CD was also reported. Data are very variable for all groups. There is a variation from 0 to 100% of MAP by PCR detection (Abubakar et al., 2008).

Another argument against a possible etiology of MAP in CD is related to the observation of a good response from patients with CD undergoing immunosuppressive therapy.

### **5.3 Conditions in Brazil that could facilitate the transmission from MAP to humans**

MAP has been detected in several states in the Southeast, South, Midwest and Northeast regions of Brazil. Despite reports of paratuberculosis in several states, few research groups working in this field are still doing surveys on the disease. In addition to bovine paratuberculosis, there are some groups in the country researching the presence of MAP in goats and sheep.

Although there is a strong and effective control program for tuberculosis in the country, there is not a control program for paratuberculosis. Research into this disease is still preliminary and there has been no survey even made, even superficially, about the economic losses caused by the disease.

Currently, Brazil is the fifth largest milk producing country, with a volume that corresponds to approximately 4.5% of world production (IBGE, 2010). The Southeast region is the largest producing region, accounting for 38.4% of all domestic production. Despite the high production, productivity of dairy herds in Brazil is low. The milk industrial chain is important from the standpoint of economic and social development, generating significant income and jobs in all sectors. Despite all these factors, no studies in the country are aimed at detection of MAP in dairy products and the first detection of MAP in milk was quite recent. MAP was detected by PCR using primers based on the IS900 sequence in an initial survey about the disease in the Southeast region (Carvalho et al., 2009).

Brazil has also not been reported the presence of MAP in pasteurized milk. A survey in this sense, in the same region where it was detected MAP DNA in raw milk samples, is already in the early stages. In parallel, the resistance of MAP to pasteurization temperatures in the laboratory is being tested.

Brazil has a volume estimated at around 112 billion cubic meters of fresh water of the planet. Moreover, in Brazilian subsoil there is the Brazilian Guarani aquifer, the largest subsoil reservoir of freshwater on the planet. This enormous underground wealth extends over an area of 1.6 million square kilometers, of which two thirds are in Brazilian territory. Even with all that water, there have been no studies in the country to verify the presence of MAP in water.

There are no studies about the possible association between MAP infection and Crohn's disease (CD). A first attempt of an association between MAP infection and CD is being performed. Intestinal biopsies of patients with CD, ulcerative colitis patients and patients without inflammatory bowel disease are being collected with the aim of isolating MAP and/or detection of DNA of the microorganism for molecular tests.

## **6. Future prospects**

Historically, one of the ways to make the connection between a potential agent of an infectious disease and the disease itself was considering whether Koch's postulates were true. For the relationship between MAP infection and Crohn's disease (CD), postulates "The organism can be isolated from a sick patient" and "The organism can be cultured in the laboratory" can still be controversial, but are true. "If the causative microorganism is introduced into another susceptible host, the same disease should be generated" is a postulate a little harder to prove. Disease results from interaction between infection and immune response by the microorganism that causes it. Clinical manifestations of infection depend on several variables such as genetics of the host and the state of the immune system, among others (Rosenfeld and Bressler, 2010).

CD certainly involves host genetic influences and environmental influences that interact to cause clinically evident disease. It is known that MAP is widely present in the human food chain and that MAP DNA can be recovered from intestinal samples of patients with CD.

Although existing data do not necessarily involve MAP as a causative agent of CD, this possibility cannot be definitively excluded.

Besides this, there is the difficulty in obtaining experimental models for studies involving human pathogens or potential human pathogens. Such studies are

complicated to performed, since although the relationship between MAP and CD is not yet established, it is reckless inoculate MAP in human patients for testing.

There is still much to be learned about MAP and diseases that it can cause in humans. CD may be a syndrome with multiple etiologies that result in clinical, endoscopic, radiological and pathological findings that define the disease. MAP may be one of the etiologies of this syndrome.

## **7. Conclusion**

Some studies have shown that the etiology of Crohn's disease (CD) may involve a variety of viral and bacterial agents, including MAP, or an immunological origin. Evidence supports an interaction between a persistent environmental stimulus, such as a microbial antigen, and genetic factors that regulate an immune response or a function of the intestinal mucosa. Recent discoveries in many research fields have generated favorable results suggesting an association between CD and MAP, but that does not necessarily indicate that the microorganism is involved in the etiology of the disease. It is not possible to conclude that a single agent is solely responsible for the cause of CD as a multifactorial cause is more likely. For patients and their physicians, a clear answer on this association is an important step toward establishing control measures and treatment for this debilitating disease. Theories of mycobacterial and autoimmune etiologies of CD should be seen as complementary rather than mutually exclusive. The causal association between CD and MAP infection remains unanswered.

If MAP is responsible for a subset of CD, public health measures should be implemented to eliminate the source of infection in the human food chain and food processing practices must be modified. If there is no evidence of a causal association of MAP and CD, we must direct resources to other research fields. This controversy has persisted for too long and needs to be resolved.

When appropriate methods are used, most patients with CD are detected and there is no data showing that MAP is harmless to human patients. More epidemiological studies seeking to rigorously analyze both diseases are needed. While CD is likely to be a multifactorial condition, MAP can be a primary etiologic agent or a significant secondary invading agent. It is well documented that MAP is present in humans and that there are many routes of transmission and consumption

of milk and dairy products are the main, and perhaps the biggest, problem link on the subject, since it has been shown that the microorganism can resist milk pasteurization processes and also the chlorination processes of drinking water. Therefore, until MAP is declared as a non-pathogenic agent for humans, it should be treated as such.

## **8. Acknowledgments**

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**CHAPTER II. Comparative evaluation among  
36 combinations of decontamination and  
isolation protocols for *Mycobacterium avium*  
subspecies *paratuberculosis* (MAP) from raw  
milk samples**

Manuscript to be submitted

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## **Abstract**

Most protocols regarding sample decontamination for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) isolation are based on the MAP detection from feces and not milk. The choice of the best decontamination protocol is crucial to a successful MAP isolation. In this study, 36 combinations of variables for sample decontamination and MAP isolation from raw milk presented in the literature were carried out on milk samples artificially contaminated which were then inoculated into tubes with three different culture media: Herrold Egg Yolk Medium (HEYM) prepared with fresh egg yolk, HEYM prepared with commercial egg yolk and Lowenstein-Jensen medium (LJ). Each treatment was performed in triplicate for each medium, with a total of 324 observations. The protocol combination which provided higher MAP growth and lower nonspecific contamination in a shorter period of time was considered the best. In this study, the protocol involving 0.75% HPC at room temperature for 24h, using centrifuge at  $2500 \times g$  for 15 minutes and addition of antimicrobial solution immediately before inoculation into tubes with HEYM prepared with fresh egg yolk provided the greatest MAP isolation from raw milk samples.

**Keywords:** *Mycobacterium avium* subsp. *paratuberculosis*; milk; decontamination protocols

## **Introduction**

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis, a chronic granulomatous enteritis that affects all ruminants and has been proposed as one of the etiologic agents of Crohn's disease, a chronic granulomatous enteritis seen in humans (Abubakar et al., 2008). The transmission vehicle could be milk and dairy products. Despite the ban on the marketing of raw milk in Brazil, it is known that the sale of these products is held freely in several cities (Abrahão et al., 2005), and this is a significant public health problem.

Animals infected with paratuberculosis eliminate MAP in feces and, in smaller quantities, in milk. Most protocols regarding sample decontamination for MAP isolation are based on MAP detection in feces and not in milk (Stabel, 1997; Whitlock et al., 2000). Due to the characteristics of each type of sample, different protocols must be followed. Therefore, it is necessary to develop methods accordingly.

MAP isolation also depends on chemical decontamination to inactivate other microorganisms in the sample that could inhibit the growth of MAP, since this presents a very slow rate of growth (Collins, 2003). Chemical decontamination, however, is known to affect the viability of MAP and therefore increases the likelihood of a false-negative culture result (Grant and Rowe, 2004). In addition, the existing culture protocols take from 12 to 18 weeks to isolate a suspect colony (Grant et al., 2001). Thus, a balance between an efficient inactivation of undesirable microorganisms and low environment toxicity for MAP is needed. The choice of the best decontamination protocol is crucial to a successful isolation of this potential zoonotic organism.

This study compared protocol combinations for sample decontamination and MAP isolation from raw milk, aiming at further isolation, less contamination and facility of application.

## **Material and Methods**

### ***MAP K10 strain***

A MAP K10 strain certified by genetic sequencing was grown in Middlebrook 7H9 supplemented with OADC. After that, the prepared suspension at a concentration of  $10^6$  CFU/ml was inoculated into 40mL raw milk aliquots, collected from a bulk tank from a historically paratuberculosis-free farm, which also tested negative for MAP presence by IS900-PCR using the primers BN1 (5' GTT ATT AAC GAC GCC CAG C 3') and BN2 (5' ACG ATG CTG TGT TGG GCG TTA G 3') accordingly Sivakumar et al. (2005).

### ***Combinations of variables***

A total of 36 combinations of variables for MAP isolation presented in the literature (Collins et al., 1993; Grant et al., 1996; Dundee et al., 2001; Pillai and Jayarao, 2002; Stabel et al., 2002) were carried out on milk samples artificially contaminated which were the inoculated into tubes with three different culture media: Herrold Egg Yolk Medium (HEYM) prepared with fresh egg yolk, HEYM prepared with commercial egg yolk and Lowenstein-Jensen medium (LJ) (Table 1).

**Table 1.** Protocol combinations for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) isolation carried out on artificially contaminated milk samples. Each combination was inoculated into tubes with Herrold Egg Yolk Medium (HEYM) prepared with fresh egg yolk, HEYM prepared with commercial egg yolk and Lowenstein-Jensen medium (LJ).

