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Original Article

Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Iranian patients with type 1 diabetes mellitus by PCR and ELISA

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Abstract

Introduction: *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis or Johne's disease in ruminants. Its role in triggering autoimmunity, including type 1 diabetes mellitus (T1DM), has been reported in recent years. Due to the high contamination rate of MAP in Iran's livestock and the increasing outbreak of T1DM, we investigated this association in a small group of patients with T1DM in Iran.

Methodology: Blood samples of 29 T1DM patients and 29 healthy control subjects were tested through enzyme-linked immunosorbent assay (ELISA) to detect antibodies against MAP3865c and ZnT8 homologous epitopes and the presence of MAP DNA. Blood samples were also cultured in mycobacterial growth indicator tubes and Herrold's egg yolk medium containing mycobactin J.

Results: The results of ELISA showed a significant difference between T1DM patients and healthy group. IS900 was also detected in 51.72% of T1DM patients but in none of the control group. None of the samples grew in culture media.

Conclusions: Due to the presence of MAP DNA and antibodies against MAP peptides in a significant number of T1DM patients compared with healthy control subjects, we may consider MAP as a possible trigger of T1DM in Iran. This indicates that exposure to MAP occurred in the positive subjects. Identifying the sources of contamination and routes of MAP transmission to humans seems necessary to prevent and reduce the burden of MAP infection in Iran.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*; T1DM; ELISA; IS900; PCR.

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Introduction

Type 1 diabetes mellitus (T1DM) is a metabolic disorder caused by autoimmune destruction of insulin-producing pancreatic β-cells [1]. Multifactorial etiology of the disease cannot be explained by genetic predisposition alone. There is evidence showing only 30% concordance for T1DM in monozygotic twins with genetic susceptibilities [2]. Several environmental factors that might trigger T1DM have been reported; these include viruses, bacteria, and dietary factors such as wheat proteins, cow's milk, coffee and tea, and toxins [3-5].

While T1DM has increased worldwide, the role of environmental factors in triggering T1DM seems necessary to prevent or delay the disease. Recent studies have suggested that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is associated with T1DM [6-8].

MAP is the causative agent of Johne's disease or paratuberculosis in ruminants [9]. Infected animals shed MAP through their milk and feces. These organisms can survive for extended periods of time in soil and water and can infect other animals. MAP is also resistant to chlorination of water and milk pasteurization [10]; therefore, it can be transmitted to humans through contaminated drinking water, milk, or even meat [11]. In fact, different studies in Iran have shown a high prevalence of MAP in animal feces and raw milk [12-17]. Contamination of bulk-tank milk in some areas of Iran has also been reported [13]. These

data may indicate that Iranian people are at risk of exposure to MAP.

Although it has not been proven that MAP infection is symptomatic in humans, the association of MAP with Crohn's disease has been extensively reported [18]. In addition, MAP has been suggested as a possible cause of T1DM. The association of T1DM and MAP has been observed in the Sardinian population (Italy) [19,20], but a recent study could not replicate this finding in India, probably due to the small number of investigated samples [21].

The present study was conducted to find a possible association between MAP and T1DM in Iran by bacterial culture, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA).

Methodology

Clinical samples

Twenty-nine T1DM patients (mean age: 17.2 years) (Table 1) and 29 healthy controls (mean age: 16.3 years) were investigated. Before blood sampling, written informed consent was obtained from all subjects or their parents. The study was approved by the ethics committee of Tehran University of Medical Sciences, Iran.

Blood samples of 6 mL were collected from patients and healthy controls; 4 mL of the blood was transferred into sodium heparinized tubes, and 2 mL was transferred into clot tubes.

Clot tubes were centrifuged for 10 minutes at 400×g (2,000 rpm) to separate the supernatant (serum) for further ELISA. Serum was aliquoted in microtubes and kept at -20°C for short-term storage (< 6 months) or at -80°C for long-term storage (> 6 months).

