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The possible involvement of *Mycobacterium avium* spp. *paratuberculosis* in the aetiology of Crohn's Disease

A case control study in the Netherlands

A.A.P.M. Herrewegh^{1,5}, P. Overduin¹, P.J.M. Roholl², F.K. Gielis², J.E. Robinson²,
N. Mahmmod³, R.J. Lieverse³, R.J. Robijn³, A.G.M. van der Zanden⁴ and
D. van Soolingen¹

Diagnostic Laboratory for Infectious Diseases and Perinatal Screening (LIS)¹ and
Laboratory for Toxicology, Pathology and Genetics (TOX, RIVM)², RIVM, Bilthoven,
the Netherlands. Department of Gastroenterology³ and Department of Medical
Microbiology and Infectious Diseases⁴, Gelre Hospitals, Apeldoorn, the Netherlands.
BioMedics Scientific Consultancy, Maarssen, the Netherlands⁵

Contact: Dr. D. van Soolingen

Laboratory for Infectious Diseases and Perinatal Screening (LIS)

email: d.van.soolingen@rivm.nl



Abstract

The possible involvement of *Mycobacterium avium* ssp. *paratuberculosis* in the aetiology of Crohn's Disease: a case control study in the Netherlands.

A case control study was performed to investigate the possible role of *Mycobacterium avium* ssp. *paratuberculosis* (*Map*) in the aetiology of Crohn's disease (CD). Biopsy samples were collected from the ileum and colon of CD patients, Ulcerative Colitis (UC) patients and control persons. The biopsy samples were either cultured in MGIT and BACTEC medium, formaline-fixed, or immediately snap-frozen in liquid N₂.

The presence of *Map* bacteria in the cultures was determined using a nested PCR assay targeted to the conserved IS900 DNA-sequence of *Map*. 27% of CD patients, 6% of UC patients, and 28% of control persons were positive for MGIT-PCR. The BACTEC cultures resulted in a slightly smaller percentage of PCR positive patients, 22% for CD patients, 7% for UC patients and 25% for control persons. The presence of *Map* was further studied applying the IS900-PCR directly on DNA from frozen biopsy samples. 7% of CD patients, 8% of UC patients and 5% of control persons were PCR positive for *Map*. The presence of *Map* was also investigated in formalin-fixed biopsies samples using a hyperimmune antiserum to the *M. avium*-complex. With immunoperoxidase (IP) staining, *M. avium*-complex antigens were observed in biopsy samples from 20% of CD patients, 13% of UC patients and 29% of control persons. Surprisingly, these data show even a 50% higher presence of *Map*-antigens in control persons (29%) compared to CD patients (20%). Our data clearly show that *Map* is present both in IBD patients (CD and UC) and in control persons. The results of culturing and IP staining show a good correlation; with both techniques, an average of 23% of CD patients and 27% of control persons is positive for *Map*. Remarkably, the results of culturing and IP staining show an even higher prevalence of *Map* in control persons compared to CD patients. Moreover, only in a few instances patients were positive in two different tests (PCR, IP, MGIT and BACTEC), and even with both culturing assays almost no overlap in positive results is observed. This result indicates that the number of *Map* cells in human tissue is very low, and that the prevalence of *Map* in the population of IBD patients and of control persons is difficult to estimate. Unexpectedly, we obtained evidence that members of the *Chlamydiales* family are present in high amounts in most of the CD and UC patients, whereas they were of low incidence in control persons.

In conclusion, our data do not support the hypothesis that *Map* is directly involved in Crohn's disease, but does not exclude that *Map* infection may play a role in the aetiology of Crohn's Disease for a susceptible subset of the population.

Keywords: *Mycobacterium avium* ssp. *paratuberculosis*, Crohn's disease, Ulcerative colitis, biopsy, culturing, PCR, immunoperoxidase staining, Chlamydiae.