Treatment	Time-speed of centrifuge	HPC <sup>1</sup> Concentration	Time of contact with HPC	Temperature of contact with HPC	Time of contact with antimicrobial solution
1	3100 × g / 15'	0.75%	2h	room	immed <sup>2</sup>
2	3100 × g / 15'	0.75%	2h	room	2h
3	3100 × g / 15'	0.75%	2h	37°C	2h
4	3100 × g / 15'	0.75%	5h	room	immed
5	3100 × g / 15'	0.75%	5h	room	2h
6	3100 × g / 15'	0.75%	5h	37°C	2h
7	3100 × g / 15'	0.75%	24h	room	immed
8	3100 × g / 15'	0.75%	24h	room	2h
9	3100 × g / 15'	0.75%	24h	37°C	2h
10	3100 × g / 15'	0.9%	2h	room	immed
11	3100 × g / 15'	0.9%	2h	room	2h
12	3100 × g / 15'	0.9%	2h	37°C	2h
13	3100 × g / 15'	0.9%	5h	room	immed
14	3100 × g / 15'	0.9%	5h	room	2h
15	3100 × g / 15'	0.9%	5h	37°C	2h
16	3100 × g / 15'	0.9%	24h	room	immed
17	3100 × g / 15'	0.9%	24h	room	2h
18	3100 × g / 15'	0.9%	24h	37°C	2h
19	2500 × g / 15'	0.75%	2h	room	immed
20	2500 × g / 15'	0.75%	2h	room	2h
21	2500 × g / 15'	0.75%	2h	37°C	2h
22	2500 × g / 15'	0.75%	5h	room	immed
23	2500 × g / 15'	0.75%	5h	room	2h
24	2500 × g / 15'	0.75%	5h	37°C	2h
25	2500 × g / 15'	0.75%	24h	room	immed
26	2500 × g / 15'	0.75%	24h	room	2h
27	2500 × g / 15'	0.75%	24h	37°C	2h
28	2500 × g / 15'	0.9%	2h	room	immed
29	2500 × g / 15'	0.9%	2h	room	2h
30	2500 × g / 15'	0.9%	2h	37°C	2h
31	2500 × g / 15'	0.9%	5h	room	immed
32	2500 × g / 15'	0.9%	5h	room	2h
33	2500 × g / 15'	0.9%	5h	37°C	2h
34	2500 × g / 15'	0.9%	24h	room	immed
35	2500 × g / 15'	0.9%	24h	room	2h
36	2500 × g / 15'	0.9%	24h	37°C	2h

<sup>1</sup>HPC = hexadecylpyridinium chloride; <sup>2</sup>immed = immediately

### **Statistical analysis**

Each treatment was performed in triplicate for each medium, with a total of 324 observations. Data were analyzed by ANOVA and discriminated means were compared by F test and Scott-Knott test at 5% probability. Observation of bacterial growth was made considering score 5 for optimum growth, score 3 for good growth, score 1 for no growth and score 0 for contamination.

### **Results and Discussion**

After observation of bacterial growth into tubes with HEYM prepared with fresh egg yolk, 25 (23.1%) showed score 5; 16 (14.8%) showed score 3; 29 (26.9%) showed score 1 and 38 (35.2%) showed score 0. For tubes with HEYM prepared with commercial egg yolk, none showed score 5; 10 (9.3%) showed score 3; 69 (63.9%) showed score 1 and 29 (26.9%) showed score 0. For tubes with Lowenstein-Jensen medium, 3 (2.8%) showed score 5; 2 (1.9%) showed score 3; 7 (6.5%) showed score 1 and 96 (88.9%) showed score 0.

These results showed significant differences considering the culture media and 36 treatments (Table 2). Comparing the different culture media used, HEYM with fresh egg yolk was significantly better than HEYM with commercial egg yolk and LJ (Table 3).

**Table 2.** ANOVA for comparing means between variables 'treatment' and 'culture media'

Source of Variation	DF	Mean Square
Treatment	35	0.43**
Culture media	2	20.52**
Treatment x Culture Media	70	0.23 <sup>ns</sup>

\*\* Significant differences at 1% probability

<sup>ns</sup> No significant differences

There are no studies showing differences between the use of a fresh egg yolk emulsion or commercial egg yolks in the composition of HEYM. However, these differences can be explained by the possible use of some kind of preservative in the manufacture of commercial emulsions of egg yolks which may influence on MAP growth. Unlike some studies (Juste et al., 1991; Florou et al., 2009) where there have been no reported differences between HEYM and LJ, in this study, 88% of tubes containing LJ were discarded due to contamination. However, production of LJ is

more difficult compared to HEYM and one objective of this study was the ease of handling.

Comparing the 36 treatments used for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) isolation, the protocols followed by letter 'a' were significantly better than those followed by letter 'b' (Table 4).

**Table 3.** Mean comparison among three different culture media used for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) isolation carried out on artificially contaminated milk samples

Culture media	Mean
HEYM prepared with fresh egg yolk	1.67 <sup>a</sup>
HEYM prepared with commercial egg yolk	1.15 <sup>b</sup>
Lowenstein-Jensen medium	0.81 <sup>c</sup>

Means followed by the same letters does not differ statistically by Scott-Knott test at 5% probability

**Table 4.** Mean comparison among 36 treatment used for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) isolation carried out on artificially contaminated milk samples

Treatment	Mean	Treatment	Mean
1	1.06 <sup>b</sup>	19	1.01 <sup>b</sup>
2	1.13 <sup>b</sup>	20	1.02 <sup>b</sup>
3	1.37 <sup>a</sup>	21	1.07 <sup>b</sup>
4	0.97 <sup>b</sup>	22	0.89 <sup>b</sup>
5	1.35 <sup>a</sup>	23	1.31 <sup>a</sup>
6	1.3 <sup>a</sup>	24	1.07 <sup>b</sup>
7	1.61 <sup>a</sup>	25	1.43 <sup>a</sup>
8	1.61 <sup>a</sup>	26	1.5 <sup>a</sup>
9	1.19 <sup>b</sup>	27	1.37 <sup>a</sup>
10	1.19 <sup>b</sup>	28	0.84 <sup>b</sup>
11	1.15 <sup>b</sup>	29	0.89 <sup>b</sup>
12	1.14 <sup>b</sup>	30	1.18 <sup>b</sup>
13	1.32 <sup>a</sup>	31	0.77 <sup>b</sup>
14	1.28 <sup>a</sup>	32	1 <sup>b</sup>
15	1.27 <sup>a</sup>	33	1.24 <sup>a</sup>
16	1.12 <sup>b</sup>	34	1.2 <sup>b</sup>
17	1.19 <sup>b</sup>	35	1.62 <sup>a</sup>
18	1.56 <sup>a</sup>	36	1.3 <sup>a</sup>

Means followed by the same letters does not differ statistically by Scott-Knott test at 5% probability

Similar to some studies (Dundee et al., 2001; Gao et al., 2005) which have shown that treatment of milk with 0.75% HPC is better for the detection of MAP, in this study 0.75% HPC was used in the best protocol. Some studies have used other products for chemical decontamination, such as BHI with HPC and CB-18™ (Dundee et al., 2001; Ozbek et al., 2003; Ruzante et al., 2006). However, this study aimed at a high isolation rate of MAP and ease of application, and considering that these other reagents are more costly compared to HPC and that more work is necessary for the implementation of these protocols, this study used only HPC. The other agents would be greatly disadvantageous if a large number of samples needed to be tested. Meanwhile, studies carried out by Dundee et al. (2001) indicated that treatment with HPC for 5h was more effective, while in this study HPC for 24h was used in the best protocol.

Although in this study we used just K10 strain for comparing protocol combinations for sample decontamination, it is important highlight that the types of culture media could determine differences in the growth of MAP strains (Cernicchiaro et al., 2008)

It was also observed that a significant proportion of MAP cells present in the initial sample of milk were not recovered after decontamination, regardless of the method used. There is a consensus that decontamination methods may also affect MAP cells, resulting in false negatives (Reddacliff et al., 2003). These researchers have found that, during decontamination, the number of microorganisms is greatly reduced as well as in subsequent removal of aliquots for inoculation into media. This increases the necessity of using other diagnostic methods, for example molecular tools, as complementary instruments in MAP detection, although isolation is considered the gold standard.

## **Conclusion**

In this study, it was considered that protocol involving 0.75% HPC at room temperature for 24h, using a centrifuge at 2500 × g for 15 minutes and an antimicrobial solution immediately before inoculation into tubes with HEYM prepared with fresh egg yolk provided the optimal MAP isolation from raw milk samples. This protocol was also less laborious, an ideal quality for the simultaneous processing of

large quantities of raw milk samples, although the protocol was somewhat time consuming, requiring 24 hours.

### **Acknowledgements**

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**CHAPTER III. Resistance of *Mycobacterium avium* subspecies *paratuberculosis* to different pasteurization temperatures**

Manuscript to be submitted

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## **Abstract**

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, a chronic granulomatous enteritis that affects all species of ruminants. There is evidence to suggest that MAP may be involved in the etiology of Crohn's disease (CD), which presents pathophysiological similarities with paratuberculosis. However, this is controversial in the scientific community. Several studies have shown that MAP can survive extreme conditions such as high temperatures or low pH, thus milk and dairy products may be potential sources of MAP to humans. This study aimed to verify what MAP concentrations remain viable in milk after pasteurization. Raw milk known to be MAP-free was artificially spiked with MAP (K10 strain) at different concentrations ( $5 \times 10^1$  to  $5 \times 10^7$ ). All samples were pasteurized in the laboratory slowly for 10, 15, 20, and 30 minutes, and rapidly for 5, 10, 15, 20, and 25 seconds. All samples were processed, inoculated on Herrold Egg Yolk Medium (HEYM) with mycobactin J, and incubated at 37°C for 16 weeks. Growth of MAP was observed when spiked into milk at high concentrations ( $5 \times 10^6$  and  $5 \times 10^7$ ), regardless of the time-temperature binomial used. These results confirm that pasteurization is not sufficient to eliminate viable MAP in milk samples, when it is present in high concentrations, which is a concern considering the possible role of MAP in the etiology of CD.

