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ORIGINAL INVESTIGATION

Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* and *Escherichia coli* in blood samples from patients with inflammatory bowel disease

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Abstract *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and adherent-invasive *Escherichia coli* (AIEC) have been implicated as primary triggers in Crohn's disease (CD). In this study, we evaluated the prevalence of MAP and *E. coli* (EC) DNA in peripheral blood from 202 inflammatory bowel disease (IBD) patients at various disease periods and compared against 24 cirrhotic patients with ascites (CIR) (non-IBD controls) and 29 healthy controls (HC). MAP DNA was detected by IS900-specific nested PCR, EC DNA by *mal*B-specific nested PCR and AIEC identity, in selected samples, by sequencing of *fimH* gene. CD patients with active disease showed the highest MAP DNA prevalence among IBD patients (68 %). Infliximab treatment resulted in decreased MAP detection. CIR patients had high individual and coinfection rates (75 % MAP, 88 % EC and 67 % MAP and EC), whilst HC controls had lower MAP prevalence (38 %) and EC was undetectable in this control group. EC DNA prevalence in IBD patients was highly associated with CD, and 80 % of EC from the selected samples of CD patients analyzed carried the *fimH30* allele, with a mutation strongly associated with AIEC. Our results show that coinfection with MAP and AIEC is common and persistent in CD, although the high MAP and EC detection in CIR patients suggested that colonization is, at least, partially dependent on increased gut permeability. Nevertheless, facilitative mechanisms between a susceptible host and these two potential human pathogens may allow their implication in CD pathogenesis.

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Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBD) characterized by chronicity and high morbidity, with a complex pathogenesis that is still unclear [1]. For CD, the role of microorganisms, acting as a single or combined species in triggering primary etiology, has long been suggested [2, 3]. Recent interest has been focused on *Mycobacterium avium* subsp. *paratuberculosis* (MAP) or adherent-invasive *Escherichia coli* (AIEC), which have been detected in higher numbers among CD patients than control groups [4, 5].

The *Mycobacterium avium* complex (MAC) is widely distributed in the environment, mainly in water and soil [6, 7], and may cause infectious diseases both in humans and animals [6, 8, 9]. MAC includes MAP, the etiological agent of Johne's disease (JD), a chronic granulomatous inflammation of the intestine that is an important and widespread disease of domestic ruminants and other mammals, including primates [10–12].

Since the initial isolation of MAP from CD patients by Chiodini et al. [13] in the 1980s, a possible link between JD and CD has been widely debated and investigated. JD exhibits many pathological similarities with CD [10, 11, 14, 15], although some differences are also evident, e.g., the more localized pattern of lesions and the absence of certain CD complications, such as fissures and fistulae [15]. A number of studies show MAP to be more frequently detected from CD patients than from healthy controls or UC patients [14, 16–18], and successful MAP culture from intestinal tissues, blood and breast milk of CD patients has proven that a viable form of the bacteria is present [18–21]. Juste and collaborators [22] have detected a lower frequency of MAP DNA in IBD patients from Spain undergoing therapies with known anti-mycobacterial activity [azathioprine, 5-aminosalicylic acid (5-ASA) and methotrexate]. Conversely, some studies from geographically separated populations have not found a positive correlation between MAP detection and CD or UC [23]. Whilst the presence of viable MAP infection in IBD is thus not disputed, the evidence for MAP as a primary causative agent remains inconclusive with the possibility remaining that MAP presence represents secondary invasion as a result of an inflamed gut mucosal barrier selectively increasing permeability, or the indirect inability of CD macrophages to neutralize and clear these particular bacilli.

Diverse evidence also supports a role for distinct *Escherichia coli* (EC) types in the pathogenesis of IBD [24, 25]. Studies performed in IBD patients, particularly CD, have identified an increased occurrence of AIEC (adherent-invasive *E. coli*) isolates in gut mucosal samples [26–30]. They have been more frequently isolated from ileal mucosa of CD patients than from healthy controls or UC patients [24, 31, 32]. AIEC lack known virulence determinants present in other intestinal pathogenic EC strains including entero-invasive, entero-pathogenic or entero-toxigenic *E. coli* [33]. AIEC genomes include regional homologies with extra-intestinal pathogenic EC (ExPEC) but are phenotypically distinct [34, 35]. AIEC contain factors allowing adherence to specific receptors in the intestinal epithelium, promoting colonization of gut mucosa, invasion of intestinal epithelial cells and macrophages accompanied by intracellular replication without triggering host cell death [27, 28, 33, 36, 37]. Adhesion is mediated by type 1 fimbriae expressing the adhesive subunit FimH, located at the tip of the fimbriae [38, 39]. Specific mutations in the gene *fimH* of AIEC result in a FimH protein with enhanced specific adherence [40] to the carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), a glycosylated receptor located on the apical side of enterocytes, that is abnormally expressed in CD patients as compared to control subjects [39–42].