Rapport in het kort

***Mycobacterium avium* ssp. *paratuberculosis* als mogelijke oorzaak van de ziekte van Crohn: resultaten van een patiëntenstudie in Nederland**

De bacterie *Mycobacterium avium* ssp. *paratuberculosis* (*Map*) wordt beschouwd als een mogelijke oorzaak van de ziekte van Crohn (morbus Crohn, MC). In samenwerking met Gelre ziekenhuizen heeft het RIVM een onderzoek uitgevoerd naar het voorkomen van *Map* in darmbiopten van patiënten met MC, patiënten met Ulcerative Colitis (UC) en controlepersonen zonder darmontsteking. De aanwezigheid van *Map* in de darmbiopten werd onderzocht met behulp van kweekmethoden, een DNA amplificatiemethode (PCR) en een immunologische detectiemethode (immunoperoxidase kleuring, IP-kleuring). Met de PCR-methode werd *Map* aangetoond in 7% van de MC-patiënten, 8% van de UC-patiënten en 5% van de controlepersonen. Met de gecombineerde kweek- en PCR-methoden waren gemiddeld 25% van de MC-patiënten, 7% van de UC patiënten en 27% van de controlepersonen positief. Met behulp van de IP-kleuring werd *Map* aangetoond in 20% van de MC-patiënten, 13% van de UC-patiënten en 29% van de controlepersonen. De resultaten van de kweek- en PCR-methoden tonen geen significant verschil tussen de aanwezigheid van *Map* in MC-patiënten vergeleken met controlepersonen. De resultaten van de IP-kleuring tonen zelfs een hoger percentage *Map*-positieve controlepersonen vergeleken bij MC-patiënten. Onze resultaten tonen duidelijk aan dat *Map* zowel bij MC-patiënten als bij UC-patiënten en controlepersonen voorkomt. Naast de aanwezigheid van *Map* is ook gekeken naar de aanwezigheid van Chlamydia in de biopten. Op grond van IP en *in situ* hybridisatie kleuringen bleken *Chlamydiae* in grote aantallen aanwezig te zijn in de biopten van MC- en UC-patiënten en nauwelijks aanwezig in de biopten van controle patiënten.

In conclusie: onze resultaten ondersteunen niet de hypothese dat *Map* direct betrokken is bij de ziekte van Crohn, maar sluiten ook niet uit dat *Map* een rol speelt bij het ontstaan van de ziekte van Crohn in een gevoelig deel van de populatie.

Trefwoorden: *Mycobacterium avium* ssp. *paratuberculosis*, ziekte van Crohn, Ulcerative Colitis, biopten, kweek, PCR, immunoperoxidase kleuring, Chlamydia.

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Samenvatting

In samenwerking met Gelre ziekenhuizen heeft het RIVM een onderzoek uitgevoerd naar het voorkomen van *Mycobacterium avium* ssp. *paratuberculosis* (*Map*) bij patiënten met de ziekte van Crohn (CD) en bij patiënten met Colitis Ulcerosa (UC). Als controle werden personen onderzocht die niet leden aan CD of UC. Tijdens routine coloscopie werden minimaal zes darmslijmvliesbiopten afgenomen uit het terminale ileum en het colon. Bij CD- en UC-patiënten werden deze biopten genomen uit ontstoken delen van de darm -indien aanwezig- en uit niet ontstoken delen.

Twee biopten werden gekweekt in MGIT en BACTEC medium, twee biopten werden gefixeerd in formaline, en twee of meer biopten werden direct ingevroren in vloeibare stikstof. Na een jaar incuberen bij 37 °C werd de aanwezigheid van *Map* in de kweken onderzocht met behulp van een nested-PCR gericht tegen het geconserveerde IS900 element dat aanwezig is in *Map* DNA. Dertien van de 49 CD-patiënten (27%), één van de 16 UC patiënten en 17 van de 61 controlepersonen waren positief in de MGIT-PCR. De BACTEC-PCR leverde iets lagere percentages positieve patiënten op, 22% voor CD, 7% voor UC en 25% voor controlepersonen. Daarnaast werd de aanwezigheid van *Map* bestudeerd in de vriesbiopten met behulp van de nested-PCR. Vier van de 59 CD-patiënten (7%), 2 van de 25 UC-patiënten (8%) en vier van de 79 controlepersonen (5%) waren PCR positief voor *Map*. Deze resultaten tonen geen significant verschil tussen de aanwezigheid van *Map* in CD-patiënten vergeleken met controlepersonen. De aanwezigheid van *Map* werd ook bestudeerd in de formaline gefixeerde biopten met behulp van een immunoperoxidase (IP) kleuring en een serum dat immuno-reactief is tegen *M. avium*-complex antigenen. Biopten van 11 van de 55 CD-patiënten (20%), drie van de 23 UC-patiënten (13%) en 21 van de 73 controlepersonen (29%) waren positief in de IP kleuring. De resultaten van deze kleuring tonen zelfs een hoger percentage *Map* positieve controlepersonen (29%) vergeleken bij CD-patiënten (20%). *Map* bleek opmerkelijk vaker voor te komen in ontstoken biopten dan in niet ontstoken biopten; dit was onafhankelijk van de diagnose.