**Keywords:** *Mycobacterium avium* subsp. *paratuberculosis*; pasteurization; milk.

## **Introduction**

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, a chronic granulomatous enteritis that affects all species of ruminants (Chiodini et al., 1984). MAP may be associated with Crohn's disease (CD), a human chronic enteritis, and this fact is increasingly debated in the scientific community. Milk and dairy products would be the major source of MAP transmission to humans (Uzoigwe et al., 2007; Behr and Kapur, 2008; Hermon-Taylor, 2009; Over et al., 2011; Chiodini et al., 2012).

Pasteurization was introduced as a public health measure at the end of the 19<sup>th</sup> century and the time and temperature conditions were established to kill most pathogenic, heat-resistant, non-spore-forming bacteria that could be present in raw

milk, such as *Mycobacterium bovis* (etiological agent of tuberculosis) and *Coxiella burnetii* (etiological agent of Q fever) (Grant, 2006).

Under Brazilian law, milk intended for human consumption must be treated by heat between 72°C and 75°C for 15 to 20 seconds and then kept at 4°C (BRASIL, 2011). MAP occurrence in milk is well documented and several studies have shown that this microorganism may be viable after pasteurization (Millar et al., 1996; Gao et al., 2002; Ayele et al., 2005; Ellingson et al., 2005; Grant, 2006; Shankar et al., 2010; Carvalho et al., 2012).

Since Brazil is the fifth largest milk producer (IBGE, 2010) and MAP has been detected in raw milk by PCR (Carvalho et al., 2009) and in pasteurized milk by culture (Carvalho et al., 2012), this study aimed to verify what concentrations of viable MAP remain in milk after pasteurization.

## **Material and Methods**

### ***Preparation of MAP inoculum***

A MAP K10 strain confirmed by PCR was grown in Middlebrook 7H9 broth (HIMEDIA) supplemented with OADC (oleic acid, bovine albumin, dextrose, and catalase) for 12 weeks. Raw milk was collected from the tank of a property of healthy cattle with no history of paratuberculosis and tested by PCR (Sivakumar et al., 2005). Different concentrations of MAP K10 ( $5 \times 10^1$  to  $5 \times 10^7$ ) were inoculated into 40ml of raw milk and then all samples were slowly or rapidly pasteurized in the laboratory.

### ***Pasteurization process***

During slow pasteurization, each sample was heated by full immersion in a waterbath operating at  $63.5 \pm 0.5^\circ\text{C}$ . The tubes were shaken until the temperature of pasteurization and then the temperature was maintained for 10, 15, 20, or 30 minutes. After the appropriate time, all samples were cooled to 8°C. In the HTST method, samples were heated at  $72 \pm 0.5^\circ\text{C}$ , and then the temperature was held constant for 5, 10, 15, 20, and 25s, and then cooled to 8°C. Throughout the process, a thermometer was kept in a tube containing the same amount of milk and subjected to the same treatment to check the temperature maintenance. Once cooled,

pasteurized milk samples were kept at 8°C for no more than 12 hours until the analysis.

### ***Decontamination and culture of MAP***

All samples were decontaminated and cultured as described below. Each sample was centrifuged at 2500 × *g* for 15min, the supernatant was discarded, and 15ml of 0.75% HPC was added to the sediment. The suspension was left for 24 hours at room temperature and then centrifuged at 2500 × *g* for 15min. The supernatant was discarded and the pellet resuspended in 1ml of antimicrobial solution containing nalidixic acid (50mg/l), vancomycin (50mg/l) and amphotericin B (150mg/l). Aliquots of 200µl of this suspension were inoculated into four tubes containing medium HEYM (two with mycobactin *J* (Allied Monitor, Inc., Fayette, MO, USA) and two without mycobactin *J*) prepared so that the medium formed a slant to increase its surface area (Grant et al., 2002a).

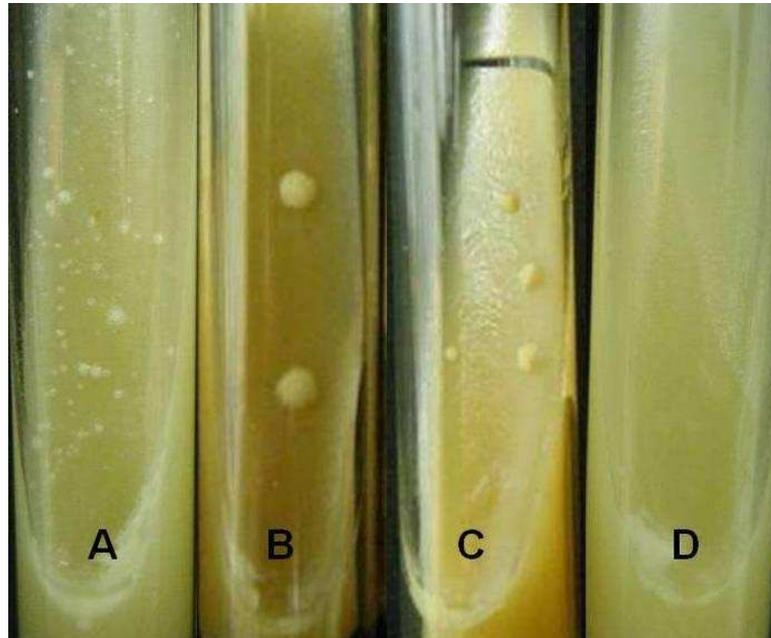
After inoculation, the tubes were incubated at 37°C for one week with the lid slightly loose and in an inclined position. After this period, the tubes were raised, threaded and kept at a temperature of 37°C for 16 weeks (Kalis et al., 1999). Observations of colony growth were made weekly.

### **Results and Discussion**

MAP colonies were observed in the tubes with mycobactin *J* inoculated with samples containing high MAP concentrations ( $5 \times 10^6$  and  $5 \times 10^7$ ), regardless of the time-temperature binomial used (Figure 1) and there were no colonies in the tubes without mycobactin *J*.

Several studies about MAP resistance to pasteurization have been performed in recent years. As in the present study, the MAP survival to pasteurization when present in concentrations at  $10^6$  and  $10^7$  in the original sample has been observed in most studies, both those involving naturally infected milk (Grant et al., 2002b; Ayele et al., 2005; Ellingson et al., 2005), and those where the milk was artificially contaminated (Gao et al., 2002; Stabel and Lambertz, 2004; Grant et al., 2005; Mcdonald et al., 2005). In Brazil, however, research on the topic is only just beginning and although there are studies showing detection of MAP in raw and

pasteurized milk (Carvalho et al., 2009; Carvalho et al., 2012), there are no studies about resistance of MAP to pasteurization.



**Figure 1.** A) MAP colonies grew in HEYM with mycobactin *J*, from raw milk containing MAP at concentration  $5 \times 10^2$ ; MAP colonies grew in HEYM with mycobactin *J*, from milk pasteurized in the laboratory containing MAP at concentrations B)  $5 \times 10^6$  and C)  $5 \times 10^7$ ; D) HEYM with mycobactin *J*: no growth from milk after laboratorial pasteurization at concentrations less than  $5 \times 10^5$ .

Some authors affirm that MAP resistance to pasteurization is caused by clumping, which occurs when the microorganism is subjected to high temperatures. Under commercial pasteurization conditions, however, there is turbulence in the milk flow, which likely prevents formation of clumpings and permits MAP inactivation (Patel and Shah, 2012). This fact, together with the small bacterial load resulting from dilution of milk, is a basis for an optimistic view, but it is still worrisome.

Another study suggests that sporulation may be a viable route by which MAP accomplishes persistence in the environment. According Lamont et al. (2012), heat treated MAP spore structures retained macrophage infectivity as well as acid-fast characteristics upon germination.

In addition to the effects of pasteurization, it is also necessary to take into consideration factors such as volume of milk tested, application of chemical decontamination in the sample before cultivation, and if the milk was processed for

cultivation immediately after being pasteurized or if it was cooled, which may influence the recovery of MAP from pasteurized milk samples (Grant, 2006).

It is known that animals with clinical signs of paratuberculosis can excrete MAP in milk; however, animals without clinical signs can also shed this microorganism that likely enters the milk production chain. It is estimated that levels of MAP present in pasteurized milk samples are low (Grant, 2006). However, the consequences of periodic ingestion of MAP in pasteurized milk by susceptible individuals, even at low levels, are not known.

Some studies suggest that a slightly longer time of pasteurization (25s instead of 15s) at 72°C may be more effective in inactivating high numbers of MAP in raw milk. As a direct result of these studies, most of the milk processing establishments in the United Kingdom voluntarily changed its pasteurization equipment in mid-1998 to adopt a 25s time of pasteurization (Grant et al., 2002a). This measure was taken in the hope of ensuring the complete inactivation of MAP that may be present in raw milk.

## **Conclusion**

The results of this study confirm that pasteurization is not sufficient to eliminate viable MAP in milk samples when this microorganism is present in high concentrations in the initial sample. This is a concern considering the possible role of MAP in the etiology of CD. Further research in this field is required using commercial conditions of pasteurization. Additionally, as animals without clinical signs of paratuberculosis may also eliminate MAP in milk, it emphasizes the importance of animal health and the need to establish a control program of paratuberculosis in Brazil.

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**CHAPTER IV. *Short Communication: Recovery of viable *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from retail pasteurized whole milk in Brazil***

Paper accepted for publication in the “**Journal of Dairy Science**”

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*In Press*

## **Abstract**

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, chronic granulomatous enteritis that affects all ruminants worldwide. Some researchers indicate a possible role of MAP in Crohn's disease. Despite extensive research and large and important advances in the past few decades, the etiology of Crohn's disease remains indefinite. The most probable transmission route of MAP from animals to humans is milk and dairy products. MAP has already been detected in milk samples worldwide and some studies have reported that MAP is resistant to pasteurization. In Brazil, MAP has already been reported in raw milk samples, however, Brazilian retail pasteurized milk has not yet been tested for viable MAP. The aim of this study was to investigate MAP in pasteurized milk in the region of Viçosa, Minas Gerais State, Brazil. Thirty seven samples were collected and processed for culture of MAP. One colony similar to MAP was observed and confirmed by IS900-Nested-PCR and sequencing. Analysis revealed 97-99 % of identity with the MAP K-10 strain. This study is the first report of the presence of MAP in retail pasteurized whole milk in Brazil.

