



Genomic Detection of *Mycobacterium Avium Subspecies Paratuberculosis* in Blood Samples of Patients with Inflammatory Bowel Disease in Southern Iran

Saeid Amirzadehfard^{1,2}, MSc; 
Mohammadreza Mahzounieh¹, PhD; 
Alireza Safarpour², MD, PhD; Maryam
Nejabat², MSc; Nazanin Nazari³, MSc

¹Department of Pathobiology,
Shahrekord University, Shahrekord, Iran;

²Department of Internal Medicine,
Gastroenterology Research Center,
Shiraz University of Medical Sciences,
Shiraz, Iran;

³Department of Immunology, Shiraz
University of Medical Sciences, Shiraz,
Iran

Correspondence:

Mohammadreza Mahzounieh, PhD;
Department of Pathobiology, Shahrekord
University, P. O. Box: 88186-34141,
Shahrekord, Iran

Tel: +98 38 32324401

Email:

mahzouniehmohammadreza@gmail.com

Received: 26 June 2018

Revised: 27 August 2018

Accepted: 09 September 2018

What's Known

- The prevalence of *Mycobacterium avium subspecies paratuberculosis* (MAP) is widespread and its DNA can be detected in the blood samples of Inflammatory bowel disease (IBD) patients and non-IBD people.
- The presence of MAP in IBD patients varies in different regions of the world.

What's New

- The presence of MAP in IBD patients and non-IBD people is higher in the South of Iran compared to Tehran in the North.
- The high prevalence of MAP indicates a possible role of MAP in stimulating IBD.

Abstract

Background: Inflammatory bowel disease (IBD), of which Crohn's disease (CD) and ulcerative colitis (UC) are the two main clinicopathological subtypes, is a group of digestive system diseases of unknown etiology. Risk factors for IBD are environmental factors, genetics, and immune system agents. *Mycobacterium avium subspecies paratuberculosis* (MAP) is one of the most important infectious factors and a suspected cause of IBD. The present study aimed to determine the prevalence of MAP in both IBD patients and non-IBD people as well as to investigate the relationship between the presence of this bacterium and IBD.

Methods: A cross-sectional study was conducted during May-December 2017 among 146 IBD patients (32 with CD and 114 with UC) at the Motahari Clinic affiliated to Shiraz University of Medical Sciences, Shiraz, Iran. For comparison, the blood samples of 146 non-IBD volunteers (the control group) were tested for the presence of MAP using the polymerase chain reaction method (specific IS900 fragment). The data were analyzed using the SPSS software (version 19.0). The Kolmogorov-Smirnov test was used to evaluate the normal distribution of variables. The χ^2 test was used to compare the qualitative variables between the groups.

Results: MAP was present in 104 (71.2%) IBD patients out of which 24 (75%) had CD and 80 (70.2%) had UC. In the control group, MAP was present in 63 (43.2%) non-IBD volunteers. There was a significant association between the presence of IBD and MAP ($P < 0.001$).

Conclusion: A high prevalence of MAP was observed in the South of Iran. MAP DNA was detected in the blood samples of CD and UC patients as well as non-IBD volunteers. The high prevalence of MAP indicated a possible role of MAP in stimulating IBD.

Please cite this article as: Amirzadehfard S, Mahzounieh MR, Safarpour AR, Nejabat M, Nazari N. Genomic Detection of *Mycobacterium Avium Subspecies Paratuberculosis* in Blood Samples of Patients with Inflammatory Bowel Disease in Southern Iran. 2020;45(3):214-219. doi: 10.30476/ijms.2020.72403.0.

Keywords • *Mycobacterium avium subspecies paratuberculosis*
• Crohn disease • Ulcerative colitis • Inflammatory bowel disease • Polymerase chain reaction

Introduction

Inflammatory bowel disease (IBD) is a type of digestive system disease of unknown etiology. The two main clinicopathological subtypes of IBD are Crohn's disease (CD) and ulcerative colitis

