Paucity of mycobacteria in mucosal bowel biopsies from adults and children with early inflammatory bowel disease

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Abstract

Background: The presence of Mycobacterium avium subspecies paratuberculosis (MAP) has previously been inferred in the genesis of Crohn's disease (CD), and a higher incidence of MAP PCR positivity has been demonstrated in the gut and peripheral blood of CD patients than in healthy individuals. The objective of this prospective study was to assess the potential etiological role of MAP in the pathogenesis of CD.

Methods: The presence of mycobacteria was assessed in bowel biopsies from newly diagnosed, treatment naïve Norwegian patients with IBD, including CD and ulcerative colitis (UC), as compared to a hospital-based cohort of CD and UC patients. Biopsies were collected from the small and large bowel in 354 individuals with suspected IBD. Detection of mycobacteria was performed by long-term cultivation in combination with direct detection by MAP IS900-specific PCR.

Results: Among the specimens included from the patients with early IBD, samples from only two of the patients with CD (2.7%) and two of the non-IBD controls (1.5%) exhibited a positive growth...
Inflammatory bowel disease (IBD) refers to ulcerative colitis (UC) and Crohn's disease (CD), which are two different clinical phenotypes, both considered to be a result of inappropriate chronic activation of the immune system. CD was first described in 1913 and recognized as a clinical entity in 1932. It has been hypothesized that CD is a multifactorial disease, elicited by a combination of genetic predisposition and immunologic disturbances, however, environmental factors, such as infections, have been suggested to unleash this disease. In particular, Mycobacterium avium subspecies paratuberculosis (MAP) has been detected in association to CD in several studies. In addition, selected bacterial species have been suggested to play a role in IBD pathogenesis, including Salmonella, Shigella, Escherichia coli, Yersinia and some commensal microbiota.

Over the years, clinical studies have investigated various gut tissues, including mucosa, Peyer plaques, lymph nodes and per-operative specimens, as well as blood and serum, for the presence of bacteria or antibacterial antibodies. Many of these reports have shown a correlation between the presence of MAP and CD, while other studies have not been able to reproduce these findings. A higher incidence of viable MAP or presence of MAP DNA in patients with CD than in controls has been detected by using bacterial culture or polymerase chain reaction (PCR) analysis on biopsy tissue, resected tissues or peripheral blood.

The potential association between CD and the presence of MAP is still a topic of controversy. In this context, several questions have arisen on the relationship between CD and MAP. Is MAP the causative agent of, or only a factor contributing to, CD? Is MAP merely a commensal due to secondary colonization in a CD-compromised host? Addressing IBD patients in CD and non-CD categories, approximately 80% and 34–50% MAP positivity rates were found for intestinal and blood specimens in CD, respectively, and 0–22% positive rates in non-CD IBD specimens. Recently, a paper describing the finding of MAP IS900 positivity in gut tissue and peripheral blood mononuclear cells from paediatric IBD patients prior to treatment was published, showing a significantly higher identification of MAP in CD patients compared to non-IBD control patients. Another recent study where whole blood of adult CD patients and healthy controls was tested for the presence of MAP by culture and IS900-specific PCR, however, did not recover MAP.

In order to delineate the potential role of MAP in CD versus other forms of IBD, numerous studies have addressed this topic by subjecting bowel biopsies to analysis by culture and PCR. While many reports describe high rates of MAP in CD compared to UC patients and healthy controls, this correlation was not found in other studies.

The aim of the present investigation was to study the presence of MAP in bowel biopsies from a new population-based cohort of treatment naïve patients with IBD in South-Eastern Norway, included in the Inflammatory Bowel South-Eastern Norway (IBSEN) II study, with the aim to define etiologic factors leading to IBD. We extended our investigation by asking the question if there is an increased prevalence of mycobacteria present in bowel biopsies from patients with IBD.

Conclusions: These findings demonstrate the paucity of MAP in the gut of treatment naïve CD patients. This study does not provide evidence for a role of MAP in early IBD.