**Keywords:** *Mycobacterium avium* subspecies *paratuberculosis*, insertion sequence IS900, PCR, milk.

The *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the etiological agent of paratuberculosis that affects all ruminants (Chiodini et al., 1984). Contamination can occur by the ingestion of milk or food that has been contaminated with feces. It is suspected that MAP is correlated with Crohn's disease, a chronic human enteritis, thus the intake of milk with MAP has been considered a potential factor in the transmission of this agent to humans (Over et al.).

Under Brazilian law, milk intended for human consumption must be subjected to heat treatment between 72 and 75°C for 15 to 20 seconds and then maintained at 4°C (BRASIL, 2011). Some researchers have shown that MAP can survive pasteurization conditions (Ellingson et al., 2005), and MAP has already been identified in pasteurized milk in the United Kingdom, United States, Canada, India and the Czech Republic (Millar et al., 1996; Gao et al., 2002; Ayele et al., 2005; Ellingson et al., 2005; Shankar et al., 2010).

Although Brazil is the fifth largest producer of milk (IBGE, 2010), and MAP has already been detected in raw milk (Carvalho et al., 2009), Brazilian retail pasteurized milk has not yet been tested for viable MAP. The aim of this study was to investigate the presence of viable MAP in retail pasteurized milk in Viçosa, Minas Gerais State, Brazil.

From July 2010 to May 2011, 37 plastic bags of cow's milk treated by commercial pasteurization (minimum of 72 °C for 15 s) were obtained at random from supermarkets in Viçosa. Samples were brought directly to the Bacterial Diseases Laboratory (LDBAC), where they were processed. All samples were subjected to the phosphatase test as previously described by Grant et al. (2002a). In order to obtain a positive control sample of milk, the MAP K-10 strain was inoculated in milk powder Molico® (Nestlé, São Paulo, Brazil) reconstituted with ultrapure water. A negative control sample was also obtained by repeating the same procedure, but without the addition of MAP.

All samples and controls were subjected to the process outlined by Jayarao et al. (2004), with some modifications. Briefly, 40 mL of each sample was centrifuged for 15 min at 2,500 × *g* at room temperature. The resulting pellet was resuspended in 15 mL of 0.9 % hexadecylpyridinium chloride (HPC) (Sigma, Mumbai, India). Decontamination was performed overnight and, after this, tubes were centrifuged again for 15 min at 2,500 × *g* at room temperature. After centrifugation, the pellet was resuspended in 1 mL of antimicrobial solution (nalidixic acid + vancomycin chloride + amphotericin). A 150µl portion of the resuspended pellet was used to inoculate Herrold's egg yolk medium (HEYM) with and without mycobactin J. The incubation period at 37 °C was 4 months. Colonies resembling MAP were stained by the Ziehl-Neelsen (ZN) method for the presence of acid-fast bacilli.

DNA was extracted from primary colonies. Briefly, a small loop full of bacteria was suspended in 50µl of ultrapure water in a polypropylene tube. After spinning down for 5 min at 16,000 × *g* at room temperature, supernatant was discarded and the solutions of the Wizard® Genomic DNA Purification Kit were added to the bacteria according to the protocol recommended by the manufacturer. In order to perform the IS900-Nested-PCR tests, the Go Taq® Green Master Mix (Promega, Madison, WI, EUA) was used. The first-round PCR was performed using the following primers: s204 (5'-TGA TCT GGA CAA TGA CGG TTA CGG A-3') and s749

(5'-CGC GGC ACG GCT CTT GTT-3'), which amplified a fragment of 563bp. The nested-PCR was performed using the primers s347 (5'-GCC GCG CTG CTG GAG TTG A-3') and s535 (5'-AGC GTC TTT GGC GTC GGT CTT G-3'), which amplified an internal fragment of 210bp (Englund et al., 1999). PCR tests were performed according to the authors' recommendations. Ultrapure water and the MAP K-10 strain were used as negative and positive controls, respectively, and a 100bp DNA Ladder (Invitrogen, Washington, DC, USA) was used as a molecular marker.

In order to confirm the identity of the amplified fragments (210bp), the amplicons were purified using the Wizard Plus SV Miniprep DNA Purification System Kit (Promega), sequenced in triplicate in both directions and the obtained sequences were aligned, edited and compared with other sequences deposited in the GenBank using the Basic Local Alignment Search Tool (BLAST) software (<http://www.ncbi.nlm.nih.gov>).

In 1 of the 37 (2.7 %) analyzed samples of commercially pasteurized milk, suspected MAP colonies (based on colony morphology) were detected on HEYM with mycobactin J, and no colonies were observed on HEYM without mycobactin J. Microscopically, colonies were found to consist of typical ZN-positive acid-fast bacilli showing characteristic clumping. All commercially pasteurized retail milk samples tested phosphatase negative.

The IS900- Nested-PCR was positive for these colonies. The fragment of 210bp was sequenced and the genetic analysis revealed 97-99 % identity between the amplified sequence and the MAP strain K-10 sequence available in the NCBI database.

MAP may be cultured from milk after High Temperature Short Time (HTST) pasteurization if the organism is present in raw milk in sufficient numbers (Millar et al., 1996; Grant et al., 1998; Grant et al., 2002b). In Brazil there are no studies regarding the enumeration of MAP in raw milk samples, and studies about paratuberculosis in this country are only just beginning to appear.

The detection of viable MAP in retail milk destined for consumers in Viçosa is further confirmatory evidence that this microorganism can survive the minimum HTST pasteurization temperatures accepted by Brazilian legislation. Taken together, the results of research from several countries confirm that human populations are

exposed to this chronic enteric pathogen in retail milk supplies (Grant et al., 2002a; Ayele et al., 2005).

The successful recovery of MAP from milk samples depends on many factors, such as number of MAP organisms in the original sample, the differences between strains, the impact of HPC decontamination on MAP viability, the antagonistic interference from non-acid-fast microorganisms during incubation, and others (Dundee et al., 2001; O'Reilly et al., 2004). Thus, there is a risk of false-negative results and a lower culture detection rate of MAP in milk treated by small-scale and commercial pasteurization. Therefore, although the culture detection rate of MAP in this study is comparable to rates from the Czech Republic (1.6 %) and the United Kingdom (2 %), it can be underestimated (Grant et al., 2002a; Ayele et al., 2005).

MAP detection peaks were observed in many studies (Millar et al., 1996; Grant et al., 2002a; Ayele et al., 2005) and this study was designed with sample collection over a year to enable observation of these peaks. However, MAP detection was observed only in 1 sample. Thus, it was not possible to make inferences about peaks of MAP detection in Brazil.

The most effective way of reducing any potential human health risk of exposure to MAP through the consumption of cow's milk is to control paratuberculosis in the national dairy herd (O'Reilly et al., 2004). However, in Brazil there is no national program for the control of paratuberculosis yet.

This study provides evidence that MAP is present in commercial pasteurized milk in the Minas Gerais State and in Brazil. In other words, it is further confirmatory evidence that this microorganism can survive the minimum HTST pasteurization temperatures outlined by the legislation. This result has important implications, since human exposure to MAP is a potential risk for Crohn's disease. This is the first report of viable MAP in retail pasteurized whole milk in Brazil.

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**CHAPTER V. Presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Brazilian patients with inflammatory bowel diseases and controls**

Manuscript to be submitted

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## Abstract

Since the first isolation of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from intestinal tissue of human patients bearing Crohn's disease (CD), the possibility of an infectious etiology for CD has been widely discussed. Consequently, MAP has attracted the interest of many researchers because of the similarities between paratuberculosis and CD, and these studies have produced conflicting results regarding the presence of MAP in the tissues of CD patients. The aims of this study were, firstly to verify the presence of MAP in formalin-fixed paraffin-embedded and fresh human intestinal biopsies, by molecular techniques and culture, from patients attending a referral center for treatment of intestinal diseases, in Minas Gerais State, Brazil. Secondly, we aimed to determine the relationships between the detected amounts of MAP and histological results within the groups CD, ulcerative colitis (UC) and non-inflammatory bowel disease controls (nIBD). Fresh samples were collected from 25 patients; five with CD, eight with UC and 12 with nIBD. Formalin-fixed paraffin-embedded samples were collected from 149 patients, comprising 44 with CD, 49 with UC, and 56 with nIBD. None of the fresh samples from CD, UC or nIBD patients were positive for viable MAP with any culture medium used in this study. There were no statistically significant differences between groups in the detection of MAP by IS900 PCR. The mean number of acid-fast bacilli in the CD group was 10 times higher than the UC group ( $p < 0.05$ ); no significant differences were found between the CD and nIBD or UC and nIBD groups for formalin-fixed paraffin-embedded samples. In fresh samples, no statistically significant differences between groups were observed. CD and UC groups did not show statistically significant differences in their scores for degrees of injury in formalin-fixed paraffin-embedded samples; these groups had mean scores higher than the nIBD group. In fresh samples, there were no statistically significant differences. We could not make any statistical inferences about quantities of DNA found by qRT-PCR in the three groups due to the small sample size; however, there was a greater bacterial load in the CD group. The pathogenic role of MAP remains controversial and inconclusive. This is the first report of MAP presence in human intestinal biopsy tissues in Brazil.

## Introduction

Crohn's disease (CD) is a chronic inflammatory disease of the human gut. It has an increasing incidence worldwide, and an unknown etiology (Shanahan, 2002). Paratuberculosis is a chronic granulomatous enteritis, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which affects all species of ruminants worldwide. (Chiodini et al., 1984). Due to the similarities between paratuberculosis and CD, the possibility of an infectious etiology for this disease has been widely discussed and MAP has attracted the interest of many researchers (Feller et al., 2007; Abubakar et al., 2008).