(UC). In CD, parts of the intestine are healthy while other parts are inflamed, whereas UC is limited to the colon and is a chronic inflammatory disease. UC only affects the innermost lining of the colon while CD can occur in all the layers of the bowel walls. Environmental factors, genetics, and immune system agents contribute to the disease. The prevalence of IBD in Western Europe and North America has reached a steady level, whereas it is on the rise in developing countries.¹ In previous studies, some infectious factors such as *Listeria monocytogenes*,² *Pseudomonas melophilia*, *Mycobacterium kansasii*, and *Bacterioides fragilis* have been examined, but they reported no evidence of a significant correlation with IBD.³ However, *mycobacterium avium* subspecies *paratuberculosis* (MAP) is one of the most important infectious factors that influence the outbreak and increase in IBD. MAP is a fastidious bacterium for which no specific immune response has been identified. It is the main cause of John's disease in cattle. A similarity in clinical and pathological results between John's disease and IBD has been proven. Detection of MAP in the intestinal tissues of IBD patients is an indication of a possible association between MAP and IBD.^{4,5}

MAP is an obligate intracellular parasite that seems to need both a host genetic and immunological deficiency to survive and to release bacteria in phagocytes. For example, the genetic defect of caspase recruitment domain-containing protein 15 (CARD15) may indicate an inability to deal with intracellular pathogens.⁶ It can be isolated from the blood culture of CD patients, using the polymerase chain reaction (PCR), to detect MAP DNA. However, bacterial culture has not been successful in non-IBD people with positive PCR for MAP. These results may indicate an active infection with MAP in IBD patients, ranging from colonization to latent stage of infection. Unaffected IBD individuals are better able to cope with MAP infection.⁷ According to Marks and colleagues, inherent immune deficiency in CD patients causes the accumulation of substances in the intestine, which can lead to the destruction of the mucous membrane of the intestinal wall. The lack of a sufficient number of functional neutrophils to effectively remove bacteria and the killing of these bacteria by macrophages lead to chronic granulomatous disease and IBD.⁸

MAP in CD has been shown to be present in a protease-resistant form. It can evade recognition by the immune system and may cause impairment in the regulation of the immune system. Similar to *Mycobacterium*

avium complex (MAC), MAP is also resistant to most standard anti-tuberculous drugs. A previous study showed that CD treatment with anti-MAC drugs (Rifabutin and Clarithromycin) could significantly improve IBD treatment and eventually lead to a complete cure.⁹

Rath and colleagues reported that 20% of the UC patients, 7% of the CD patients, and 38% of non-IBD people in Norway tested positive for MAP.¹⁰ Lee and colleagues also reported that 35% of the CD patients, 5% of the UC patients, and 5.2% of non-IBD people tested positive for MAP.¹¹ Although infection due to the presence of MAP in industrial dairy plants has been reported, there is limited information available on the relationship between IBD and MAP in Iran. Zamani and colleagues reported that the prevalence of DNA MAP and the increase in anti-MAP antibodies in patients with CD disease were significantly associated with CD progression.¹² However, due to the role of various factors involved in IBD, it can be said that the diagnosis of DNA MAP and anti-MAP antibodies in patients with Crohn's disease can indicate the transfer of MAP through dairy and other livestock products to humans.¹² Considering the above, the present study aimed to determine the prevalence of MAP in both the IBD patients and non-IBD people as well as to investigate the relationship between the presence of this bacterium and IBD.

Patients and Methods

A cross-sectional study was conducted during May-December 2017 among 146 IBD patients (32 with CD and 114 with UC) at the Motahari Clinic affiliated to Shiraz University of Medical Sciences, Shiraz, Iran. For comparison, the blood samples of 146 non-IBD volunteers (the control group) were obtained from the Shiraz Blood Transfusion Organization, Shiraz, Iran. The IBD patients were randomly selected from the list of 1236 IBD registered patients (as sampling frame) using command "sample", that can be used to take a random sample from data set in STATA software (version 14), to select 146 participants of the study. The disease was confirmed by clinical diagnosis of two gastrointestinal specialists and colonoscopy results. Demographic and disease details were extracted from the IBD registry of Shiraz University of Medical Sciences, Shiraz, Iran. The exclusion criterion for the control group was any kind of clinical or histopathological diagnosis of IBD.

Blood Test

For complete blood count, blood samples (4 ml) from all IBD patients were collected in

tubes containing K2-EDTA anti-coagulant. The specimens were transferred to a laboratory 2 hours after separation of the buffy coats. The samples were centrifuged at 3500 rpm for 10 minutes, the buffy coats were divided into two batches after separation, and the Eppendorf® microtubes containing 100 µL of Tris-EDTA (TE) buffer were stored at -20 °C in a freezer.