Onze resultaten tonen duidelijk aan dat *Map* zowel bij CD-patiënten als bij UC-patiënten en controlepersonen voorkomt. De resultaten van de kweek en IP kleuring tonen een goede overeenkomst. Met beide technieken is ongeveer 23% van de CD-patiënten en 27% van de controlepersonen positief voor *Map*. Opmerkelijk is dat de resultaten van kweek en IP kleuring zelfs een nog hogere prevalentie van *Map* aantonen in controlepersonen dan in CD-patiënten. Ofschoon in totaal respectievelijk 10, 35, 31 en 25 personen PCR, IP, MGIT en BACTEC-positief zijn, werden slechts een paar personen positief bevonden in twee of meer tests. Zelfs met beide kweekmethoden is een zeer beperkte overlap in positieve resultaten te zien. Dit toont aan dat het aantal *Map* bacteriën in de onderzochte weefsels erg laag is, en daarmee is de prevalentie van *Map* in weefsel van de verschillende groepen moeilijk in te schatten. Naast de aanwezigheid van *Map* is ook gekeken naar de aanwezigheid van *Chlamydia* in de biopten. Op grond van IP en *in situ* hybridisatie kleuringen bleken *Chlamydiae* in grote aantallen aanwezig te zijn in de biopten van CD- en UC-patiënten en nauwelijks aanwezig in de biopten van controle patiënten. In conclusie: onze resultaten

ondersteunen niet de hypothese dat *Map* direct betrokken is bij de ziekte van Crohn, maar sluiten ook niet uit dat *Map* een rol speelt bij het ontstaan van de ziekte van Crohn in een gevoelig deel van de populatie.

Introduction

A case control study was performed to investigate the possible role of *Mycobacterium avium* ssp. *paratuberculosis* (*Map*) in the aetiology of Crohn's disease (CD). Intestinal biopsy samples were collected from CD patients, Ulcerative Colitis (UC) patients and control persons with intestinal disorder other than CD or UC. Patients attending for routine ileocolonoscopy as a normal part of their clinical care were enrolled prospectively in the study. Informed consent was obtained in each case. The case definition of CD was established on the basis of standard clinical, radiological, endoscopic and histopathological criteria. The control group comprised patients attending for ileocolonoscopy who did not have a clinicopathological diagnosis of IBD. Inclusion criteria were compliance with case definitions and consent. Mucosal biopsies (2-8) were taken during colonoscopy from the terminal ileum and colon of all CD, UC and control patients. In CD and UC, biopsies were obtained from inflamed mucosa, if present, and from non-inflamed parts of the intestine. Two mucosal sample were fixed in phosphate buffered formaldehyde and embedded in paraffin for pathology and stained with immuno-peroxidase (IP) for the presence of *Map*-antigens using *M. avium* -complex specific antibodies. Two samples were stored in phosphate buffered saline (PBS) for culturing, and 2 to 4 samples were transferred to a cryo-vial and snap frozen in liquid N₂ for DNA isolation to investigate the presence of *Map* DNA with a nested-PCR using *Map*-specific primers targeted to the IS900 sequence. All patients and their physician in attendance were requested to fill in a detailed questionnaire, including questions about general features, clinical picture and symptoms of their complaints, medication, (family) background, life-style, eating, drinking and smoking habits and physical condition.

1. Materials and Methods

1.1 Patients and control persons

All patients were obtained from the outpatient clinic of Gelre Teaching Hospital in Apeldoorn, the Netherlands. The mean age, height and weight of the persons in the three subgroups were comparable. The majority of the CD patients was in the age group of 20 to 30 years.