Until now, studies have focused on the possible implications of either MAP or AIEC in CD development. Both are detected in higher proportion in CD patients [19, 43], share invasive ability [27, 28, 33, 36, 37, 44, 45] and are resistant to elimination by macrophages [35, 46–48]. No studies have as yet considered a joint contribution to CD pathogenesis from both bacteria.

The aim of the present study was to determine the prevalence of both MAP and EC in patients with IBD and confirm AIEC isolate identities using specific *fimH* sequencing. To our knowledge, this is the first study of co-detection of MAP and EC in the peripheral blood of IBD patients and provides novel insights into the involvement of both MAP and AIEC in CD pathogenesis.

Materials and methods

Patients

This prospective study included a total of 255 subjects. These included 66 IBD patients with active disease [40 CD patients (CDA) and 26 UC patients (UCA)], 39 IBD patients in remission [25 CD patients (CDR) and 14 UC patients (UCR)], 51 IBD patients with active disease and under infliximab therapy [43 CD patients (CDA-IFX) and 8 UC patients (UCA-IFX)], 46 IBD patients in remission and under infliximab therapy [34 CD patients (CDR-IFX) and 12 UC patients (UCR-IFX)], 24 cirrhotic patients with

ascites (CIR) and 29 healthy controls (HC). IBD activity was evaluated by the level of C-reactive protein and either the Harvey–Bradshaw index for CD [49] or the partial Mayo score for UC [50]. A level of C-reactive protein >3 and either a Harvey–Bradshaw index >4 for CD or a partial Mayo score >2 for UC were indicative of disease activity. Patients given infliximab were identified as a specific group because this drug is recognized as a major regulator of gut barrier by reducing gut inflammation [51, 52].

Patients were recruited from the Gastroenterology Department at Centro Hospitalar São João (Porto, Portugal). Diagnosis was based on standard clinical, endoscopic, histologic and radiographic criteria [53, 54]. HC were recruited from the academic community of the Health Sciences Faculty, University Fernando Pessoa (Porto, Portugal). Age, gender and therapeutic regimen of the subjects enrolled in this study are represented in Table 1. Informed consent was obtained in accordance with the institutional review board regulations at Centro Hospitalar São João and University Fernando Pessoa, after approval of the corresponding ethics committees (studies number 105/2007 and 02/2007, respectively).

Sample collection and processing

Whole blood samples (13.5 mL) were obtained from each individual, diluted 1:2 in phosphate-buffered saline (PBS, Sigma) and layered onto Histopaque®-1077 (Sigma, St. Louis, MO, USA) in 15 mL sterile tubes. After centrifugation for 30 min at 400×*g* (room temperature), the mononuclear cells present in the monolayer were collected, washed three times in Hank's balanced salt solution (HBSS, Sigma) and re-suspended in a mycobacterial lysis solution [2 mM sodium EDTA (Sigma), 400 mM NaCl (Sigma), 10 mM Tris–HCl (Sigma), 0.6 % SDS (Sigma) and 33 μg proteinase K (Sigma)].