1. Introduction

2. Materials and methods

2.1. Patients

Patients were recruited from the prospective IBSEN II study (2005–2007) investigating genetic, immunologic and environmental factors that contribute to IBD etiology. Inclusion of the 321 individuals (221 adults and 100 children <18 years) in the study was based upon a history of abdominal symptoms, including diarrhea and/or blood in faeces for more than 10 days and typical endoscopic and histologic findings. Patients with infection, other kinds of chronic inflammation or cancer were excluded. Individuals without pathologic endoscopy or histology were defined as non-IBD controls. Detailed phenotypic data were available on members of this cohort through the IBSEN II study. We calculated the disease activity of the CD patients using the Harvey Bradshaw index with a median score of 4 (range 0 to 29). Forty-five (20.4%) out of the 221 study subjects had received some form of antibiotic treatment within the last 12 months prior to inclusion in the study. In the different subgroups, the percentage of the subjects that had received antibiotics were as follows: CD 27.8%, UC 16.4%, IBDU 33.3%, possible IBD 20.0% and non-IBD 18.6%. Among the 45 subjects, fifteen patients received antibiotics up to four weeks prior to inclusion in the study (two metronidazole, one metronidazole and ciprofloxacin in combination, and one ciprofloxacin alone). Nine of the patients received antibiotics between four and eight weeks (one metronidazole) prior to inclusion and 17 patients received antibiotics more than eight weeks prior to inclusion. For four of the patients, the time course was not recorded. A majority of these 45 patients received antibiotics in the form of penicillins or macrolids due to upper airway infections. As a reference material, we used a cohort of patients with long-standing IBD.
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and non-IBD controls \(n=33\) at the Oslo University Hospital (Rikshospitalet) (OUH/RH), a third line referral center. In addition to the main study, a cohort of patients with primary sclerosing cholangitis and colitis \(n=195\) at OUH/RH, were included.

2.2. Clinical specimens

Colonoscopy was performed and mucosal biopsies were retrieved from the terminal ileum and five colonic segments (coecum, ascending colon, descending colon, sigmoid colon and rectum). Regarding the CD patients, biopsies were taken from inflamed, adjacent and non-inflamed areas. The biopsies were taken with biopsy forceps used for routine biopsies, transferred to tubes containing a sterile saline solution, kept at room temperature and sent immediately to the mycobacterial laboratory at the OUH/RH. Transport times ranged from 1 to 8 h, with a median of 3.5 h.

2.3. Processing of bowel biopsies, growth conditions and conventional characterization

Each bowel biopsy was resuspended in 1 ml of sterile phosphate buffer saline, pH 6.8. Aliquots of the 0.5 ml suspension of each colon biopsy, which were NALC decontaminated and used to inoculate one MGIT vial (Becton, Dickinson and Company, Franklin Lake, NJ, US), each containing 7 ml of modified Middlebrook 7H9 broth base medium (Becton, Dickinson and Company) enriched with 0.5 ml fastidious organism supplement (FOS) (Becton, Dickinson and Company) which contains iron and 10 μl mycobactin J (Caltech, Pasadena, CA, US) supplement to an end concentration of 2 mg/ml. All inoculated culture media were incubated at 37 °C in the BACTEC MGIT 960 semi-automated system for at least 52 weeks. The MGIT vials were continuously assessed by the MGIT fluorescence-quenching activity, but also submitted to manual monitoring. MGIT vials with positive signal, and all MGIT cultures incubated for more than 52 weeks, were assessed for the presence of MAP by auramine staining and nested PCR as described below. The positive control material for the cultivation of MAP from bowel biopsies, were mucosal bowel biopsies from MAP-infected goats. One heavily infected goat with pluribacillary disease and one goat with paucibacillary disease, were included. Negative controls were colon biopsies from healthy goats and human controls.

2.4. DNA extraction

All biopsy specimens in the MGIT vials were investigated for the presence of mycobacteria by IS900-specific nested PCR after prolonged cultivation using enriched media in the BACTEC MGIT 960 continuous culture system. Specimens yielding a positive reaction in the BACTEC MGIT 960 semi-automated vial monitoring were assessed with repeated IS900 PCR testing. From the cultures that yielded a positive MGIT signal, a volume of 0.5 ml was subjected to heat inactivation at 98 °C for 10 min. The tubes were then centrifuged at 12,000 rpm (18,500 g) for 10 min, the supernatant removed and the pellet suspended in 100 μl of sterile Tris EDTA buffer (pH=7.5). The resuspended pellet was used for the extraction of genomic DNA.