The percentage of female to male was relatively comparable in the CD and control group, while in the ulcerative colitis (UC) group (consistent with the epidemiological studies) were more males than females (14 and 12, respectively). The majority of the population in the study was born in the Netherlands (95%). The major indication for coloscopy in the control group was the screening for colon cancer. The second major indication was abdominal pain and polyps' control. Coloscopy showed signs of inflammation in 71% of the CD patients (n = 59), 64% of UC patients (n = 25) and 6.1% of control persons (n = 82). The inflammation was located in the ileum in 30.4 % of the CD patients. Two CD patients were also associated with colon inflammation. The ileum was not affected in any of the UC patients.

Histopathological examination of the persons revealed that the correlation between the macroscopic inflammation and the histopathological examination was very high. All persons showing signs of macroscopic inflammation by coloscopy also had signs of microscopic inflammation by histopathological examination. However, the appearance of a non-inflamed mucosa by coloscopy does not exclude a microscopical inflammation. Histopathological examination of mucosa with a normal macroscopic appearance showed different ranges of microscopical inflammation in samples from 75% of CD patients, 44% of UC patients and 36% of control persons. The majority of these patients had a mild form of inflammation: 93% of the biopsies from CD patients, 80% of UC patients and 37% of control persons showed signs of inflammation of variable gradation.

1.2 *Map* culturing

The fresh biopsies were decontaminated and subsequently cultured in MGIT (BD Diagnostics) and BACTEC (BioMerieux) culture tubes at 37°C for up till now. Briefly, 0.5 ml NaOH-N-acetyl-L-cysteine (NALC: BBL Mycoprep, Becton Dickinson,) was added to each sample in 0.5 ml saline and incubated at room temperature for 20 min with occasional mixing by inversion. Samples were then centrifuged (10,000 x g for 10 min) and the supernatant was removed by pipetting. The pellet was resuspended in 0.5 ml TEN buffer (2 mM NaEDTA; 400 mM NaCl; 10 mM TrisHCl pH 8.0: Sigma, UK) and transferred to the tubes containing MGIT and Bactec medium supplemented with 2 µg/ml Mycobactin J (Allied

Monitor, USA). After one year of incubation at 37°C, the culture media were harvested and the presence of *Map* bacteria was determined using the IS900 PCR as described henceforward.

1.2 DNA extraction

DNA extraction was performed using the High Pure PCR Template Preparation Kit (Roche, Cat. No. 1.796.828) in combination with the protocol of Bull *et al.*, 2003¹. Frozen biopsy sample were resuspended in 200 µl Tissue Lysis Buffer and 40 µg/ml Proteinase K (Roche kit) and incubated for 2 h at 55°C. Subsequently, 200 µl Binding buffer (Roche kit) was added and the mixture was transferred to a Lysing Matrix B ribolyser tube (Qbiogene, Cat. No. 6911-100). Tubes were chilled on ice for 5 min and mechanically disrupted in the FastPrep Ribolyser (Qbiogene) at a setting of 6.5 m/s² for 45 s, centrifuged for 1 min at 8000 rpm and incubated for 10 min at 72°C. Subsequently, 100 µl isopropanol was added and the mixture was added to the upper reservoir of a High Pure filter tube, followed by 1 min centrifugation at 8000 rpm. Three consecutive washing steps with Inhibitor Removal buffer (Roche kit), Wash buffer and Wash buffer (Roche kit) were performed, respectively. Finally the DNA was eluted from the filter tube with 100 µl pre-warmed (70°C) Elution buffer (Roche kit).

1.4 IS900 nested PCR

A nested PCR test was developed targeting IS900. The primers AV1 and S535 were used for the first PCR and the primers S204 and AV2 for the nested PCR (Table 1). PCR comprised a first round PCR mix containing 5 µl of DNA extract in a final 50 µl volume with 2µM primers AV1 and S535, 1x HotStar reaction buffer, 200µM dATP, dGTP, dCTP, dTTP and 2.5U HotStar *Taq* polymerase (Qiagen, Cat. No. 203445). Cycling conditions were: 1 cycle of 95°C for 15 min then 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 3 min then 1 cycle of 72°C for 7 min. Nested PCR used 5 µl of the first round reaction added to a 50 µl reaction mix as in the primary mix but containing 2 µM primers S204 and AV2. Cycling conditions were: 1 cycle of 95°C for 15 min then 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 3 min then 1 cycle of 72°C for 7 min. Positive and negative controls were processed in parallel. Amplicons of expected size were visualised with ethidium bromide on 1.5% agarose gels.