MAP has been detected by culture and PCR in patients with CD (Bull et al., 2003; Naser et al., 2004; Autschbach et al., 2005; Sechi et al., 2005; Abubakar et al., 2008). Numerous theories about a possible cause of CD have been postulated over the years (Prantera, 2007). Scientific evidence supports the theory of an interaction between a persistent environmental stimulus (such as a microbial antigen) and genetic factors that regulate the immune response and/or function of intestinal mucosa (Chiodini and Hermon-Taylor, 1993; Chamberlin et al., 2001). It would be simplistic, however, to conclude that one agent is solely responsible for the etiology of CD; a multifactorial cause is more likely (Feller et al., 2007; Sibartie et al., 2010).

Several studies investigating these causes have been conducted, and they suggest that, although further research is required, the association between MAP and CD cannot be excluded (Feller et al., 2007; Abubakar et al., 2008; Chiodini et al., 2012). The aim of this study was to verify the presence of MAP in fresh and formalin-fixed paraffin-embedded intestinal biopsies, through cultivation and molecular techniques, collected from patients attending a referral center for treatment of intestinal diseases in Minas Gerais State, Brazil. We also aimed to determine the relationship between the amount of MAP detected in samples, and scores established for degrees of injury within the patient groups: CD, ulcerative colitis (UC) and non-inflammatory bowel disease controls (nIBD).

## **Material and Methods**

### ***Patients and Samples***

#### *Fresh tissues*

Fresh samples were collected, using sterile biopsy forceps, from patients undergoing routine ileocolonoscopy as part of their normal clinical treatment in the Instituto Alfa de Gastroenterologia (IAG), Hospital das Clínicas (HC) of Universidade Federal de Minas Gerais (UFMG). Prior to sample collection, informed consent was obtained from each individual. The confirmation of CD in each patient was based on clinical, radiologic, endoscopic and histopathological findings. Patients with other inflammatory bowel diseases were included in the UC group and patients diagnosed without inflammatory bowel disease (nIBD) were those undergoing ileocolonoscopy without a clinicopathological diagnosis of any inflammatory disease. Inclusion criteria for the study were identification of the patient condition and informed consent of patient.

Samples were collected from 25 patients, comprising five patients with CD, eight with UC and 12 with nIBD. Patient details are shown in Table 1. From each patient, six biopsy specimens were collected: three samples of terminal ileum and three samples of ascending colon. Four samples from each patient (two ileum and two colon) were placed in 1.5ml microcentrifuge tubes containing Middlebrook OADC broth supplemented with 20% autoclaved glycerol, and then stored in liquid nitrogen for subsequent microbiological cultivation and DNA extraction. The remaining two samples (one ileum and one colon) were placed in 1.5ml microtubes containing buffered formalin for subsequent histological analysis. For patients with CD and UC, samples were collected from inflamed and uninfamed parts of the mucosa (Bull et al., 2003).

#### *Formalin-fixed, paraffin-embedded tissues*

We obtained 259 paraffin blocks, derived from biopsies of 149 patients, in the Pathology Laboratory of IAG-HC/UFMG. As with fresh samples, paraffin blocks were also categorized into three groups: 89 samples from patients with nIBD, 71 from patients with CD and 99 from patients with UC. Patient details are shown in Table 2. All blocks were derived from patients attended to between 2009 and 2011 in the IAG-

HC/UFMG. From each block, two slides for histopathological analysis were prepared and three slices of 10-20µm thickness were used for DNA extraction.

### ***Tissue processing and MAP culture***

Samples for cultivation were taken to LDBAC/UFV, where they were macerated and decontaminated, according to Bull et al. (2003) and Sechi et al. (2005). Briefly, for decontamination, 0.5ml of NaOH 2% was added to samples, and they were kept at rest for 20 min at room temperature. Subsequently, samples were centrifuged at 3000 x *g* for 30 min; the supernatant was discarded, and the pellet was washed with 10ml of PBS. After washing, the pellet was resuspended in 0.5ml of TEN buffer (50µM Tris-HCl, 100mM EDTA, 150mM NaCl, pH 8). Aliquots of 100µl of the suspension were inoculated into four tubes of Herrold Egg Yolk Medium (HEYM), two with mycobactin *J* and two without mycobactin *J*. The remaining 100µl were inoculated into a tube containing Middlebrook 7H9 broth, supplemented with OADC and mycobactin *J*, and all the tubes were incubated at 37°C for up to 30 weeks.

### ***DNA extraction and PCR***

For DNA extraction, the Wizard® Genomic DNA Purification Kit was used according to the manufacturer's recommendations and the extracted DNA was stored at 8°C for later use. For PCR, Go Taq® Green Master Mix was used according to the manufacturer's instructions and the primers BN1 (5'-GTT ATT AAC GAC GCC CAG C-3') and BN2 (5'-ACG ATG CTG TGT TGG GCG TTA G-3') (Sivakumar et al., 2005), based on the insertion sequence IS900 which amplifies a fragment of 626bp, were used. Each reaction had a total volume of 25µl, comprising: 12.5µl of mix, 1µl of each primer, 6.5µl of ultrapure water and 4µl of DNA extracted at a concentration of approximately 200ng/µL. PCR was carried out as recommended by the authors: initial denaturation at 94°C/4 min, 30 cycles of 94°C/1 min, 60°C/1 min, 72°C/1 min and a final extension step at 72°C/4 min.

To confirm the process of DNA extraction, PCR reactions were performed to target a region of human *APC* gene, using the primers F (5'-CCC CTC CAA ATG AGT TAG CTG C-3') and R (5'-CTC TGC TTT ATT GTC ATC CAA TTC A-3') (Rivero et al., 2006).

Amplified fragments were visualized by electrophoresis, in 1% agarose gel in Tris-Borate-EDTA (TBE) stained with GelRed™ Nucleic Acid Gel Stain, using a UV transilluminator. *Ladder* 100bp was used as molecular size marker and ultrapure water as a negative control.

Results were analyzed by relating the MAP detection within the groups CD, UC and nIBD.

### ***Sequencing and genetic analysis***

Amplified fragments were extracted and purified from agarose gel using Wizard® SV Gel and PCR Clean-Up System (according to manufacturer's instructions); subsequently, both strands were sequenced in triplicate. Sequences were edited with software DNAMAN, and then compared with the sequence of MAP K10 strain deposited in GenBank, using the software Basic Local Alignment Search Tool (BLAST), available at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>).

### ***Histopathological analysis***

Samples stored in buffered formalin were processed according to routine procedures of the Laboratory of Histopathology, DVT/UFV. Paraffin-embedded material was used in the preparation of slides (in duplicate) In each pair of duplicate slides, one slide was stained with hematoxylin-eosin (HE) to determine the score for degree of injury, and the other was stained with Ziehl-Neelsen (ZN) to verify the presence of acid-fast bacilli.

To analyze degrees of injury, tissues were scored histologically by three blinded investigators who applied the following scores adapted from Dieleman et al. (1998): 0 (no inflammation), 1 (mild inflammatory activity), 2 (most severe inflammatory activity, with distortion of crypts or crypt abscesses), and 3 (severe inflammatory activity ulcerations). Then, the mean score for each sample was used. Results were analyzed by determining the relationships between the scores for degrees of injury and the presence of acid-fast bacilli with the groups CD, UC and nIBD.

### **qRT-PCR**

qRT-PCR reactions in absolute quantitation were performed in duplicates on plates with 48 wells using the TaqMan® Universal Master Mix II (Applied Biosystems, Foster City, CA, USA). Genomic quantitation of each sample was generated by the software for detection from Eco™ Real-Time PCR System, and compared to the standard curve of the bacterial genome ( $10^6$  to  $10^1$  copies) using the values of the quantification cycle (Cq) for each reaction. The reaction used the primers MPF (5'-CCG CTA ATT GAG AGA TGC GAT T-3') and MPR (5'-CCA GAC AGG TTG TGC CAC AA-3'), based on the insertion sequence IS900, and the specific probe (5'-FAM-ACC TCC GTA ACC GTC ATT GTC CAG ATC A-TAMRA-3') (Herthnek et al., 2006). The initial concentration of the sample to construct the standard curve was determined by the following formula according to the manual QuantiFast™ SYBR® Green PCR Handbook (Qiagen, Valencia, CA, USA):

$$\text{molecules}/\mu\text{L} = \frac{\text{concentration of DNA (g}/\mu\text{L)}}{\text{size of DNA (bp)} \times 660 \times 6.022 \times 10^{-23}}$$

For each reaction (total 20 $\mu$ l) we used 10 $\mu$ l of Mix II, 1 $\mu$ l of each primer at the initial concentration of 10pmol/ $\mu$ l, 5.5 $\mu$ l of nuclease-free water, 2 $\mu$ l of DNA and 0.5 $\mu$ l of specific probe at the initial concentration of 10pmol/ $\mu$ l. Amplifications were performed according Herthnek et al. (2006), and are briefly described here: incubation for 2 min at 50°C followed by activation of polymerase for 10 min at 95°C; after this pretreatment, samples were subjected to 45 cycles of 95°C for 15 s and 60°C for 1 min.

Results were analyzed by comparing the amount of MAP within the three patient groups (CD, UC and nIBD).

### **Statistical analysis**

All statistical analysis were performed using the software Statistica 7.0 (StatSoft Inc, 2007). Data were subjected to analysis of variance (ANOVA) and means were compared by F test. In cases of significant differences Tukey's test was used at 5% of probability ( $p < 0.05$ ).

### **Ethical Considerations**

This study was approved by the Committee of Ethics in Research (COEP) of the Federal University of Minas Gerais (UFMG) (ETIC nº 0471.0.203.000-10 – Supplement I). All participants provided documented informed consent prior to taking part in this study.

### **Results and Discussion**

#### **Patients**

149 formalin-fixed, paraffin-embedded samples were collected from 143 patients: 56 male and 87 female, whose mean age was 40.5 years (range 2-83 years). Six samples were collected from patients from whom samples were collected at two different times. All samples were divided into three groups: 44 CD, 49 UC and 56 nIBD (Table 1). Fresh samples were collected from 14 male and 7 female patients, whose mean age was 46.5 years, (range 23- 74 years; Table 2).