DNA extraction

DNA extraction was performed as described previously [19]. Briefly, mononuclear cells in mycobacterial lysis solution were incubated at 37 °C for 2 h with shaking (200 rpm). Tubes were chilled on ice, and 100 μL of zirconium beads (0.5 mm) (Biospec Products, Bartlesville, OK, USA) were added to each tube. Cells were mechanically disrupted by shaking in a Mini Beadbeater™ (Biospec Products, Bartlesville, OK, USA) and chilled on ice, and 600 μL of phenol saturated in TE (Sigma) was added. The mixture was vortexed for 20 s and then centrifuged at 10,000×*g* for 1 min. The aqueous layer was transferred to a new tube containing phenol–chloroform–isoamyl alcohol (25:24:1) in TE (Sigma), vortexed and centrifuged at 10,000×*g* for 1 min. The aqueous layer was again transferred to a new tube containing equal volume of chloroform–isoamyl alcohol (24:1) (Sigma), vortexed for 30 s and centrifuged (10,000×*g* for 1 min). The final aqueous layer was then transferred to a new tube containing 10 M ammonium acetate (Sigma) and mixed. One milliliter of absolute ethanol was added to allow DNA precipitation. After centrifugation (10,000×*g*, 20 min), pellets were washed with ethanol 70 %, incubated 30 min to dry, re-suspended in TE buffer (10 mM Tris–HCl, 1 mM sodium EDTA, pH 8) and allowed to dissolve at 4 °C overnight. All DNA solutions were stored at −20 °C for further studies.

MAP insertion sequence (IS)*900*-specific nested PCR and sequencing

The presence of MAP DNA was detected by nested PCR, using primers and conditions for amplification of IS*900*, detailed in Table 2. The primary PCR round used primers L1 and L2, which amplified a 398-bp fragment of the IS*900* gene. In the second round, primers AV1 and AV2 were used for amplification of a 298-bp internal nucleotide sequence from the 398-bp amplicon obtained from the first PCR. The first PCR mixture (final volume of 50 μL) consisted of 1X reaction buffer with 1.5 mM $MgCl_2$ (Promega, Madison, WI, USA), 10 % DMSO (Sigma), 2 μM of each primer, 200 μM of each dNTP (dATP, dCTP, dGTP, dTTP) (Finnzymes, Espoo, Finland), 3.5 U GoTaq® Flexi DNA polymerase (Promega) and 5 μl of sample DNA. The nested PCR mixture included the same components described for the primary reaction, with exception of the template DNA (5 μL of the PCR product from the first round was used) and the primers (AV1 and AV2; 2 μM). The amplification conditions for both PCRs are described in Table 2. Amplicons (including a lambda DNA/HindIII marker (125–23,130 bp) (Promega)) were visualized with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) on a 3 % agarose gel using an UV transilluminator (BioRad, Hercules, CA, USA), and their identity was confirmed by sequencing, using AV1 and AV2 primers. Positive and negative reagent and lysis controls were included with all reaction runs, and the specificity of the reaction was confirmed by sequencing of some amplified fragments.

Detection of EC and *fimH* sequencing

The identification of EC DNA was also carried out by a nested PCR, using primers and amplification conditions detailed in Table 2. The first PCR was carried out in a final volume of 25 μL using the following reaction conditions: 1X reaction buffer (Green GoTaq® Flexi Buffer) containing 1.5 mM $MgCl_2$ (Promega, Madison, WI, USA), 0.8 μM of each primer (ECO-1 and ECO-2), 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP) (Finnzymes), 1.0 U GoTaq®

Table 1 Age, gender, Montreal classification, therapy and bacterial DNA detection results of the subject groups enrolled in the study