Table 1 Primers used for nested PCR

Primer sequence (5' to 3')	Orientation	Reference
AV1: ATG TGG TTG CTG TGT TGG ATG G	sense	Bull <i>et al.</i> , 2003 ¹
S535: AGCGTCTTTGGCGTCGGTCTTG	antisense	Englund <i>et al.</i> , 1999 ³
S204: TGATCTGGACAATGACGGTTACGGA	sense	Englund <i>et al.</i> , 1999 ³
AV2: CCG CCG CAA TCA ACT CCA G	antisense	Bull <i>et al.</i> , 2003 ¹

1.5 Immunoperoxidase staining (IP)

The presence of *Map* was studied on two formalin fixed biopsies from the ileum and/or the colon of patients with Crohn's disease or ulcerative colitis and from control persons. Formalin fixed biopsies were embedded in paraffin according to procedures routinely used in pathology laboratories. Presence of *Map* was determined on paraffin embedded material using a rabbit hyperimmune antiserum to *M. avium*-complex. This serum reacts with most members of the *M. avium* complex, including *M. avium* ssp. *paratuberculosis*, *M. avium* ssp. *avium* and *M. avium* ssp. *intracellulare*, but not with other members of *Mycobacterium* genus or with cells of the intestinal flora. Per biopsy, four paraffin sections were stained for *Map*. Sections were deparaffinized and endogenous peroxidase was blocked with methanol containing 0.3% H₂O₂. Next, sections were pre-treated with 0.5% blocking solution and incubated with a rabbit anti-*Map* in an optimal concentration. Peroxidase labelled swine anti-rabbit polyclonal antibody (DAKO) was used as secondary antibody. For detection, sections were incubated with diaminobenzidine/nickel substrate. The sections were counterstained with Nuclear Fast Red (Merck). Adjacent sections were incubated with an irrelevant rabbit antiserum (RIVM). Paraffin sections from ileum tissue from cow with Paratuberculosis disease were used as positive control. A cell was considered positive when cellular immunoreactivity was observed.

2 Results

2.1 Results nested PCR on biopsy samples

Fifty-nine CD patients, 25 UC patients and 79 control persons were examined by nested-PCR for the presence of *Map* DNA. From most patients, 2 biopsy samples were examined. The results are shown in Table 2. Four out of 59 (7%) CD patients, 2 of 25 (8%) UC patients and 4 of 79 (5%) control persons were PCR positive for *Map*. These data show no significant difference between the presences of *Map*-DNA in CD patients (7%) compared to UC patients (8%) and control persons (5%).

Table 2 Results nested PCR on biopsy samples

Patient group	Patients ^a	Biopsies ^b	Positive Patients ^c	Inflamed ^d	PCR positive		PCR negative	
					Inflamed ^e	Not inflamed ^f	Inflamed ^g	Not inflamed ^h
Crohn	59	118	4 (7%)	47 (40%)	4 (3%)	0	43 (36%)	71 (60%)
UC	25	52	2 (8%)	7 (13%)	0	2 (4%)	7 (13%)	43 (83%)
Control	79	164	5 (6%)	0	0	5 (3%)	0	159 (97%)

^aTotal number of patients examined; ^bTotal number of biopsies examined; ^cTotal number of PCR positive patients; ^dTotal number of biopsies taken from involved (inflamed) areas of disease; ^eTotal number of PCR positive biopsies from inflamed areas; ^fTotal number of PCR positive biopsies from non-inflamed areas; ^gTotal number of PCR negative biopsies from inflamed areas; ^hTotal number of PCR negative biopsies from non-inflamed areas.

2.2 Results immunoperoxidase staining (IP)

Immunoperoxidase staining (IP) of *Map* was observed in macrophages. Positive macrophages were low in number and very often located in the muscularis mucosa. A cytoplasmic granular staining pattern was frequently seen, but sometimes the cytoplasm was diffusely stained. Figure 1 shows that in infected areas variable amounts of *Map*-associated staining were observed within these cells. Macrophages with tiny speckles but also with moderate to large dots could be discriminated (Figure 1A – B). Macrophages in infected areas in the ileal of a cow with Paratuberculosis were often multi-nucleated and showed an intense immunoreactivity for *Map* (Figure 1D). The results are presented in Table 3.