Heparinized tubes were centrifuged at 400×g (2,000 rpm) for 20 minutes, and the white ring formed containing peripheral blood mononuclear cells (PBMCs) was collected. The PBMCs were washed with sterile phosphate-buffered saline (PBS) twice and then used for culturing. The remaining PBMC fraction was stored at -20°C for DNA extraction.

Table 1. Presence of *Mycobacterium avium* subsp. *paratuberculosis* DNA and antibodies against MAP3865c and ZnT8 in type 1 diabetes mellitus patients.

Patient number	Gender	Age (years)	Duration of the disease (years)	Antibodies against MAP3865c	Antibodies against ZnT8	IS900 PCR
1	F	22	11	-	-	+
2	F	9	4	+	+	-
3	M	9	1	-	-	+
4	F	25	1	-	-	+
5	M	30	15	+	+	-
6	F	18	2	-	-	+
7	F	16	2	-	-	-
8	F	12	2	+	+	+
9	F	14	4	+	-	-
10	F	14	3	-	-	+
11	F	18	1	-	-	-
12	M	10	5	+	+	-
13	F	23	6	-	-	-
14	M	20	7	+	+	+
15	M	8	4	-	-	-
16	M	9	8	+	+	+
17	M	18	10	+	+	-
18	F	20	13	-	-	-
19	F	12	5	-	-	-
20	F	17	2	-	-	+
21	F	29	14	+	+	-
22	F	20	10	-	-	-
23	F	18	3	-	-	+
24	F	18	4	-	-	+
25	F	12	7	-	-	+
26	F	29	1	-	-	+
27	F	27	11	-	-	+
28	F	10	1	-	-	-
29	F	12	2	-	-	+
Total				**9**	**8**	**15**

DNA extraction

The tissue biopsies protocol of the RTP Mycobacteria Kit (Invitek, Berlin, Germany) was used to extract DNA from PBMCs. DNA was stored at -20°C.