It was observed that studies including children were more likely to report a positive result than those with an adult population (Abubakar et al., 2008). Dell'Isola et al. (1994) suggested that if the initial MAP infection occurs in childhood, its detection in studies among children would be more likely. However, in this study, the inclusion of children did not influence on results.

**Table 1.** Characteristics of patients with Crohn's disease (CD), ulcerative colitis (UC) and non-inflammatory controls (nIBD): fresh samples

	CD	UC	nIBD	Total
n	5	8	12	25
Age at time of biopsy, mean (range)	26 (23-29)	49.2 (31-74)	49.5 (32-70)	
Sex				
Female	3 (60%)	4 (50%)	4 (33%)	11
Male	2 (40%)	4 (50%)	8 (67%)	14

**Table 2.** Characteristics of patients with Crohn’s disease (CD), ulcerative colitis (UC) and non-inflammatory controls (nIBD): formalin-fixed, paraffin-embedded samples

	CD	UC	nIBD	Total
n	44	49	56	149
Age at time of biopsy, mean (range)	40.5 (11-77)	37 (2-77)	44.5 (13-83)	
Sex				
Female	24 (54.5%)	32 (65.3%)	34 (60.7%)	87
Male	20 (45.5%)	17 (34.7%)	22 (39.3%)	56

### **MAP Culture**

Fresh samples from CD, UC or nIBD patients did not provide a positive result for viable MAP with any culture media used in this study, even after 30 weeks. MAP culture from intestinal human biopsies is quite difficult, even under optimal conditions; however, several research groups were able grow MAP from tissues of patients with CD, by classical methods of culture, with success rates ranging from 0-40% (Chiodini et al., 2012).

MAP isolates from humans, in addition to the usual requirements of sample decontamination and very slow growth of the microorganism, present themselves in spheroplasts (a cell wall-deficient form), which extremely hard to isolate, recover and maintain in sufficient numbers for studies (Hines and Styer, 2003).

Additionally, the isolation of MAP may also have been negatively affected by the freezing of the samples. It was not possible to work with fresh samples due to the distance between HC/UFMG and LDBAC. The freezing of samples before processing was necessitated and even though a cryoprotectant had been used, the ideal is that tissues had been processed immediately (Bull et al., 2003).

Although MAP was not isolated in this study, it is important to highlight that MAP has only been recovered from human tissues with CD. The microorganism has never been isolated from patients with UC or nIBD (Chiodini et al., 2012).

### **PCR**

DNA was extracted successfully from all samples. In five formalin-fixed paraffin-embedded samples, fragments of similar size to expected were amplified by PCR: 1/44 (2.3%) from patients with CD, 2/49 (4%) from patients with UC and 2/56 (3.5%) from patients with nIBD (Table 3). These differences between groups,

however, were not statistically significant. In three fresh samples, fragments of similar size to expected were amplified: 1/5 (20%) from patients with CD, 1/8 (12.5%) from patients with UC and 1/12 (8.3%) from patients with nIBD (Table 3). These differences between groups were not statistically significant.

**Table 3.** Relationship between IS900 PCR results and clinical groups of patients

Group	Paraffin-embedded samples		Fresh samples		Total
	n	IS900 PCR +	n	IS900 PCR +	
CD	44	1 (2.3%) <sup>a</sup>	5	1 (20%) <sup>b</sup>	49
UC	49	2 (4%) <sup>a</sup>	8	1 (12.5%) <sup>b</sup>	57
nIBD	56	2 (3.5%) <sup>a</sup>	12	1 (8.3%) <sup>b</sup>	68
Total	149	5 (3.3%)	25	3 (12%)	174

Results followed by the same letters did not differ statistically by Tukey's test at 5% probability. Paraffin samples: F = 0.1211, p = 0.8860; fresh samples: F = 0.2051, p = 0.8161. CD = Crohn's disease; UC = ulcerative colitis; nIBD = non-inflammatory bowel diseases

PCR results for MAP detection are inconclusive and conflicting; reports ranged from 0-100% detection each in CD, UC and nIBD using a variety of different methodologies and target sequences (Feller et al., 2007; Abubakar et al., 2008). Contrary to other studies in this field (Bull et al., 2003; Naser et al., 2004; Autschbach et al., 2005), MAP was not detected more frequently among patients with CD compared to patients with UC or nIBD. Other research groups, however, have shown that the detection of MAP in patients with CD is more frequent than in patients with UC or nIBD (Ricanek et al., 2010; Rath et al., 2011).

In this study, we verified the presence of MAP in intestinal biopsy specimens from eight patients among 174 samples tested. Previous studies have demonstrated that MAP is difficult to detect reliably and reproducibly by PCR in DNA extracted from human tissues (Bull et al., 2003). Thus, the use of non-optimal procedures in the processing of samples may result in false-negative results. Bull et al. (2003) indicated several important steps in the process of DNA extraction, such as the processing of fresh tissues (which have never been frozen); the mechanical disturbance to ensure access to MAP DNA; resuspension of the DNA overnight at 4°C; and nested-PCR. In this study, we followed these recommendations wherever possible. However, as previously mentioned, it was necessary for us to freeze the samples, and this could be one reason for the low detection rate of MAP in our results.

### ***Sequencing and genetic analysis***

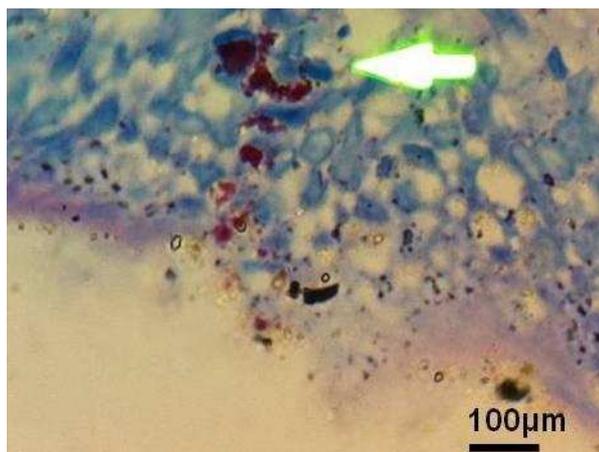
All 626bp amplicons were sequenced; genetic analysis revealed 97-99% of identity between the amplicons and the sequence of MAP K-10 strain available on NCBI database.

### ***Histopathological analysis***

In formalin-fixed paraffin-embedded samples, acid-fast bacilli were identified (Figure 1) in 15/149 (10%) slides stained with ZN; comprising 9/44 (20.4%) from patients with CD, 1/49 (2 %) from patients with UC and 5/56 (8.9%) from patients with nIBD. The mean detection of acid-fast bacilli in the CD group was 10 times higher than in the UC group ( $p < 0.01$ ); there were no statistically significant differences between CD and nIBD, or between UC and nIBD groups (Table 4). In fresh samples, acid-fast bacilli were detected in 1/25 (4%) slide from one patient with UC. Acid-fast bacilli were not identified on slides from patients with CD or nIBD. Among the fresh samples, the differences between groups were not statistically significant.

Few studies have shown a higher frequency of detection of MAP or acid-fast bacilli in patients with CD (Sechi et al., 2001; Ellingson et al., 2003; Romero et al., 2005; Jeyanathan et al., 2007). This is due to the difficulty in detecting MAP in tissue as they occur in spheroplast form, and only a small amount of the microorganism is present in the tissues. While these technical limitations are unresolved, demonstrating the presence of MAP in tissues of patients with CD will be a challenge (Chiodini et al., 2012). Moreover, Koch's postulates cannot be fully satisfied since for ethical reasons, the inoculation of MAP in human patients is not possible.

We analyzed the degree of injury in formalin-fixed paraffin-embedded samples, and observed a mean score of 2.44 for patients with CD, 2.34 for patients with UC and 1.86 for the nIBD group (Table 5). There were no statistically significant differences between the CD and UC groups and they had higher scores than those of the nIBD group ( $p < 0.01$ ). In fresh samples, we observed a mean score of 1.25 for patients with CD, 0.92 for patients with UC and 0.58 for the nIBD group; however, these differences were not statistically significant. On Figure 2 degrees of injury observed in this study are showed.



**Figure 1.** Acid-fast bacilli in the intestinal mucosa of a patient with Crohn's disease, stained by Ziehl-Neelsen.

**Table 4.** Relationship between acid-fast bacilli presence and clinical groups of patients

Group	Paraffin-embedded samples		Fresh samples		Total
	n	acid-fast bacilli +	n	acid-fast bacilli +	
CD	44	9 (20.4%) <sup>a</sup>	5	0 (0%) <sup>c</sup>	49
UC	49	1 (2%) <sup>b</sup>	8	1 (12.5%) <sup>c</sup>	57
nIBD	56	5 (8.9%) <sup>a,b</sup>	12	0 (0%) <sup>c</sup>	68
Total	149	15	25	1	174