Table 3 *Results immunoperoxidase staining of paraffin biopsy samples*

Diagnosis	Patients ^a	IP positive patients ^b	Inflamed ^c	IP positive and inflamed ^d	% inflamed of IP positive ^e	IP negative and inflamed ^f
CD	55	11 (20%)	43 (78%)	11 (20%)	100% (11/11)	32 (58%)
UC	23	3 (13%)	12 (52%)	1 (4%)	33% (1/3)	11 (48%)
Control	73	21 (29%)	20 (27%)	11 (15%)	55% (11/20)	9 (12%)

^aTotal number of patients examined; ^bTotal number patients (biopsies) who are IP positive; ^cTotal number of biopsies taken from involved (inflamed) areas of disease; ^dTotal number of IP positive biopsies from inflamed areas; ^ePercentage of IP positive biopsies that are inflamed; ^fTotal number of IP negative biopsies from inflamed areas.

Immuno-reactivity for *M. avium*-complex antigens was observed in biopsy samples from 11 out of 55 (20%) CD patients, 3 of 23 (13%) UC patients and 21 of 73 (29%) control persons. The data of IP staining show even a 50% higher presences of *Map*-antigens in control persons (29%) compared to CD patients (20%). The presence of *Map*-antigens is more often present in biopsies with tissue inflammation. For example all IP positive CD biopsies showed inflammation, whereas only one third and half of the IP positive biopsies of respectively UC and control patients showed signs of inflammation. It should be noted that a correlation between tissue inflammation and the presence of Chlamydia-antigens and -DNA, which served as methodological controls for the detection of *Map* in this study, was also found.

2.3 Results *Map* culturing

The results of *Map* culturing in MGIT and BACTEC media is presented in Table 4 and 5.

Table 4 *Results MGIT culturing from biopsy samples followed by PCR*

Diagnosis	Patients ^a	Positive patients ^b	Inflamed ^c	MGIT-PCR positive		MGIT-PCR negative	
				Inflamed ^d	Not inflamed ^e	Inflamed ^f	Not inflamed ^g
CD	49	13 (27%)	14 (29%)	2 (4%)	11 (22%)	12 (24%)	24 (49%)
UC	16	1 (6%)	3 (19%)	0	1 (6%)	3 (19%)	12 (75%)
Control	61	17 (28%)	0	0	17 (28%)	0	44 (72%)

^aTotal number of patients examined; ^bTotal number of MGIT positive patients; ^cTotal number of biopsies taken from involved (inflamed) areas of disease; ^dTotal number of MGIT positive biopsies from inflamed areas; ^eTotal number of MGIT positive biopsies from non-inflamed areas; ^fTotal number of MGIT negative biopsies from inflamed areas; ^gTotal number of MGIT negative biopsies from non-inflamed areas;

Table 5 Results BACTEC culturing from biopsy samples followed by PCR

Diagnosis	Patients ^a	Positive patients ^b	Inflamed ^c	Bactec positive		Bactec negative	
				Inflamed ^d	Not inflamed ^e	Inflamed ^f	Not inflamed ^g
CD	45	10 (22%)	11 (24%)	2 (4%)	8 (18%)	9 (20%)	26 (58%)
UC	15	1 (7%)	2 (13%)	0	1 (7%)	2 (13%)	12 (80%)
Control	55	14 (25%)	0	0	14 (25%)	0	41 (75%)

^aTotal number of patients examined; ^bTotal number of BACTEC positive patients; ^cTotal number of biopsies taken from involved (inflamed) areas of disease; ^dTotal number of BACTEC positive biopsies from inflamed areas; ^eTotal number of BACTEC positive biopsies from non-inflamed areas; ^fTotal number of BACTEC negative biopsies from inflamed areas; ^gTotal number of BACTEC negative biopsies from non-inflamed areas;

In Table 6, the combined data from PCR, culturing and IP staining are depicted. This table shows how many patients were positive in two different assays. Since none of the patients were positive in all three tests, combined data for three tests (PCR, IP and culturing) are not depicted.