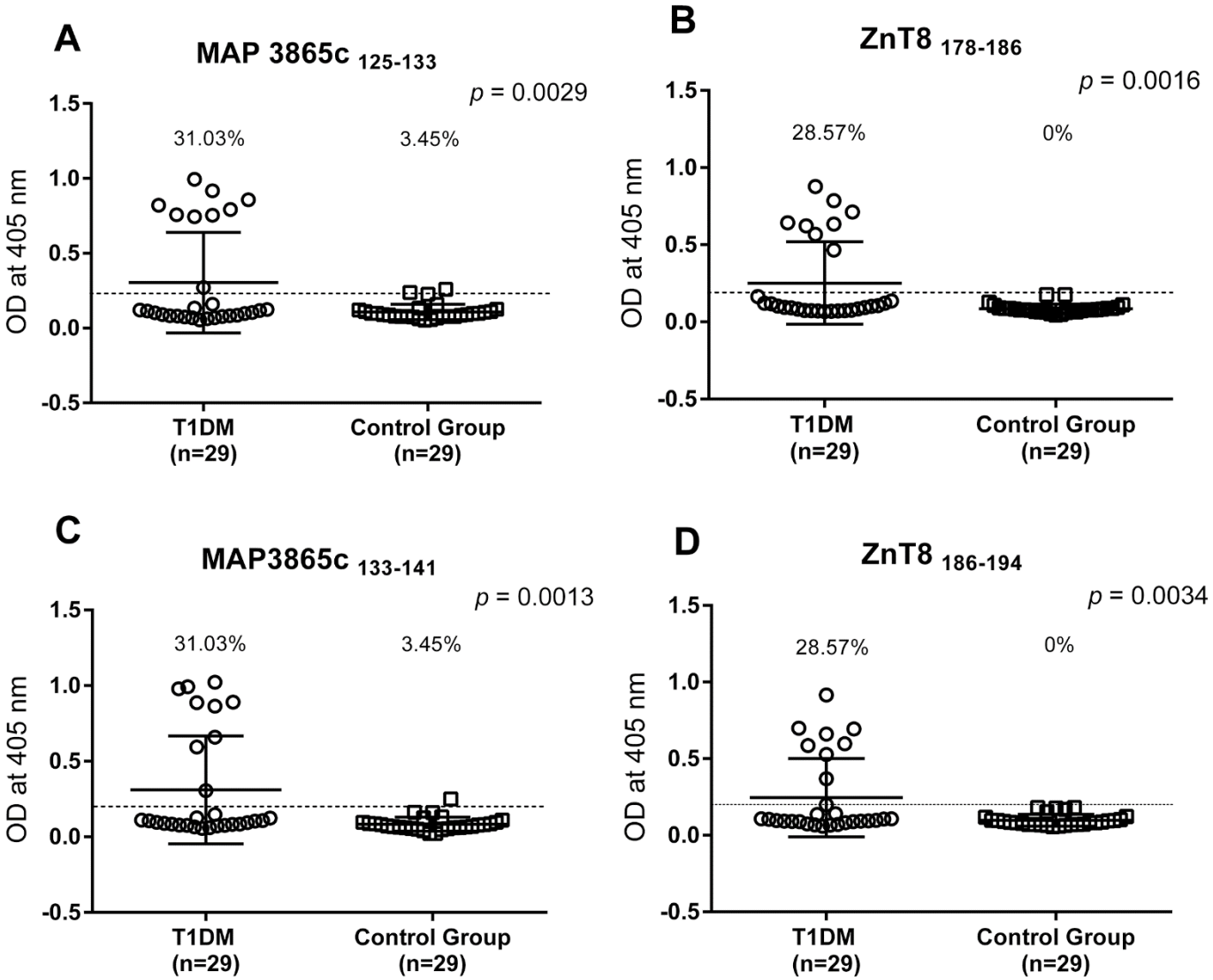
IS900 amplification and sequencing

Nested PCR was used to amplify IS900 [L/AV] as previously described [22]. The PCR products were sequenced to confirm the identity of amplicons.

Culture of samples

The PBMCs of all the samples were cultured in Herrold's egg yolk medium (HEY) (Razi Vaccine and Serum Research Institute, Karaj, Iran) containing mycobactin J (Razi Vaccine and Serum Research Institute, Karaj, Iran) and HEY without mycobactin J. In addition, the PBMCs were inoculated into mycobacterial growth indicator tubes (MGIT) (Becton-Dickinson, Oxford, England), enriched with mycobactin J. All cultures were incubated at 37°C for about six months.

Figure 1. Prevalence of Abs (in optical density [OD]) against MAP3865c and its human homologous peptides ZnT8 in type 1 diabetes mellitus (T1DM) patients and healthy control subjects.



Rate of Abs against MAP3865c $_{125-133}$ (A) and its homologous, ZnT8 $_{178-186}$ (B); and MAP3865c $_{133-141}$ (C), and its homologous ZnT8 $_{186-194}$ (D) in T1DM patients and healthy control subjects. The dotted lines indicate the cut-off for positivity used in each assay. Percent of antibody positivity is indicated on the top of each distribution.

ELISA

Peptides MAP3865c$_{125–133}$ (MIAVALAGL) and MAP3865c$_{133–141}$ (LAANFVVAL) along with their respective homologous peptides ZnT8$_{178–186}$ (MIIVSSCAV) and ZnT8$_{186–194}$ (VAANIVLTV) were synthesized at > 85% purity (GL Biochem, Shanghai, China). The peptides were re-suspended in 10 mM of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Darmstadt, Germany), aliquoted into sterile vials, and stored at -20°C until use.

An indirect ELISA was applied to detect antibodies against peptides. The procedure was performed as previously described [19]. The optical density (OD) was read at a 405 nm wavelength using VersaTunable MAX microplate reader (Molecular Devices, Sunnyvale, USA).

Statistical analysis

GraphPad Prism version 6 (www.graphpad.com) was used for analysis of the data, determination of positive subject, and calculation of p values. The cut-off for positivity in each assay was calculated by (mean value + 3 × standard deviation + 10%). The associations of the values were evaluated by Fisher's exact tests (2 × 2 tables).