DNA Extraction

The MAP DNA was extracted from the buffy coat using the DNA extraction kit (QIAGEN Inc., USA).

Polymerase Chain Reaction Assay

The amplification of IS900 was done in accordance with the procedure as described by Naser and colleagues.¹³ The product obtained from the last stage of the PCR test was examined using the gel electrophoresis technique. For this purpose, 2% agarose gel and TE buffer or sterile water were used for negative control in all electrophoresis steps. The DNA samples were extracted from MAP (College of Veterinary Medicine, University of Shiraz, Iran) as a positive control in all stages of the PCR test. Primer P90 and P91 were used in the first stage of PCR to propagate a 398bp fragment of the IS900 gene (figure 1). This method has been described in a previous study by Sibartie and colleagues.¹⁴

The specific portion of the MAP DNA was obtained using a dedicated pair of AV1 and AV2 primers on the previous 398bp fragment with a size of 298bp (figure 2). For final confirmation, a sample was tested in the second round of the PCR product which was tested positive. The gene sequencing was determined using Eurofins GeneScan (Eurofins Analytik, Germany). Finally, alignment and BLAST were performed using the BioEdit software.

Statistical Analysis

The data were analyzed using the SPSS software (version 19.0). The Kolmogorov-Smirnov test was used to evaluate the normal distribution of variables. The χ^2 test was used to compare the qualitative variables between the groups.

Results

The group of IBD patients consisted of 73 women (13 [17.8%] with CD and 60 [82.2%] with UC) and 73 men (19 [26%] with CD and 54 [74%] with UC). The age range of the participants was 21-68 years. In addition, the blood samples of 146 non-IBD volunteers were used as the control group. The age and sex of the IBD patients and

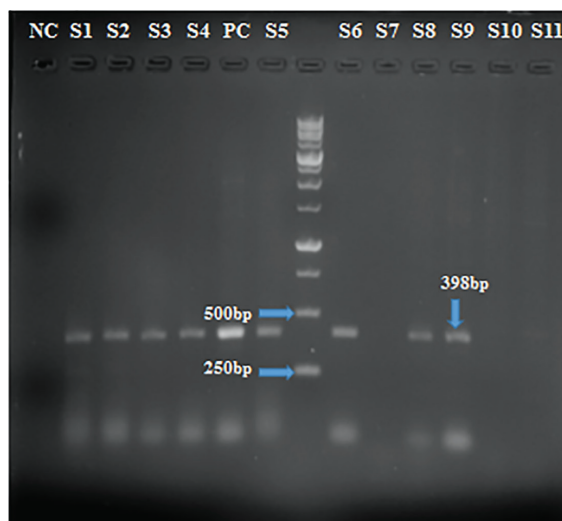


Figure 1: The first round of polymerase chain reaction for the detection of *Mycobacterium avium subspecies paratuberculosis* DNA. Positive results are indicated by a bright 398 bp band (blue arrows) on a 2% agarose gel electrophoresis by P90 and P91 primers. PC: Positive control; NC: Negative control; S1-S11: Patient's sample

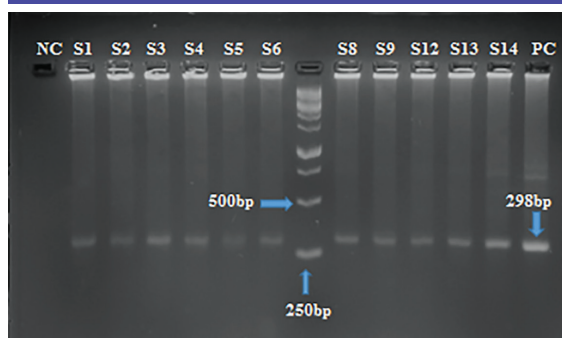


Figure 2: The second round of polymerase chain reaction for the detection of *mycobacterium avium subspecies paratuberculosis* DNA. Positive results are indicated by a bright 298 bp band (blue arrows) on a 2% agarose gel electrophoresis by AV1 and AV2 primers. PC: Positive control; NC: Negative control; S1-S14: Patient's sample

non-IBD volunteers were similar. Demographic characteristics of both groups are presented in table 1.