	HC ($n = 29$)	CIR ($n = 24$)	CDA ($n = 40$)	CDA-IFX ($n = 43$)	CDR ($n = 25$)	CDR-IFX ($n = 34$)	UCA ($n = 26$)	UCA-IFX ($n = 8$)	UCR ($n = 14$)	UCR-IFX ($n = 12$)
Age	37 (23–69)	65 (40–85)	43 (21–73)	40 (25–69)	45 (26–67)	39 (22–64)	48 (21–84)	42 (24–66)	42 (23–63)	45 (32–64)
Gender (m/f)	15/14	18/6	23/17	18/25	15/10	21/13	16/10	3/5	5/9	8/4
Montreal classification										
Crohn's disease										
A1	–	–	2	3	1	2	–	–	–	–
A2	–	–	30	33	14	31	–	–	–	–
A3	–	–	8	4	10	2	–	–	–	–
L1	–	–	12	14	17	10	–	–	–	–
L2	–	–	4	7	2	8	–	–	–	–
L3	–	–	24	22	6	16	–	–	–	–
B1	–	–	20	24	17	19	–	–	–	–
B2	–	–	9	5	5	7	–	–	–	–
B2	–	–	11	14	3	8	–	–	–	–
Ulcerative colitis										
E1	–	–	–	–	–		2	0	2	0
E2	–	–	–	–	–		11	4	2	5
E3	–	–	–	–	–		13	4	10	7
Therapy										
5ASA	0	0	12	6	19	5	9	1	12	4
AZA	0	0	9	8	17	13	6	4	6	8
PRED	0	0	18	4	0	2	6	0	1	2
AB	0	15	16	4	0	0	3	0	0	0
IFX	0	0	0	43	0	35	0	8	0	12
MAP DNA (*n* pos/*n* total)	38 % (11/29)	75 % (18/24)	68 % (27/40)	49 % (21/43)	60 % (15/25)	50 % (17/34)	62 % (16/26)	0 % (0/8)	43 % (6/14)	33 % (4/12)
EC DNA (*n* pos/*n* total)	0 % (0/29)	88 % (21/24)	50 % (20/40)	66 % (28/43)	72 % (18/25)	71 % (24/34)	12 % (3/26)	0 % (0/8)	14 % (2/14)	0 % (0/12)
MAP+EC+(*n* pos/*n* total)	0 % (0/29)	67 % (16/24)	43 % (17/40)	33 % (14/43)	44 % (11/25)	41 % (14/34)	8 % (2/26)	0 % (0/8)	14 % (2/14)	0 % (0/12)
MAP+EC− (*n* pos/*n* total)	38 % (11/29)	8 % (2/24)	25 % (10/40)	16 % (7/43)	16 % (4/25)	9 % (3/34)	54 % (14/26)	0 % (0/8)	29 % (4/14)	33 % (4/12)
MAP− EC+(*n* pos/*n* total)	0 % (0/29)	21 % (5/24)	7 % (3/40)	33 % (14/43)	28 % (7/25)	30 % (10/34)	4 % (1/26)	0 % (0/8)	0 % (0/14)	0 % (0/12)

HC healthy controls, *CIR* cirrhotic inpatients with ascites, *CDA and UCA* CD and UC patients with active disease, *CDA-IFX and UCA-IFX* patients with active disease, under infliximab treatment, *CDR* and *UCR* patients in remission for more than 12 months, *CDR-IFX* and *UCR-IFX* patients in remission for more than 12 months, under infliximab treatment. Montreal classification in Crohn's disease: *A* age at diagnosis—A1 (below 16 years); A2 (between 17 and 40 years); A3 (above 40 years), *L* disease localization—L1 (ileal); L2 (colonic); L3 (ileocolonic); *B* behavior—B1 (non-structuring, non-penetrating); B2 (stricturing); B3 (penetrating). Montreal classification in ulcerative colitis: E1 (ulcerative proctitis), E2 (left-sided UC), E3 (pancolitis). *5-ASA* 5-aminosalicylate, *AZA* azathioprine, *PRED* prednisolone, *AB* antibiotics (one of the following: ciprofloxacin, metronidazole or ceftriaxone), *IFX* infliximab

Flexi DNA polymerase (Promega) and 2 μL of template DNA. The amplified product obtained (585 bp, *malB* gene) was further used in the second PCR, which was performed under the same reaction conditions described above and using as primers ECO-7 and ECO-8, amplifying a 581-bp DNA sequence. The PCR products (including the lambda

Table 2 Primers and amplification conditions used in this study

Primer	Oligonucleotide sequence (5′–3′)	Gene	Amplification conditions	Product size (bp)	Reference(s)
L1	CTT TCT TGA AGG GTG TTC GG	IS*900*	1 cycle of 5 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 58 °C, 1 min at 72 °C; 1 cycle of 7 min at 72 °C	398	Bull et al. [19]
L2	ACG TGA CCT CGC CTC CAT				
AV1	ATG TGG TTG CTG TGT TGG ATG G	IS*900*	1 cycle of 5 min at 94 °C; 40 cycles of 1 min at 94 °C, 1 min at 58 °C, 1 min at 72 °C; 1 cycle of 7 min at 72 °C	298	Bull et al. [19]
AV2	CCG CCG CAA TCA ACT CCA G				
ECO-1	GAC CTC GGT TTA GTT CAC AGA	*mal*B (promoter)	1 cycle of 10 min at 94 °C; 35 cycles of 50 s at 94 °C, 50 s at 59 °C, 50 s at 72 °C; 1 cycle of 10 min at 72 °C	585	Wang et al. [69]
ECO-2	CAC ACG CTG ACG CTG ACC A				
ECO-7	CCT CGG TTT AGT TCA CAG AAG C	*mal*B (promoter)	1 cycle of 10 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 65 °C, 1 min at 72 °C; 1 cycle of 10 min at 72 °C	581	This study
ECO-8	CAC GCT GAC GCT GAC CAC				
fimH-1F	GAA ACG AGT TAT TAC CCT GTT TGC T	*fimH*	1 cycle of 10 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 64 °C, 1 min at 72 °C; 1 cycle of 10 min at 72 °C	901	This study
fimH-1R	TTA TTG ATA AAC AAA AGT CAC GCC AAT				
fimH-3F	GGT CAT TCG CCT GTA AAA CC	*fimH*	1 cycle of 10 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 59 °C, 1 min at 72 °C; 1 cycle of 10 min at 72 °C	826	This study
fimH-3R	ACG CCA ATA ATC GAT TGC AC				