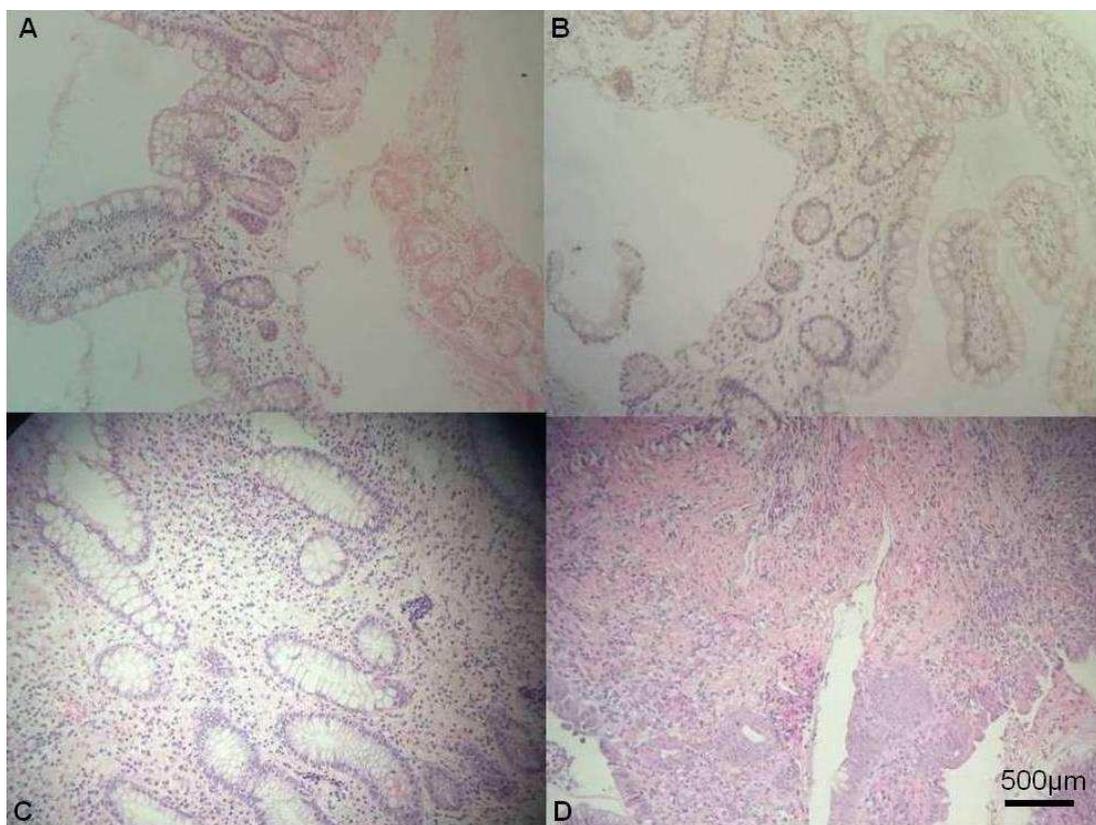
Results followed by the same letters did not differ statistically by Tukey's test at 5% probability. Paraffin samples:  $F = 4.5879$ ,  $p = 0.0117$ ; fresh samples:  $F = 1.0686$ ,  $p = 0.3607$ . CD = Crohn's disease; UC = ulcerative colitis; nIBD = non-inflammatory bowel diseases

**Table 5.** Association between mean scores and clinical groups of patients

Group	Paraffin-embedded samples		Fresh samples	
	n	mean score for degree of injury	n	mean score for degree of injury
CD	44	2.44 <sup>a</sup>	5	1.25 <sup>c</sup>
UC	49	2.34 <sup>a</sup>	8	0.92 <sup>c</sup>
nIBD	56	1.86 <sup>b</sup>	12	0.58 <sup>c</sup>

Results followed by the same letters did not differ statistically by Tukey's test at 5% probability. Paraffin samples:  $F = 7.8287$ ,  $p = 0.0006$ ; fresh samples:  $F = 2.26473$ ,  $p = 0.0933$ . CD = Crohn's disease; UC = ulcerative colitis; nIBD = non-inflammatory bowel diseases

There were no statistically significant differences between mean scores of injury in formalin-fixed paraffin-embedded samples for CD and UC groups, and they had higher mean scores than the nIBD group ( $p < 0,01$ ). We expected this result, since inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract characterized by infiltration of neutrophils as well as mast cells and eosinophilic granulocytes into the colonic wall, accompanied by epithelial cell necrosis and ulceration (Podolsky, 1991). In fresh samples, there were no statistically significant differences among scores of injury for the three groups. The small sample size of fresh samples may have influenced this result.



**Figure 2.** Histological sections stained with hematoxylin and eosin with different degrees of injury. A) score 0, showing villi organized without changes, and inflammatory cells infiltrated irrelevant; B) score 1, showing mild inflammatory activity; C) score 2 showing mild disorganization of crypts and crypt abscesses; D) score 3, showing absence/disruption of crypts, loss of continuity of the epithelium and inflammatory infiltration

### **qRT-PCR**

qRT-PCR was performed for eight samples that were positive by PCR (five formalin-fixed paraffin-embedded and three fresh). In formalin-fixed paraffin-embedded samples we observed values of 192.12 copies/ $\mu$ l in the CD group, 72.28 copies/ $\mu$ l in the UC group and 81.43 copies/ $\mu$ l in the nIBD group. In fresh samples we observed values of 432.99 copies/ $\mu$ l, 167.92 copies/ $\mu$ l and 249.73 copies/ $\mu$ l in the CD, UC and nIBD groups, respectively (Table 6).

Considering the small number of PCR-positive samples that were tested for qRT-PCR, we could not make any statistical inferences about the quantities of DNA found in the three groups (CD, UC and nIBD), although we observed that CD patients had higher bacterial loads in both formalin-fixed paraffin-embedded and fresh samples.

**Table 6.** Relationship between qRT PCR results and clinical groups of patients

Group	Paraffin-embedded samples		Fresh samples		Total
	n	qRT PCR	n	qRT PCR	
CD	1	192.12	1	432.99	2
UC	2	72.28	1	167.92	3
nIBD	2	81.43	1	249.73	3
Total	5		3		8

CD = Crohn's disease; UC = ulcerative colitis; nIBD = non-inflammatory bowel diseases

Some studies support the theory that MAP is present in most individuals, but there are found in greater quantities in people with CD, suggesting that MAP is a ubiquitous environmental organism that is an opportunistic pathogen and not a primary cause of CD (Chiodini et al., 2012).

In this study, the frequency of MAP detection by PCR did not differ between CD, UC or nIBD patients, and although the bacterial load was higher in patients with CD, is unknown if a high bacterial load causes higher scores of inflammation or if higher scores of inflammation cause higher bacterial loads. A possible explanation may be that the microorganism finds better conditions for replication in patients with CD than in patients with UC or nIBD. Immunological factors related to MAP, in susceptible patients, may allow MAP to replicate in larger quantities, increasing the bacterial load in patients with CD. This fact is corroborated by Lee et al (2011), who showed general mucosal colonization by MAP and suggested that there is simply

increased mucosal surface colonization (dysbiosis) in CD, that is unassociated with causality. The dysbiosis and reduced bacterial diversity of the intestinal microbiome in CD likely promotes MAP growth and detection (Chiodini et al., 2012).

Further investigations into the etiological role of MAP in CD are also needed. CD remains to be a debilitating disease that severely affects the quality of life of its sufferers. Further research is required to definitively answer the questions regarding the etiological nature of the disease.

### **Conclusion**

MAP was present in all groups of patients analyzed, although the greatest bacterial load were observed in the CD group. This study supports the view that MAP is a ubiquitous organism that colonizes the mucosal surfaces of the gut resulting in increased detection in CD patients. The pathogenic role of MAP remains controversial and inconclusive. This is the first report of the presence of MAP in biopsy specimens of human gut in Brazil.

### **Acknowledgments**

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**CHAPTER VI. Genetic evaluation of IS900  
partial sequence of *Mycobacterium avium*  
subspecies *paratuberculosis* Brazilian isolates  
from bovine milk**

Paper published in the “**Tropical Animal Health and Production**”

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## **Abstract**

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis. Insertion sequence *IS900* is used for the identification of MAP. The objective of this study was to verify the genetic conservation of *IS900* sequences in raw milk samples. To evaluate genetic conservation, 206 quarter milk samples and 16 bulk tank milk samples were collected. DNA extraction and *IS900*-PCR were performed in all samples. Six samples amplified the expected fragment. To confirm the identity of the amplified fragments, PCR products were cloned and sequenced. The resulting sequences were compared with other MAP sequences from GenBank and it was possible to identify eight polymorphic regions and to form five distinct haplotypes. The number of mutations in each haplotype was verified. *IS900* sequence is a very well-conserved sequence that could be used as tool for the molecular detection of this agent and epidemiological purposes. The results showed the first genetic analysis on Brazilian isolates of MAP.

**Keywords:** *Mycobacterium avium* subspecies *paratuberculosis*, Johne's disease, *IS900*-PCR, milk

## **Introduction**

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, incurable chronic granulomatous enteritis that can affect all ruminants, domestic or wild, worldwide. The disease is characterized by a malabsorption and consequently diarrhea and weight loss. Infected animals shed MAP in both milk and feces (Okura et al., 2012)

Economic losses attributable to paratuberculosis are very considerable and result of premature culling, reduced milk production, and loss of body weight in cattle sold for slaughter (Hasonova and Pavlik, 2006). Successful prevention and control depends on animal health authorities and livestock industries acquiring a good understanding of the nature and epidemiology of infection, and of the application of tools for diagnosis and control (Kennedy and Benedictus, 2001). Certain trade restrictions lead to losses at a national and international level.

In addition to the economic importance, the disease has a public health importance because of its direct association with Crohn's disease in human (Feller et al., 2007; Mendoza et al., 2009).

Fecal culture is the gold standard diagnostic test for MAP, however, it may require 8–16 weeks to confirm that a sample is negative (Whittington and Sergeant, 2001). DNA tests have been developed to address the drawbacks of other tests, detecting and identifying MAP with greater speed and safety (Sivakumar et al., 2005). Most of PCR protocols target the *IS900*, an insertion element generally accepted as the standard marker for MAP (Green et al., 1989). The technique allows detection of the disease at the early stages, which are the most important considering the transmission (Nielsen and Toft, 2008).

Some studies have compared the *IS900* sequence from different MAP isolates aiming the genetic evaluation of this sequence (Sivakumar et al., 2005; Bhide et al., 2006; Castellanos et al., 2009). The heterogeneity of MAP isolates has been exploited for epidemiological purposes.

Genetic evaluation of an infectious organism is an important tool in disease surveillance, tracing the origin and transmission of infection, identification of virulent strains, evaluation of treatment failure, and in designing of diagnostics or vaccines (Kaur et al., 2010). The present study aimed to verify the genetic conservation of the *IS900* sequences used in the identification of MAP in raw milk samples.

