MYCOBACTERIUM AVIUM SUBSP PARATUBERCULOSIS DETECTION FROM BLOOD OF CROHN'S DISEASE PATIENTS IN A PORTUGUESE HOSPITAL

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ABSTRACT
Mycobacterium avium subsp *paratuberculosis* (MAP) has been associated with Crohn's disease (CD). We performed MAP DNA detection in the blood of controls and CD patients followed at Hospital de São João, by IS*900*-based nested PCR. We found that 60% (6 out of 10) of CD patients were positive for MAP DNA, against 38% (8 out of 21) of control subjects. MAP detection in CD patients will allow us to correlate MAP infection with different pathological and immunological patterns of the disease.

KEYWORDS
Mycobacterium avium subsp *paratuberculosis*, nested PCR, Crohn's disease.

RESUMO
Mycobacterium avium subsp *paratuberculosis* (MAP) tem vindo a ser associada com a doença de Crohn (CD). Neste trabalho realizamos a detecção por nested PCR de DNA de MAP, no sangue de controlos e de doentes com CD seguidos no Hospital de São João. Detectámos seis (em 10) pacientes positivos para DNA de MAP (60%), e sete (em 20) controlos positivos (35%). A detecção de MAP em doentes com CD permitirá estudar possíveis correlações entre a infecção por MAP e diferentes características patológicas e imunológicas da CD.

PALAVRAS-CHAVE
Mycobacterium avium subsp *paratuberculosis*, PCR, doença de Crohn.

1. **INTRODUCTION**

Crohn's disease (CD) is an inflammatory bowel disease associated with considerable morbidity. It is estimated that in the western world the incidence is about 0,1-0,2% of the adult population (Casanova and Abel) and has been increasing in the last decades. In CD the inflammation may occur in any portion of the alimentary tract and affects all linings. On the other hand, in ulcerative colitis (UC), the other major form of inflammatory bowel disease (IBD), inflammation is restricted to the colon and only the mucosal layer is affected.

The ethiology of CD has remained elusive. As CD is a complex syndrome, it is possible that it may result from multiple genetic and environmental contributions. Numerous reports have shown an association between colonization by an atypical mycobacteria, *Mycobacterium avium* subsp *paratuberculosis* (MAP), and CD (Abubakar et al.; Feller et al.). MAP is the agent of Johne's disease, which affects ruminants and other mammals and shares some characteristics with CD (Glawischnig et al.; Judge et al.; McClure et al.).

Recent reports by others and by our lab have shown that MAP may be involved in the immunopathology of CD (Clancy et al.; Campos et al., Sibartie et al.). So, it is important to detect MAP positive patients, in order to be able to associate MAP colonization status and differencial immunopathological patterns of CD.

In this work, we studied MAP colonization in a group of patients being followed at Hospital São João. MAP DNA was detected in the peripheral blood of patients (and a control population) by IS*900*-based nested PCR. IS*900* is an insertion element unique to MAP and belongs to a family including other related insertion elements found in the order *Actinomycetales*, as IS*1613* and IS*1626* (Bull et al.). About 14-18 copies of IS*900* are present in MAP genome, which favours its detection.

2. **MATERIALS AND METHODS**

2.1. **PATIENTS AND CONTROLS**

In this study, 30 individuals were included: 20 healthy controls (mean age, 29,1 years; range, 18-62 years; 7 males, 13 females) and 10 with CD (mean age, 36,6 years; range, 20-64 years; 5 males, 5 females). All CD patients were on 5-aminosalicylate (5-ASA) therapy. Informed consent was obtained in accordance with the institutional review board regulations at IBMC, University Fernando Pessoa and Hospital de São João. Patients with CD were recruited from the Gastroenterology Department at Hospital de São João. CD diagnosis was based on standard clinical, endoscopic, histologic and radiographic criteria (Sands). Healthy controls were recruited from the academic community of the Health Sciences Faculty, University Fernando Pessoa.

2.2. **DNA EXTRACTION**

Blood from CD patients and controls was collected into a 4 ml EDTA-containing tube and the mononuclear cell layer was obtained by density gradient centrifugation using Histopaque 1077 (Sigma, St. Louis, MO, USA). The mononuclear cells were ressuspended in a mycobacterial lysis solution (2 mM sodium EDTA , 400 mM NaCl, 10 mM Tris HCl, 0,6% sodium dodecyl sulfate and 33 mg proteinase K) and DNA was extracted as described in (Bull et al.). Briefly,

samples in mycobacterial lysis solution were incubated at 37 ºC for 2h with shaking. Tubes were chilled on ice and samples mechanically disrupted by shaking in a Mini Beadbeater™ (Biospec Products, Bartlesville, OK, USA). Samples were treated sequentially with phenol, phenol-chlorophorm-isoamyl alcohol, and chlorophorm-isoamyl alcohol. The aqueous layer was then transferred to a new tube containing 10M ammonium acetate and mixed. One mililiter of absolute ethanol was added to allow DNA precipitation. Samples were then centrifuged at 10 000 g for 20 minutes, pellets were washed with ethanol 70%, incubated 30 minutes to dry, and then ressuspended in TE buffer (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8) and allowed to redissolve at 4 ºC overnight. The resulting DNA solution was then aliquoted and stored frozen at -20 ºC until assayed by PCR. All reagents were purchased from Sigma, St. Louis, MO, USA.