DNA/HindIII marker) were separated by electrophoresis on 1.5 % (wt/vol) agarose gels containing Midori Green Advanced DNA Stain (Nippon Genetics Europe GmbH, Dueren, Alemanha) (0.06 μL/mL) and were visualized under UV light equipment (Bio-Rad Laboratories, Hercules, EUA). Positive and negative controls were included in the reactions, and specificity was confirmed by sequencing of selected amplified fragments.

Further investigation for AIEC presence was performed using *fimH*-specific nested PCR followed by sequencing of selected samples exhibiting a strong positive band for EC PCR. Nested PCRs were carried out on a final volume of 25 μL, using the same reagents and conditions as described for detection of EC. Primary PCRs used primers fimH1-F and fimH1-R (0.8 μM each) for amplification of 901 bp of the *fimH* gene. PCR products obtained were further amplified using a nested primer pair fimH-3F and fimH-3R (0.8 μM each) producing an amplicon of 826 bp (Table 2) which were then purified (EzWay PCR Clean-Up Kit; Koma Biotech, Seoul, Korea) and sequenced. Sequences were compared with the GenBank database by using the "BLASTN alignment" tool (http://www.ncbi.nlm.nih.gov/pubmed). The corresponding amino acid sequences were analyzed using the "CLUSTALW2 Multiple Sequence Alignment" (http://www.ebi.ac.uk/Tools/msa/clustalw2/), using FimH protein from *E. coli* K-12 (GenBank Accession number NP_418740.1) for comparison and detection of amino acid mutations frequently associated with FimH from AIEC strains [40].

Statistical analysis

Comparison of MAP and EC prevalence between different populations analyzed, particular disease patterns or therapeutic regimen was made using independence Chi-square test or exact Fisher's test, as appropriate. In all tests, a significance of $p < 5$ % was considered. The program SPSS (statistical package for the social sciences) version 19.0 was used for analysis.

Results

MAP and EC prevalence

MAP DNA was detected in 53 % (135/255) and EC DNA in 45 % (116/255) of all enrolled subjects. Considering IBD subjects, MAP was detected in 56 % CD patients and 43 % UC patients; EC was detected in 63 % CD patients and 8 % UC subjects. Representative agarose gels are shown in Fig. 1. MAP DNA was most frequently detected in CIR patients (75 %, 18/24) and CD patients with active disease (CDA: 68 %, 27/40) (Fig. 2; Table 1). These detection rates were significantly higher than HC (38 %, 11/29) (CDA, $p = 0{,}0266$; CIR, $p = 0{,}0120$). MAP DNA was also detected in CDR (60 %, 15/25), CDA-IFX (49 %, 21/43; CDR-IFX (50 %, 17/34), UCA (62 %, 16/26), UCR (43 %, 6/14) or UCR-IFX (33 %, 4/12) patients but in a proportion not significantly different from that observed