## **Material and Methods**

A total of 206 quarter milk samples were collected, and bulk tank milk samples from 16 dairy herds in the region of Viçosa, Brazil. In order to be used as control, one loopful of a sample of certified wild MAP was inoculated in 50 mL powdered milk Molico® (Nestlé, São Paulo, Brazil) reconstituted.

The Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used for DNA extraction and the Go Taq® Green Master Mix (Promega) was used for PCR analysis. The primers BN1 (5' GTTATTAACGACGCCAGC 3') and BN2 (5' ACGATGCTGTGTTGGGCGTTAG 3'), which are based on the insertion sequence *IS900*, were used for PCR amplification (Sivakumar et al., 2005). Ultrapure water was used as a negative control and  $\phi$ X174/*Hae*III (Promega) as the molecular marker. To confirm the identity of the amplified fragments (626bp), the PCR-amplified products were cloned in the pGEM vector and DNA was sequenced using M13 forward and reverse primers.

In order to determine the polymorphism of obtained sequences the same MAP genomic region was selected from other nucleotide sequences available at GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>). Twenty-five sequences were obtained and aligned and MAP sequence polymorphisms were analyzed using DnaSP v5 software. Based on the polymorphisms found, the sequences were grouped into haplotypes and a Median Joining (MJ) Network was calculated using Network 4.5.1.0 software, from the Median Joining (MJ) algorithm. Using the network obtained it was possible to determine the number of mutations in each haplotype group.

## Results

Eight quarter individual milk samples (3.8%) of the 206 evaluated samples and none of the bulk tank milk samples amplified fragments of the expected size. From these samples five isolates and a positive control were cloned and sequenced. Using the alignment it was possible to identify eight polymorphic regions (Figure 1B) and from these polymorphisms it was possible to group the sequences into five distinct haplotypes (Figure 1C) (Table 1). Based on the number of mutations we can see that the phylogenetic distance from haplotype 1 to haplotype 3, 4 and 5 is just one mutation. Haplotype 2 was the most distinct from H1 and the distance found was 5 mutations.

Using the grouped sequences it was possible to create a network and to determine the number of mutations in each group of haplotypes. Just one mutation was found in haplotypes H3, H4 and H5 when they were compared with H1. In the haplotypes H3 and H4 we found a G instead an A in the positions 598 and 131 respectively. In the haplotype H5, we found a T instead an A in the position 609. Haplotype H2 showed five mutations in relation to H1. We found an A instead a C in the positions 474, 543, 568, 588 and 594 (Figure 1B).

Isolates from different regions (India, Brazil, United States and some ones from undefined locals) have similar genetic grouping (Figure 1A). Among the analyzed sequences, the verified identity ranged between 99 and 100%.

**Table 1.** Grouping of *Mycobacterium avium* subspecies *paratuberculosis* IS900 sequences into haplotypes<sup>a</sup>, with respective origin

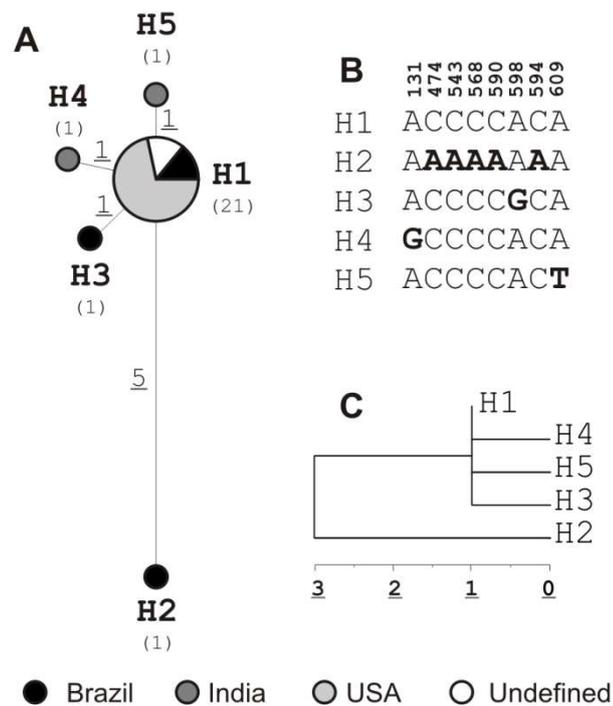
Haplotype Code	Haplotype Frequency	GenBank Access Code	Origin Country
		AE016958	USA
		AF305073	-
		AF416985	-
		AJ250015	USA
		AJ250016	USA
		AJ250017	USA
		AJ250018	USA
		AJ250019	USA
		AJ250020	USA
		AJ250021	USA
H1	21	AJ250022	USA
		AJ250023	USA
		AJ251434	USA
		AJ251435	USA
		AJ251436	USA
		AJ251437	USA
		HM015763 <sup>b</sup>	Brazil
		HM015764 <sup>b</sup>	Brazil
		HM015766 <sup>b</sup>	Brazil
		HM015768 <sup>b</sup>	Brazil
		X16293	-
H2	1	HM015765 <sup>b</sup>	Brazil
H3	1	HM015767 <sup>b</sup>	Brazil
H4	1	AY660658	India
H5	1	AY660657	India

<sup>a</sup> Group of identical sequences, considering the genomic region

<sup>b</sup> Sequenced samples in this study

## Discussion

The analyzed sequences showed some polymorphic regions, however, based on the number of mutations found, it was concluded that the MAP *IS900* sequence, which is present as 14 to 18 copies in the genome (Green et al., 1989), is highly conserved. These results are consistent with suggestions from previous studies that MAP strains tend to be clones (Whittington and Sergeant, 2001; Castellanos et al., 2009). Further investigations are also necessary in order to determine whether there are any phenotypic manifestations due to the small genomic modifications (Sohal et al., 2009).



**Figure 1.** Median-joining (MJ) network of haplotypes from *Mycobacterium avium* subspecies *paratuberculosis* *IS900* sequences. a Grouping of isolates from different regions: circle size – proportional to haplotype frequency; circle colors – origin; underlined values – number of mutations between haplotypes connected by lines. b Identification of eight polymorphic regions. c Grouping of sequences into five distinct haplotypes from polymorphisms

Being a very well-conserved sequence, *IS900* could continue being a target for MAP molecular diagnosis such as PCR, nested-PCR and real-time PCR. Although the standard method for MAP detection from samples is culture, molecular tests are easier and faster to perform. In Brazil, the first detection of MAP in raw milk samples was performed by *IS900* PCR and it is very recent (Carvalho et al., 2009).

There are no studies about the distribution of the MAP genotype or strain types in this country. This is the first study that reports genetic differences of MAP in the country and further studies are necessary in this sense.

The genotypic and phenotypic dissimilarities among strains of MAP may be reflected in the differences detected in the progression of paratuberculosis among infected herds (Castellanos et al., 2009). Further studies about MAP genomic diversity are needed since they may permit better understanding of host specificity of MAP and rational designing of adequate control measures.

Mutations occurring in MAP *IS900* sequence can complicate tracing the origin and transmission of infection, identification of virulent strains, evaluation of treatment failure and designing of diagnostics or vaccines. These can be an obstacle in establishing consolidated control measures for the disease.

The genetic differences between MAP strains related to geographic factors have a potential use in the epidemiological tracing of the disease (Kaur et al., 2010). In the present study we have verified that *IS900* sequence is a very well-conserved sequence even when we compared different isolates from different countries. Therefore, it is possible to affirm that the location does not determine genetic differences for the *IS900* sequence. The *IS900* sequence analysis could be used as a complementary diagnostic tool for epidemiological purposes to study the geographical distribution patterns of MAP.

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**Nucleotide sequence accession numbers**

The GenBank accession numbers for *IS900* partial sequences from *Mycobacterium avium* subsp. *paratuberculosis* are HM015763, HM015764, HM015765, HM015766, HM015767 and HM015768. Sequences will be available for public access in June, 2012.

**Declaration of conflicting interests**

The authors declare that they have no conflict of interest.

## GENERAL CONCLUSIONS

✓ For optimal MAP isolation from raw milk samples, the best protocol involved 0.75% HPC at room temperature for 24h, using a centrifuge at  $2500 \times g$  for 15 min, and the addition of antimicrobial solution immediately before inoculation into tubes with HEYM prepared with fresh egg yolk.

✓ Pasteurization was not sufficient to eliminate viable MAP at the highest concentrations used in the study, in milk samples. This is a concern considering the possible role of MAP in CD. Thus, the animal is a relevant factor and there is a need to establish a program for control of paratuberculosis in Brazil.

✓ This study shows that viable MAP is present in commercial pasteurized milk in the Minas Gerais State, Brazil. The microorganism can survive the minimum HTST pasteurization temperatures outlined by the legislation.

✓ MAP was present in all groups of patients analyzed, although the major bacterial load was detected in the CD group. This study support the view that MAP is a ubiquitous organism that colonizes the mucosal surfaces of the gut resulting in increased detection in CD patients. The pathogenic role of MAP remains controversial and inconclusive.

✓ IS900 sequence is a well-conserved sequence that could be used as tool for the molecular detection of MAP and for epidemiological purposes. The results of this study present the first genetic analysis on Brazilian isolates of MAP.

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# **SUPPLEMENTS**

## SUPPLEMENT I

### Opinion of Committee of Ethics in Research of Federal University of Minas Gerais (UFMG), approving execution of this research project (ETIC n.º 0471.0.203.000-10)



UNIVERSIDADE FEDERAL DE MINAS GERAIS  
COMITÊ DE ÉTICA EM PESQUISA - COEP

Parecer n.º. ETIC 0471.0.203.000-10

Interessado(a): Profa. Maria de Lourdes de Abreu Ferrari  
Departamento de Clínica Médica  
Faculdade de Medicina - UFMG

#### DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 21 de janeiro de 2011, após atendidas as solicitações de diligência, o projeto de pesquisa intitulado "*Mycobacterium avium paratuberculosis* (MAP): presença em amostras de leite pasteurizado e sua relação com doença de Crohn" bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.



Prof. Maria Teresa Marques Amaral  
Coordenadora do COEP-UFMG