Table 6 Combined data from PCR, Culturing and Immunoperoxidase staining

	PCR positive ^a	IP positive	Culture positive	PCR negative	IP negative
PCR positive	XXXX				
IP positive	3	XXXX			
Culture positive	3	5	XXXX		
PCR negative	XXXX	26	37	XXXX	
IP negative	5	XXXX	33	77	XXXX
Culture negative	5	17	XXXX	63	48

^aTotal number of patients

3. Discussion and Conclusion

Our data clearly show that *Map* is present both in IBD patients and in control persons. Strikingly, the results of culturing and IP staining show an even higher prevalence of *Map* in control persons compared to CD patients. With IP staining and culturing *Map* can be found in at least 20% of the CD patients and control persons. This would suggest that this percentage would also be found in UC patients. However, data from IP staining and culturing show a remarkably lower prevalence of *Map* in UC patients. The reason for this is unclear. The results of culturing and immunoperoxidase staining show a good correlation; with both techniques, an average of 23% of CD patients and 27% of control persons is positive for *Map* and *Map*-antigen, respectively. In discrepancy with this result is the low percentage of PCR positive biopsies compared to culture positives. An explanation for this is that for culturing the complete biopsy sample is spent, while for PCR only one-fifth of the biopsy sample is used. Furthermore, during DNA extraction from biopsy samples, loss of some DNA template is inevitable, while on the contrary during culturing, life bacteria may replicate and thus providing even more template for the succeeding PCR amplification. It is concluded that the percentage of cells containing *Map*-proteins in a biopsy sample is very low. Besides, of both biopsy samples from PCR positive patient, a positive PCR result occurred only with one sample. Collins *et al.* tested over five biopsy samples on average per patient, and among PCR positive patients most often positive PCR results occurred with only a single biopsy per patient². Furthermore, although a total of 10, 35, 31 and 25 persons were PCR, IP, MGIT and BACTEC positive, respectively, only in a few instances was a patient positive by two tests (Table 6). Even with both culturing assays, almost no overlap in positive results is observed: of the 115 persons who were tested in MGIT and in BACTEC, only 7 were positive in both culturing assays. These data show that the prevalence of *Map* in human tissue is very low. A very sensitive detection assay like PCR or culturing can only yield a positive result if the target (in this case *Map*-DNA or *Map*-bacteria) is present in the reaction mixture. Since *Map* is not uniformly distributed in the tissue, and apparently only present in minimal amounts, the place of sampling will be very critical. It is a matter of chance to pick the right spot. If it were possible to collect and analyse many biopsy samples per person, the percentage of *Map*-PCR, *Map*-IP and *Map*-culture positive persons would be higher.

Several investigators have detected *Map* in a higher percentage of inflamed tissues compared to non-inflamed tissues of CD patients^{4,5,6,7}. Our results from IP and PCR do confirm these observations: in these two assays the percentage of inflamed tissues that are *Map*-positive (26% and 9%, respectively) is higher than the percentage of non-inflamed tissues that are *Map*-positive (both 0%). Remarkably however, both culturing assays show quite the opposite result. In these assays the percentage of non-inflamed tissues that are *Map* positive (31% for MGIT and 24% for BACTEC) is higher than the percentage of *Map*-positive inflamed tissues (14% and 18%).

An interesting observation is the correlation between presence of *Map* antigens (MAC-antigens) and tissue inflammation in CD patients. All inflamed biopsy samples from CD

patients were IP positive (Table 3), while only 14% and 18% of inflamed tissues were MGIT and BACTEC positive, respectively. This might imply that this inflammation is not solely caused by *Map* antigens, but predominantly by other members of the MAC, or other pathogens. We obtained evidence that members of the *Chlamydiales* family are present in high amounts in most of the CD and UC patients, whereas they were of low incidence in control persons. Furthermore the presence of *Chlamydiales* was completely correlated with inflammation, not only in CD patients but also in UC patients and control persons (results not shown).

All patients have completed a detailed questionnaire, including questions about general features, clinical picture and symptoms of their complaints, medication, (family) background, life-style, eating, drinking and smoking habits and physical condition. These data will be studied in further detail, and will be combined to the data of PCR, IP and culturing to reveal correlations between epidemiological and molecular data related to CD and *Map*.

To conclude, our data do not support the hypothesis that *Map* is directly involved in Crohn's disease, but at least suggest that infection plays an important role. Further research is needed to validate a role of *Chlamydiales* in the pathogenesis of IBD.

