Detection of *Mycobacterium avium* subsp. *paratuberculosis* DNA isolated from archival formalin-fixed, paraffin-embedded tissue of Crohn's disease patients

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ABSTRACT

The objective of our study was to examine the archival formalin-fixed, paraffin-embedded tissues of Slovenian patients with Crohn's disease (CD) for the presence of *Mycobacterium avium* subsp. *paratuberculosis* (Map) by IS900 PCR. One hundred and sixty-four intestinal tissue specimens, taken from 33 CD patients, and 39 control specimens, taken from individuals with intestinal cancer, were processed. After the paraffin removal, a commercially available kit was used to extract DNA. For the detection of Map, specific primers were used to amplify a 298 bp fragment of IS900. Map was found in six of 164 (4%) specimens obtained from four patients. Three specimens belonged to a single patient and the other three to the remaining patients. In total, four out of 33 (12%) CD patients had Map DNA detected in their tissues. All of the control samples tested negative. With the method described in this study we could detect the IS900 sequence of Map in the formalin-fixed and paraffin-embedded tissues of CD patients. Our findings contribute to the current knowledge about Map being a possible etiological agent of CD in Slovenian patients.

INTRODUCTION

Crohn’s disease (CD) is a chronic, transmural inflammatory bowel disease of unknown etiology. Current theories implicate the role of genetic, microbial, immunologic, environmental, dietary, vascular, and even psychosocial factors as potential causative agents. It has been suggested that patients have an inherited susceptibility for an aberrant immunologic response to one or more of these provoking factors. The leading infectious candidate is *Mycobacterium avium* subsp. *paratuberculosis* (Map), in part because it causes a very similar disease, Johne’s disease, in animals, including primates (Chacon et al., 2004; Shanahan, 2002).

For the detection of Map in CD patients, various specimens including fresh or formalin-fixed intestinal mucosa obtained by biopsy or surgical resection, blood etc. have been analyzed using different techniques, e.g. PCR, *in situ* hybridization, cultivation of Map and cultivation of Map followed by PCR (Bull et al., 2003; Feller et al., 2007; Romero et al., 2005; Ryan et al., 2002; Schwartz et al., 2000). Different detection methods and diverse clinical material may be one of the reasons for conflicting results about the detection rate, ranging from 0 to 100% (Feller et al., 2007; Quirke, 2001). Considering solely the results obtained from archival, formalin-fixed and paraffin-embedded tissue, similar incongruence is observed (Baksh et al., 2004; Cheng et al., 2005; Ryan et al., 2002).

The present preliminary study was carried out to determine whether Map DNA, as an indicator of formerly present live bacteria, could be detected in the archival formalin-fixed, paraffin-embedded gut tissues of Slovenian patients with CD by IS900 PCR.

MATERIALS AND METHODS

*Patients and specimens.* A total of 203 formalin-fixed, paraffin-embedded archival specimens obtained from the Institute of Pathology, Faculty of Medicine, Ljubljana were included in the study. Among them, 164 were taken from 33 CD patients and 39 from patients with colorectal carcinoma without inflammatory bowel disease (IBD). The latter represented a control group. All 33 CD patients had an established diagnosis of CD based on clinical presentation,
endoscopic criteria and histopathology findings. Most patients had a history of long-standing CD. Patients underwent surgery for different reasons, usually stenosis. The disease was localized in the ileum in three patients (9%), in terminal ileum in 15 (45%), in cecum in two (6%), in ascending colon in five (15%), in transverse colon in two (6%), in descending colon in two (6%) and in rectosigmoid in four (12%) patients. In the control group, tumour was localized in cecum in three patients (8%), in ascending colon in one (3%), in transverse colon in 10 (26%), in descending colon in 2 (5%), in sigmoid colon in seven (18%) and in rectum in 16 (41%).

Specimens of the individual patients which showed the most pronounced lesions (transmural infiltration, cryptitis, crypt abscesses, epitheloid granulomas) were selected for further analysis. All specimens were stained according to Kreyberg trichrome method and histologically examined.

**DNA extraction.** Formalin-fixed, paraffin-embedded specimens were one to 19 years old. Five 6-µm sections were cut from each tissue block. The microtome was cleaned with xylene (Merck) and a new knife was used for each tissue block. All five sections were transferred into a 1.5-ml tube. Tubes were coded and processed blind. A total of 1200 µl of xylene was added to each tube and vortexed vigorously to remove the paraffin. After centrifugation at full speed for 5 min at room temperature, the supernatant was removed by pipetting. 1200 µl of absolute ethanol was added to the pellet to remove the residual xylene, mixed gently by vortexing and removed by careful pipetting after centrifugation at full speed for 5 min. Ethanol washing of the pellet was repeated once again. The tissue was then air-dried. DNA was extracted from all samples using the commercially available High Pure PCR Template Preparation Kit according to the manufacturer’s instructions (Roche Diagnostics).