2.3. **IS900-BASED NESTED PCR**

A nested PCR was undertaken, with primers derived from the DNA insertion sequence IS*900*. The primary PCR round used primers L1 (5'-CTT TCT TGA AGG GTG TTC GG-3') and L2 (5'-ACG TGA CCT CGC CTC CAT-3'), which amplified a 398 bp fragment of the IS900 gene. In the second round, primers AV1 (5'-ATG TGG TTG CTG TGT TGG ATG G-3') and AV2 (5'-CCG CCG CAA TCA ACT CCA G-3') were used. These primers amplified a 298 bp internal nucleotide sequence from the first 398 bp amplicon. The PCR reaction mixture consisted of 5 µl of DNA sample extract in a final volume of 50 µl, containing reaction buffer with 1,5 µM $MgCl_2$ (Promega, Madison, WI, USA); 10% Dimethyl sulphoxide (Sigma); 2 µM of each primers L1 and L2; 200 µM each dATP, dCTP, dGTP, dTTP; and 3,5 U GoTaq Flexi DNA polymerase (Promega). The cycling conditions were: 1 cycle of 94 ºC for 5 minutes and then 30 cycles of 94 ºC for 1 minute, 58 ºC for 1 minute, 72 ºC for 3 minutes, followed by one cycle of 72 ºC for 7 minutes. To reduce the risk of amplicon contamination, products of the first PCR round were treated with 1 U uracyl-DNA glycosylase (Roche Applied Science, Penzberg, Germany) at room temperature for 10 minutes. For the nested PCR the mixture had the same ingredients as the primary reaction mixture, except that 5 µl of PCR product from the first round was used as a template, 2 µM of primers AV1 and AV2 were used instead of primers L1 and L2, and 400 µM dUTP was used in replacement of dTTP. The cycling conditions were: 1 cycle of 94 ºC for 5 minutes and then 40 cycles of 94 ºC for 1 minute, 58 ºC for 1 minute, 72 ºC for 3 minutes, followed by one cycle of 72 ºC for 7 minutes. Amplicons of the expected size were visualized with SYBR safe (Invitrogen, Carlsbad, CA, USA) on 3% agarose gels.

3. **RESULTS**

Samples positive for the IS900 showed a bright band of 298 bp size in 3% agarose gels. The results obtained are summarized in Table 1. MAP DNA was detected in 13 (43,3%) of a total of 30 subjects enrolled in this study; 6 (60%, n=10) patients with CD and 7 (35%, n=20) control subjects.

Group	number of subjects	PCR-positive	% PCR-positive
Controls	20	7	35,0%
CD patients	10	6	60,0%
Total	30	13	43,3%

TABLE 1 - Results of MAP DNA detection in CD patients and control subjects.

4. **DISCUSSION**

This is the first report of MAP detection in a Portuguese population. We found that MAP DNA was present in 6 out of 10 (60%) CD patients tested, which agrees with a previous report (Naser et al.). Other reports also show a higher incidence of MAP in CD patients, but MAP detection was performed in intestinal biopsies (Bull et al.; Sechi et al.). However, as this work is under progress, samples collected until the present date (particularly from CD patients) are still low, and results must be analysed with caution. Furthermore, the presence of MAP DNA may not reflect an infectious status, because we cannot conclude whether live MAP is present. Cultures of the isolated mononuclear cells in MGIT medium (Mycobacterial Growth Indicator Tube) are being undertaken, and will provide additional information regarding the presence of live mycobacteria in blood.

Sequencing of the 298 bp amplicon detected in agarose gels also needs to be performed. In fact, although insertion sequence IS*900* is, to date, unique to MAP, other IS*900*-like elements may be present in rarely encountered environmental mycobacteria, and may give false positive results. Sequencing analysis is important to readily distinguish between IS900 from MAP and other IS900 related elements.

MAP detection in CD patients will allow us to find putative correlations between MAP infection and different pathological or immunological patterns of the disease. As MAP is a modifying factor in CD (Sibartie et al.; Campos), it is possible that it may be associated with immune homeostasis dysfunction in this disorder.

In summary, our results agree with previous reports, but need to be further confirmed by PCR product sequencing and by culture in MGIT medium. A better knowledge of the association between MAP infection and loss of immune homeostasis will certainly result in future new and improved therapies.

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