Sixteen (50%) CD patients and 43 (37.7%) UC patients were treated with immunosuppressive drugs. The presence of MAP was confirmed in 167 (71.2%) participants from both groups. Tests showed that 24 (75%) of the CD patients, 80 (70.1%) of the UC patients, and 63 (43.1%) of the non-IBD volunteers were MAP DNA positive (table 2).

All participants with negative MAP DNA were confirmed negative in the first and second round of the PCR test, indicating the absence of contamination at different stages of the experiment. MAP DNA was positive in 104 patients with IBD. As shown in table 2, there was a significant association between the presence of IBD and the presence of MAP DNA in the blood samples of the patients ($P < 0.001$).

Table 1: Demographic and clinical characteristics of the participants

Variables		IBD patients	Non-IBD volunteers	P value
Sex	Male	73 (50%)	81 (55.5%)	0.34
	Female	73 (50%)	65 (44.5%)	
Disease	Ulcerative colitis	114 (78.1%)	146 (100%)	0.62
	Crohn's disease	32 (21.9%)		
	Non-IBD			
Age (mean±SD)		41.09±10.83	40.53±8.74	

IBD: Inflammatory bowel disease; Chi- square (X2) test, Two sample student t test

Table 2: The prevalence of MAP DNA in IBD patients and non-IBD volunteers

MAP DNA	IBD patients		Non-IBD volunteers	P value
	Ulcerative colitis	Crohn's disease		
Positive	80 (70.2%)	24 (75%)	63 (43.2%)	<0.001
Negative	34 (29.8%)	8 (25%)	83 (56.8%)	

MAP: *Mycobacterium avium subspecies paratuberculosis*; IBD: Inflammatory bowel disease; Chi- square (X2) test

Discussion

A high prevalence of MAP DNA was found in the blood samples of IBD patients. The presence of MAP DNA was 75% (n=24) in the CD patients, 70.2% (n=80) in the UC patients, and 43.2% (n=63) in the non-IBD volunteers. Moreover, a high presence of MAP in the IBD and non-IBD population of Southern Iran was observed. A study conducted in 2017 reported a high prevalence of MAP in CD patients in Tehran (North of Iran).¹² However, compared with Tehran, we found a higher prevalence of MAP DNA in both the UC patients and the non-IBD volunteers. The higher prevalence of MAP among non-IBD could be due to the presence of livestock in close proximity to the local population as well as the lack of individual hygiene in this area.

Our results were in line with the reported prevalence of MAP DNA in IBD patients in Portugal (68% in CD patients and 38% in non-IBD), but were higher than those of some European countries.¹⁵ A study in Germany, conducted among 200 IBD patients and 100 non-IBD controls, indicated an association between MAP prevalence and CD by MAP detection in buffy coats with a rate of 52%.¹⁶ However, a rate of 7% was reported in Norway based on a study conducted among 63 IBD patients and 21 non-IBD controls.¹⁰ Similarly, a study conducted among 40 IBD patients and 19 non-IBD controls in the USA reported that the presence of MAP DNA was 35% in CD patients and 5.2% in non-IBD controls.¹¹ Note that the sample size in the present study was significantly larger than in the above-mentioned studies. Similar to other studies, we detected a significantly higher positive IS900 PCR in CD patients. This finding indicated a possible role of MAP in stimulating CD, whereas lower IS900 PCR detection rates

were observed in UC patients.

The main limitation of the present study was related to the long duration of bacterial growth (12 weeks) and the lack of a highly advanced culture system such as BACTEC (Becton Dickinson, USA) described as the gold standard for MAP culture.^{17, 18} Additionally, limited resources and equipment hindered the progress of the study. Consequently, we had to use the PCR method which is nonetheless a precise and widely-used method for detecting MAP.

Conclusion

Contrary to the role of multiple factors in IBD progression, the results of the present study indicated that transmission of mycobacterium from animal products to humans was the most important source of exposure. There is increasing evidence that IBD may have an infectious etiology for which MAP is the most plausible candidate. It can undermine the efforts by health and medical organizations in reducing MAP transmission to humans by improving health control systems at livestock farms and industrial dairy plants. Our results confirmed the presence of MAP in most people in the south of Iran, particularly in those with CD. Additionally, we established that there was a correlation between MAP and IBD. However, we could not claim that MAP was a trigger factor for IBD. It is recommended to conduct further research on people from different regions in Iran to ascertain the role of MAP in IBD.

Conflict of Interest: None declared.

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