**Detection of Map-specific IS900.** For the PCR assay, IS900-specific primers described previously, namely Av1 (5'-ATG TGG TGT TGT TGG ATG G-3') and Av2 (5'-CCG CCG CAA TCA ACT CCA G-3'), were used to amplify a 298 bp fragment of the IS900 gene (3). A 50-µl PCR reaction mixture contained 1× Expand High Fidelity reaction buffer containing 1.5 mM MgCl₂, 10% dimethyl sulfoxide, 0.2 mM of each dNTP, 200 µM of each primer and 3.5 U of Expand High Fidelity Taq polymerase (Expand High Fidelity PCR System, Roche Diagnostics). The amplification conditions were as follows: 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 3 min, with a final extension at 72°C for 7 min. Amplification products were detected by electrophoresis in 1.5% agarose gels stained by ethidium bromide and visualized by UV transilluminator and camera (Bio Imaging System, Gene Genious, Syngene). Each batch was run with a process control, i.e. archival formalin-fixed, paraffin-embedded sections from cattle with Johne’s disease, and with a positive PCR control (DNA from Map strain ATCC 43015). Negative PCR samples were tested for inhibition by spiking 200 ng of DNA positive control to one of the specimens of each CD patient and control patient after getting negative PCR Map results. Sterile PCR-grade distilled water was used as negative PCR control.

**Statistical analysis.** The Statistical Package for the Social Sciences (SPSS), v.17.0.0. for Windows, was used for the statistical analysis (t-test). Statistical significance was accepted at p<0.05.

**RESULTS**

Out of 33 CD patients, 19 (58%) were female and 14 (42%) were male. Patients were 15 to 59 years old with the mean age being 34 years. Paraffin-embedded tissue sections were cut from 164 blocks, with two to 5 specimens belonging to a single patient, except for one patient where 25 specimens were processed. In the control group, there were 39 patients, 21 (54%) male and 18 (46%) female patients. Patients were between 41 to 89 years old with the mean age of 70 years. From each patient one formalin-fixed, paraffin-embedded specimen of intestinal resection margin of surgical resection was processed.
All controls were negative to Map by IS900 PCR (Fig. 1). Map was detected in six of 164 (4%) specimens obtained from four patients between 26 and 41 years old. Three specimens belonged to a single patient and the other three to the remaining three patients. In total, Map DNA was detected in the formalin-fixed, paraffin-embedded tissue from four of 33 (12%) CD patients. Four positive samples were obtained from terminal ileum, one from rectum and one from mesenteric lymph node taken from the ileal resection specimen. Statistical significance was observed when comparing IS900-specific DNA detection rate between CD and non-IBD group (p=0.022).

**DISCUSSION**

Extraction of nucleic acids from formalin-fixed, paraffin-embedded tissues enables different retrospective studies. The technique has been used to detect Map as a possible infectious agent in the pathogenesis of CD (Baksh et al., 2004; Cheng et al., 2005; Ryan et al., 2002).

In this study we were able to detect Map DNA in the formalin-fixed, paraffin-embedded intestine samples from Slovenian CD patients using IS900 PCR. Not all CD patients tested positive to Map, but no Map DNA was detected in control specimens. The detection of Map in archival formalin-fixed and paraffin-embedded tissue sections is especially challenging. The target DNA may be fragmented or cross-linked to proteins, which leads to poor or no PCR amplification. This may be the reason for some potentially false negative results in our study. Moreover, extraction of Map DNA may also fail due to very low Map concentrations in CD patients and due to strong, and even for mycobacteria unconventional cell wall (Hermon-Taylor, 2001). To overcome this limitation, more specimens of the same CD patient were analyzed.

Individuals without Map infection are 17-times less likely to have IBD than Map-infected individuals (Scanu et al., 2007), which is in accordance with our Map-negative non-IBD control group. Since this is a preliminary study, all IS900-positive specimens are still to be confirmed by sequencing.

In conclusion, our findings contribute to the current knowledge about Map being the infectious agent proposed to play the role in the etiology of CD, also in Slovenian CD patients.

**REFERENCES**