2.5. PCR analysis

The pelleted biopsy material was resuspended in 100 μl sterile Tris EDTA buffer (pH=7.5), incubated in a dry heat bath at 98 °C for 20 min, and then centrifuged at 12,000 rpm (18,500 g) for 10 min. Nucleic acids were precipitated, washed, dried, and dissolved in 50 μl of sterile water. For detection of MAP-specific DNA, a nested PCR test targeting the DNA insertion sequence IS900 unique to MAP, was used. The primers used in the first round of the PCR in order to amplify a unique 398 bp fragment of the IS900 gene were P90 (5′-GTTCGAGCGTCCGTAGGG-3′) and P91 (5′-GAGGTCAGTGCCCACGTA-3′). The PCR mixture comprised of 5 μl of DNA sample extract in a final volume of 50 μl using PCR buffer, containing 2 μM each of the primers, 2.5 mM MgCl2, 0.2 mM dNTPs, and 1 U Taq polymerase (New England Biolabs, US). The PCR cycling conditions were: 95 °C for 5 min, 34 cycles of 95 °C for 1 min, 58 °C for 1.5 min, 72 °C for 1.5 min, and a final extension of 10 min at 72 °C. For the nested PCR, 5 μl of PCR product from the first round was used as a template and added to 50 μl of the reaction mixture using primers AV1 (5′-TTCGGTGTGCTGTGTT-3′) and AV2 (5′-CGCCGCAATGCAAATCAG-3′) in the second round of PCR. The amplification product size of 298 bp was assessed by 1% agarose gel electrophoresis. The sensitivity of the PCR reaction was estimated to be 10–20 IS900 copies. Results from the IS900-specific PCR were confirmed by a second real-time PCR method, the Artus RealArt™ Mycobact. Diff kit (Qiagen Inc, Hamburg, Germany). The positive control material in the PCR analysis were mucosal bowel biopsies from MAP-infected goats, as described above, as well as dilutions of DNA from the MAP strain ATCC 43015, Linda. Negative controls were DNA from colon biopsies from healthy goats and human controls, Mycobacterium bovis BCG and humans. IS900 PCR positive samples were subject to molecular subtyping by IS900 and IS1245 restriction fragment length polymorphism and confirmed by multiplexer sequence typing.

2.6. Ethical considerations

All patients provided informed written consent to be involved in this study and ethical approval for the study was given by the Regional Ethics Committee in Oslo, Norway (http://www.etikkom.no/REK/regionSorOst, reference numbers S-03232 and S-04209).

3. Results

3.1. MAP in Early IBD

Patients were grouped according to IBD status: Crohn’s disease (CD), ulcerative colitis (UC), IBD unclassified (IBDU), possible IBD or non-IBD (Table 1). Both bowel biopsies and cultures in the patient materials were subjected to MAP-specific PCR analysis by using the IS900 as a target for amplification. Among the specimens from the 321 individuals
positive individual was identified as *Mycobacterium avium* subsp. *hominissuis* (code 2).\(^2,3\) Samples from two goats known to have paratuberculosis were positive by both PCR for IS900 and for culture of MAP organisms. Viable replicating mycobacteria were not detected in biopsies from any other patients or healthy controls, indicating that MAP is not associated with early IBD in the specimens from this Norwegian population.

### 3.2. MAP in long-standing IBD

In comparison, bowel biopsies from a non-selected patient material with established IBD, both CD and UC, as well as non-IBD controls, from the Endoscopic unit at OUH/RH (Table 3) exhibited a higher prevalence of MGIT growth signal positivity. In this material, we recorded a MGIT positivity of 100% for Crohn’s patients, 57% for patients with ulcerative colitis and 32% for non-IBD controls, respectively. Among the IBD patients, two of the patients with CD (28.6%) and one of the patients with UC (14.3%) were also IS900 PCR positive. Thereby, a link between MGIT and IS900 positivity and CD was detected in the patients with longer-standing IBD. In another patient cohort consisting of 195 patients with primary sclerosing cholangitis (PSC) and colitis, with a median duration of IBD of more than 10 years, only a single MGIT positive growth specimen was detected, which was IS900 PCR negative (data not shown).