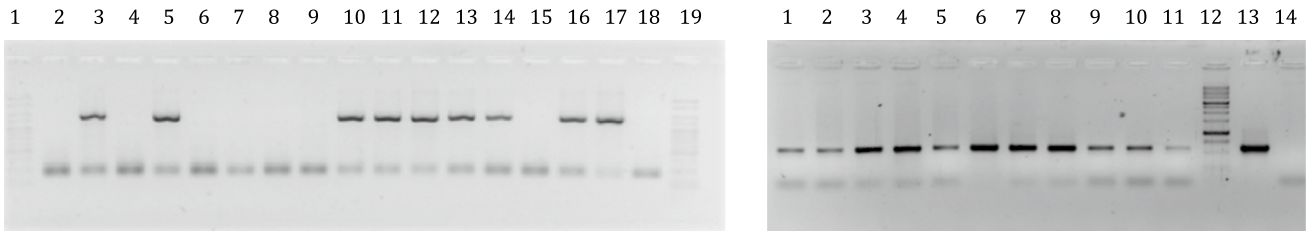

Fig. 1 Representative agarose gels, showing detection of IS900 (**a**) and EC *malB* promoter (**b**) by nested PCR. **a** *Lanes 1* and *19*, molecular weight markers; *lanes 2* and *18*, blanks; *lane 17*, positive control (MAP DNA); *lanes 3–9*, healthy control samples; *lanes 10–16*, CD patients samples. **b** *Lanes 1–11*, CD patients samples; *lane 12*, molecular weight markers; *lane 13*, positive control (EC DNA); *lane 14*, blank

in HC group. MAP was not detected in UCA-IFX group. Remission resulted in decreased MAP detection, although the frequency of MAP-positive patients was not significantly different from CDA or UCA groups (CDA vs. CDR, $p = 0.5996$; UCA vs. UCR, $p = 0.3267$). Patients receiving infliximab treatment showed the lowest MAP detection rates, with UCA-IFX patients presenting significantly lower frequency than their CD counterparts ($p = 0.0150$).

The presence of EC DNA was higher in CIR (88 %, 21/24) and in CD patients [CDR (72 %, 18/25), CDR-IFX (71 %, 24/34), CDA-IFX (66 %, 28/43) and CDA patients (50 %, 20/40)], than UC patients [UCR (14 %, 2/14), UCA, (12 %, 3/26), UCA-IFX (0 %, 0/8) and UCR-IFX (0 %, 0/12)]. EC DNA was not detected in the HC group (0 %, 0/29) (Table 1; Fig. 2).

The frequency of patients double positive for MAP and EC DNA (MAP+EC+) was higher among CIR patients (67 %, 16/24) followed by CDR (44 %, 11/25), CDA (43 %, 17/40), CDR-IFX (41 %, 14/34) and CDA-IFX (33 %, 14/43) (Fig. 3; Table 1). The frequency of patients only positive for EC DNA (MAP-EC+) was higher among CDA-IFX (33 %, 14/43), CDR-IFX (30 %, 10/34), CDR (28 %, 7/25) and CIR patients (21 %, 5/24). Among CD patients, CDA showed the lowest frequency of MAP-EC+ samples (7 %, 3/40). UC patients showed the lowest frequencies of EC DNA-positive samples, which resulted from both low MAP-EC+[UCA, 4 % (1/26), UCR, 0 % (0/14), UCA-IFX, 0 % (0/8) and UCR-IFX, 0 % 0/12)] and MAP+EC+ samples [UCR, 14 % (2/14), UCA, 8 % (2/26), UCA-IFX, 0 % (0/8) and UCR-IFX, 0 % (0/12)]. Consequently, UC patients showed the highest frequencies of IBD samples single positive for MAP DNA (MAP+EC−) [UCA, 54 % (14/26), UCR-IFX, 33 % (4–12) and UCR, 29 % (4/14)].

In CD patients, MAP prevalence showed a trend to decrease with remission and further with infliximab treatment (Figs. 2, 3), findings not observed with EC. In spite of this, double MAP and EC positives were more frequently detected among CD patients than among UC comparators (CDA vs. UCA, $p = 0.0023$; CDR-IFX vs. UCR-IFX, $p = 0.0088$) (Fig. 3).