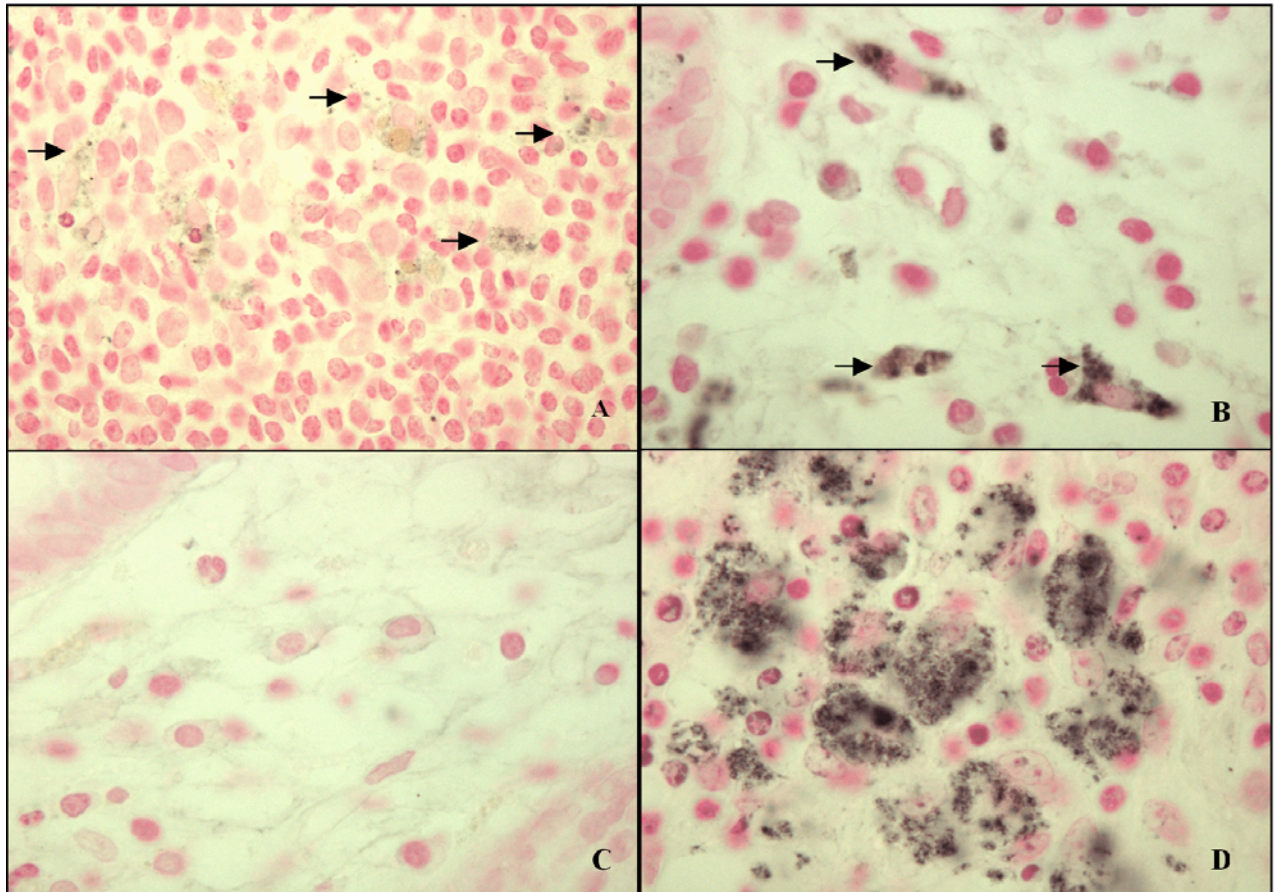


Figure 1. Immunoperoxidase staining for Map on paraffin sections of ileum from human (A-B) and cow with Johne's disease (D). Figure C shows the use of an irrelevant rabbit antiserum on ileum from human. Notice the granular staining pattern and the variation in the intensity of the staining per cell. Arrows indicate tiny (A) and moderate (B) spots intracellularly in macrophages. This staining is absent in C. Large, often multinucleated macrophages are intensely stained for Map in case of cow with Johne's disease. Pictures taken with a 40x (A) and 60x (B-D) objective.

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