### 4. Discussion

In order to elucidate the potential role of MAP in CD and in IBD in general, we compared the presence of MAP in bowel biopsies from the population based IBSEN II study with the reference cohort of IBD patients. In addition to the detection of MAP by PCR, our aim was to define the incidence of growth of mycobacteria in general in gut biopsies from IBD patients, including both CD and UC as well as non-IBD controls. Altogether, 21 out of the 354 individuals exhibited a positive MGIT growth signal, while specimens from only four out of these patients (two CD patients, one UC patient and one non-IBD control) were IS900 PCR positive. The only mycobacterial isolate cultivated in this study was, however,

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient categories included in the IBSEN II study, 321 individuals (221 adults and 100 children &lt;18 years).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical diagnosis</td>
<td>Adults</td>
</tr>
<tr>
<td>CD</td>
<td>36</td>
</tr>
<tr>
<td>UC</td>
<td>61</td>
</tr>
<tr>
<td>IBDU</td>
<td>12</td>
</tr>
<tr>
<td>Possible IBD</td>
<td>15</td>
</tr>
<tr>
<td>Non-IBD</td>
<td>97</td>
</tr>
<tr>
<td>Total</td>
<td>221</td>
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</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Outcome of monitoring bowel biopsies from the IBSEN II study for the presence of a positive MGIT signal, mycobacteria by cultivation and nested IS900-specific PCR.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient category/clinical diagnosis</td>
<td>No. of individuals</td>
</tr>
<tr>
<td>Positive tissue control</td>
<td>2/2</td>
</tr>
<tr>
<td>Colon biopsies from 2 MAP-infected goats (^b)</td>
<td>0/10</td>
</tr>
<tr>
<td>Negative tissue control</td>
<td>NA (^c)</td>
</tr>
<tr>
<td>Colon biopsies from 5 non-infected goats and 5 human control individuals</td>
<td>1(^d)</td>
</tr>
<tr>
<td>Positive PCR control</td>
<td>NA</td>
</tr>
<tr>
<td>ATCC 43015 Linda DNA</td>
<td>–</td>
</tr>
<tr>
<td>Negative PCR control</td>
<td>–</td>
</tr>
<tr>
<td>Mycobacterium avium subsp. hominissuis</td>
<td>–</td>
</tr>
<tr>
<td>BCG DNA</td>
<td>–</td>
</tr>
<tr>
<td>CD</td>
<td>2/75 (2.7%)</td>
</tr>
<tr>
<td>UC</td>
<td>0/80 (0%)</td>
</tr>
<tr>
<td>IBDU</td>
<td>0/16 (0%)</td>
</tr>
<tr>
<td>Possible IBD</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td>Non-IBD</td>
<td>2/135 (1.5%)</td>
</tr>
<tr>
<td>Total</td>
<td>4/321 (1.2%)</td>
</tr>
</tbody>
</table>

\(^a\) All specimens subjected to IS900-specific PCR were also subjected to a second *M. avium*-specific real-time PCR test, the Artus RealArt™ Mycobact. Diff kit (Qiagen Inc, Hamburg, Germany), yielding the same results.

\(^b\) One goat with pluribacillary disease and one with paucibacillary disease were included.

\(^c\) NA: Not applicable.

\(^d\) Culture and PCR-positive isolate identified as *M. avium hominissuis* (code 2).\(^2,3\) Strain isolated from healthy female control patient aged 22 (out of six biopsies from the ileum through the colon, growth of MAC/MAP in this patient was found only in two biopsies, from the sigmoid colon and from the rectum).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Outcome of monitoring bowel biopsies from a non-selected patient material in a referral hospital with long-standing IBD and non-IBD controls for the presence of a positive MGIT signal, mycobacteria by cultivation and nested IS900-specific PCR.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient category/clinical diagnosis</td>
<td>No. of individuals</td>
</tr>
<tr>
<td>CD (^a)</td>
<td>7</td>
</tr>
<tr>
<td>UC</td>
<td>7</td>
</tr>
<tr>
<td>Non-IBD</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
</tr>
</tbody>
</table>

\(^a\) In two of these patients the biopsy material was taken from the operation specimens after small bowel resection.
M. avium subsp. hominissuis, which is a ubiquitous environmental mycobacterial species and an opportunistic pathogen which is regularly isolated from pigs and humans.\(^{10,22}\) Forty-five of the 221 IBSEN II study subjects received antibiotics within the last 12 months prior to inclusion, but none of the cures given lasted for more than 10 days, and would not be expected to influence MAP presence or viability. Among the referral patients, 17 out of 33 patients were MGIT signal positive, but only three were IS900 PCR positive, potentially reflecting the multidrug resistance occurring in selective hospital environments. The general lack of MAP positivity in the PSC colitis group, which mainly consisted of patients with UC and a median disease duration of more than 10 years, does not support a relationship between disease duration of non-CD colitis and MAP positivity alone.