No association was found between MAP or EC DNA detection and gender, disease duration or Montreal classification, considering either CD or UC (results not shown). Treatment of CD patients with 5-ASA resulted in decreased MAP detection [untreated, 63 % (66/104); treated, 37 % (14/38), $p = 0.005$], whilst treatment with antibiotics (ciprofloxacin, metronidazole and/or ceftriaxone) was associated with higher MAP DNA detection [untreated, 53 % (65/123); treated, 79 % (15/19), $p = 0{,}033$] (Fig. 4). Fifteen of 20 CD patients on antibiotic therapy displayed ileocolonic disease localization, and only two of the 21 CD patients with colonic disease were on antibiotic therapy (not shown). Nevertheless, MAP detection was not significantly associated with administration of any single antibiotic. EC DNA detection was independent of therapy (results not shown).

fimH sequencing

Analysis of the *fimH* gene amplified from 11 selected samples (corresponding to those exhibiting a strong positive band in EC *mal*B PCR) (CD, $n = 10$; CIR, $n = 1$) revealed the presence of the *fimH30*-like ($n = 8$; CD), *fimH27-like* ($n = 2$; 1 CD and 1 CIR) and *fimH23*-like ($n = 1$; CD) alleles (Weissman et al. [64]). Further analysis of the deduced amino acid sequences revealed the presence of FimH proteins with the amino acid changes R166H ($n = 8$), A202 V ($n = 1$) or none ($n = 2$), in comparison with *E. coli* K12 FimH [40]. The R166H amino acid change, a mutation strongly associated with AIEC strains, was detected among 80 % (8/10) of the CD patients analyzed (2/3 CDA, 3/3 CDR and 3/4 CD-IFX). Other FimH mutations found among CD patients included the A202V, not associated with AIEC, found in one CD-IFX patient ($n = 1$). FimH sequences from 1 CD and 1 CIR patient were identical to those found in *E. coli* K12.