Results

ELISA

Anti-MAP antibodies were detected in 31% of T1DM samples (Table 1), but only one of the control subjects (3.45%) was positive ($p = 0.0119$). The humoral response against both bacterial (MAP3865c) and human homologs (ZnT8) resulted positive by ELISA (Figure 1).

IS900 PCR

A total of 15 out of 29 T1DM patients (51.72%) were positive for IS900 (Table 1), whereas all samples in the control group were negative for MAP DNA. This positivity among T1DM patients ($p < 0.0001$) was extremely statistically significant, suggesting the factitive role of MAP in T1DM autoimmunity.

Following six months of incubation, none of the samples became positive for MAP in culture media.

No specific association was found between the results of ELISA and PCR, although the percentage of positive cases was higher when evaluated by PCR than by ELISA. No correlation was found between the age at onset of T1DM, duration of the disease, and the presence of MAP.

Discussion

MAP DNA was detected in a significant number of T1DM patients compared with subjects in the control group. However, the anti-MAP humoral response did not match with the presence of DNA, but this fact does not exclude the association between MAP and T1DM. DNA-positive patients might have developed antibodies against other antigens of MAP, so a larger survey on different MAP antigens is probably needed to see the exact humoral response in these subjects. We can also hypothesize that antibodies against MAP might be present in the gut, which is the localization of the bacterium, whereas DNA could be present in blood owing to circulating MAP-infected monocytes or released from dead mycobacteria. As there are studies that show pancreatic lymph nodes highly connected with gut lymph nodes [23,24], we may speculate that the presence of MAP antibodies is restricted locally and that they may be present in stool samples.

The negative result of bacterial culture is probably due to the presence of cell-wall-modified bacteria in blood, which are extremely difficult to reconvert into a viable status [18,22]. In addition, we know that MAP is a slowly growing mycobacterium with the slowest growth rate among *Mycobacterium avium* complex (MAC), and therefore the chance of its isolation is low, especially from blood. Nested PCR permitted detection of DNA with high sensitivity, giving a specific product. We could amplify IS900, a unique insertion sequence in MAP genome, with 14–18 copy repeats [25]. Products of the positive samples were subjected to sequencing and further confirmed the presence of MAP.

Since specificity of the bacterial peptides used in our ELISA system reached 100% to MAP, we can assert with confidence that ELISA-positive subjects were exposed to MAP and not to other environmental mycobacteria. Moreover, positive response to both MAP and pancreatic peptides confirm the sequence homology of ZnT8 and MAP3865c. This similarity may cause autoimmunity by molecular mimicry between MAP antigens and pancreatic islet β-cells, and trigger islet cell depletion. These results are in line with previous studies performed in Sardinia [19,26].

The association of MAP with T1DM has been reported in Sardinia, known to have a high prevalence of type 1 diabetes and a high rate of MAP infection among sheep and cattle [6-8,19,27]. However, Rani *et al.* could not find any association despite the high prevalence of MAP and T1DM in India, probably due to the low number of patients investigated [21] or the genetic predisposition of the infected population in developing such an autoimmunity. Moreover,

Paccagnini *et al*. reported the association of the SLC11A1 gene in the Sardinian population either with T1DM or susceptibility to mycobacterial infection [20].

In this study, we showed the association of MAP and T1DM in Iran that may result from genetic predispositions in the Iranian population. Additional studies on the genetic background of patients are needed to confirm our hypothesis. With respect to the high contamination rate of MAP in Iran livestock, a wide investigation on possible contaminated foods such as milk, cheese, meat, or even water is necessary. If the role of MAP in T1DM is confirmed, prevention of the disease in genetically susceptible individuals would be possible by finding and eliminating the sources of MAP contamination.

Conclusions

We report, for the first time, a significant association of positivity to antibodies against MAP and a consistent presence of MAP DNA with T1DM in a small group of Iranian patients. A larger study in different geographical areas of Iran and with a larger sample size is needed to confirm our findings and to take steps to reduce MAP transmission to humans.

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