Numerous studies have addressed the question of how the presence of MAP in the bowel correlates with CD, and many reports have described relatively high MAP positivity rates in CD patients.\(^{6-9}\) In those studies, MAP from bowel biopsies and peripheral blood was detected by PCR directly and/or by BACTEC MGIT cultures subjected to PCR analysis. Only in very few studies, viable MAP cultures have been isolated. On the contrary, a positive signal in the MGIT system has in many studies been interpreted as a sign of MAP viability (also in the absence of a viable culture or PCR positivity), even though it is well known that the MGIT cultivation system can give a (false) positive signal for example due to the presence of human cellular material consuming oxygen.\(^{23,24}\) In our opinion, without the presence of viable replicating bacteria, the MGIT positivity rate can only marginally be regarded as a positive parameter for growth. Only in a few cases, morphological structures suggested to be spheroplasts, which cannot be recovered as a viable culture, have been observed by electron microscopy.\(^{10}\) From a technical point of view, PCR amplification, in particular nested PCR, is a common source of DNA contamination and false positive results, and even if sequences of the PCR products from different patients vary, potentially indicating that the MAP isolates occurring were independent,\(^{10}\) amplification by PCR can be error-prone enough to simulate such a result. Furthermore, a high prevalence of the presence of viable MAP in peripheral blood, even in healthy controls, would be considered as incompatible with a normal health status. On the other hand, potentially the MAP positivity rate noted in CD patients in previous studies has been overstated due to false positive MGIT readouts or false positive PCR reactions.

The strength of the present study is related to the reduced probability of these factors being false positive, due to technical advances and improved quality of controls. The controls included ensure that the assays employed were reliable and could detect small numbers of MAP organisms. Moreover, this investigation is, to our knowledge, the first to prospectively study the incidence of MAP positivity in a population-based, treatment naïve cohort of patients with IBD, the IBSEN II cohort. The low incidence of MAP positivity in this cohort compared to the non-selected material from a third-line referral center may indicate that MAP is a secondary phenomenon to the nature and duration of disease, in combination with the selective pressures of treatment and long-term contact with a hospital environment.

A possible limitation of this study might be the use of mucosal biopsies, because higher MAP positivity rates have been found in surgical resections from CD patients than in endoscopic biopsy specimens, indicating that sampling is a limiting factor for successful detection of MAP. This could either be due to the uneven sporadic distribution of organisms, which agglutinate in clumps or biofilms, or their enrichment in deeper compartments of intestinal tissue that are not available by biopsy sampling. False negative PCR results might also occur. Another weakness of the present study might be that none of the two CD cohorts presented were followed longitudinally in order to monitor changes prospectively, to assess the hypothesis of MAP positivity as a phenomenon occurring secondary to CD. We cannot rule out the possibility that MAP may contribute to the chronicity of CD (Table 3).

Potentially, MAP positivity might vary in different countries. Final evidence showing that MAP is a causative agent of IBD, and in particular CD, has so far not been provided.\(^{25}\) It is more likely that other agents might contribute to IBD development, potentially through dysbiosis.\(^{5}\) In this context, further search for other agents (microbial and/or chemical) associated with IBD is warranted.

The questions that remain are currently: Is there a general paucity of MAP in Norwegian IBD patients and in the Norwegian general population, as opposed to what is found in other geographic regions? If so, what are the microbial and other environmental factors that correlate with and potentially contribute to the particularly high incidence of IBD in Norway? Or do IS900-negative isolates of MAP occur in this region?

In conclusion, these findings demonstrate the paucity of MAP in the gut of Norwegian population-based treatment naïve IBD patients compared with a higher positivity rate in hospital-based non-selected IBD patients. The results may suggest that MAP positivity is a secondary phenomenon related to the duration of disease and treatment, rather than a primary cause eliciting IBD. This hypothesis will have to be tested further in additional prospective studies. Nevertheless, the clear difference between the population-based index cohort and the hospital-based IBD cohort was striking, motivating the search for other putative microbial factors among the exogenous and environmental stimuli contributing to the etiology of IBD, and CD in particular.

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PR, SB, GP, KJ, AR and MV performed clinical diagnostics, colonoscopy and biopsy sampling, JS and TT performed biopsy preparation and cultivation, SL and AJ performed PCR analysis, SL and TT performed bioinformatic analyses. PR, TT and MV conceived the study, participated in its design and coordination, and PR, MV and TT drafted the first version of the manuscript. Thus, all authors made substantial contributions to all of the following: (1) the conception and design of the study, acquisition of data or data analysis, and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, and (3) read and approved the final manuscript to be submitted.

References