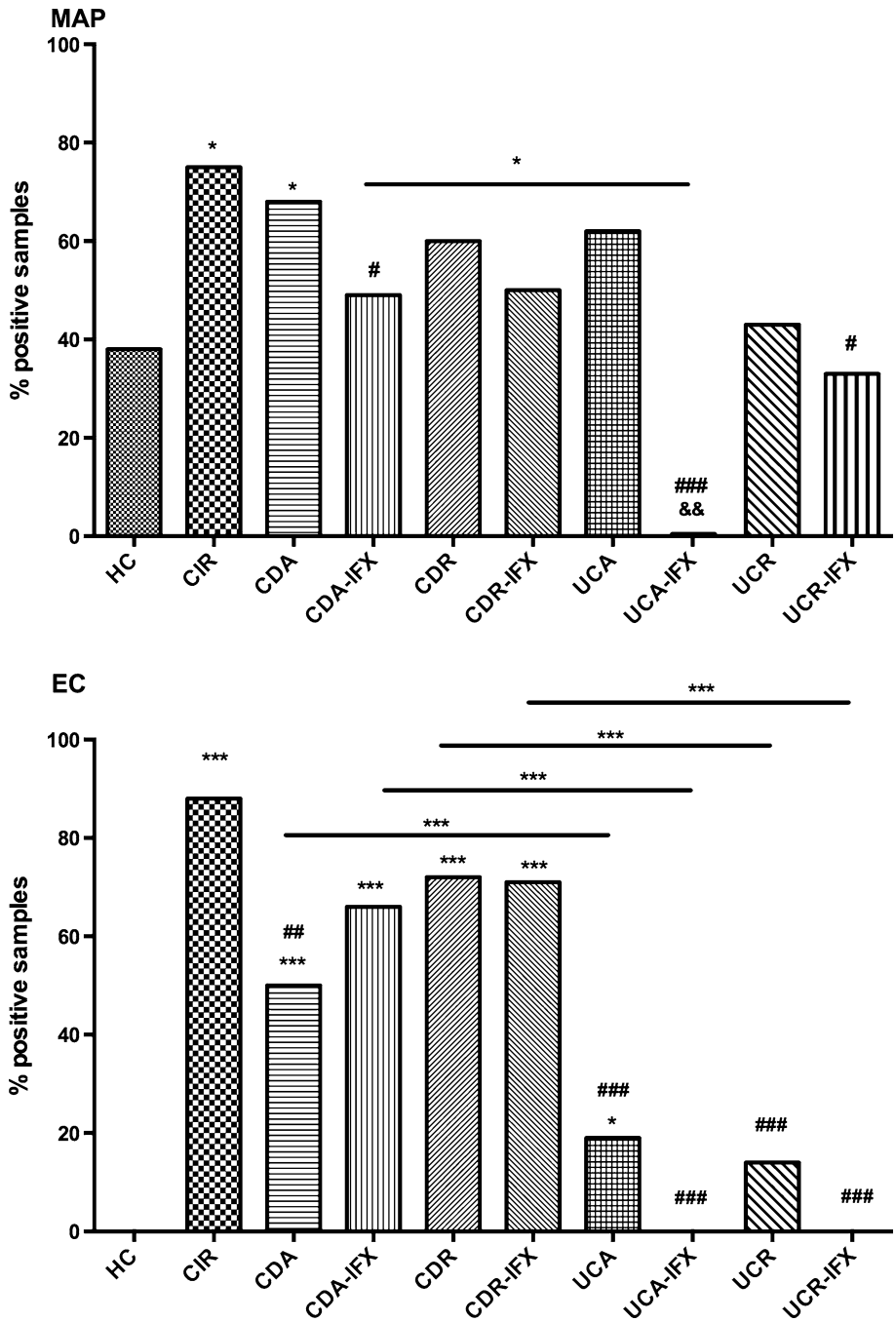

Fig. 2 MAP and EC prevalence in peripheral blood mononuclear cells isolated from healthy controls (HC), cirrhotic patients with ascites (CIR), CD or UC patients with active disease (CDA or UCA), CD or UC patients with active disease under infliximab treatment (CDA-IFX or UCA-IFX), CD or UC patients in remission (CDR or UCR) and CD or UC patients in remission, under infliximab treatment (CDR-IFX or UCR-IFX). *Significant differences with HC; $^{\&}$significant differences with the corresponding active IBD group (CDA or UCA); $^{\#}$significant differences with CIR patients. $^{*,\&}$ or $^{\#}p < 0.050$; $^{**,\&\&}$ or $^{\#\#}p < 0.010$; $^{***,\&\&\&}$ or $^{\#\#\#}p < 0.001$

Discussion

Whilst it is unclear whether any single entity can be the direct cause of IBD, the presence of potentially pathogenic intracellular organisms in blood, as opposed to gut mucosa, is indicative of successful invasion and importantly chronic persistence. Any organism that can demonstrate this capacity for entry and survival and has been allowed the opportunity to interact and manipulate normal host immune defences in such a chronic manner must be seriously considered as contributing to significant pathobiological events associated with these conditions. This study constitutes the first report describing the prevalence and distribution of two important intracellular pathogens (MAP and EC) in the blood of Portuguese IBD cohorts, using a molecular approach sufficiently sensitive to detect low load presence of both species.

Previous studies have described an association between MAP prevalence and CD through MAP detection in buffy coats [43], intestinal biopsies [19, 55, 56], resected bowel,

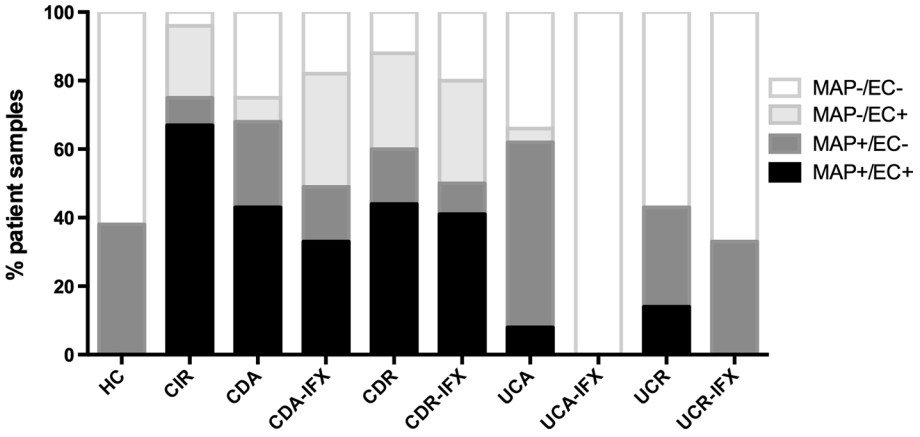

Fig. 3 Percentage of patient samples double positive for MAP and EC (MAP+EC+), single positive for MAP only (MAP+EC−) and single positive for EC only (MAP−EC+). *HC* healthy controls, *CIR* cirrhotic patients with ascites, *CDA or UCA* CD or UC patients with active disease, *CDA-IFX or UCA-IFX* CD or UC patients with active disease under infliximab treatment, *CDR or UCR* CD or UC patients in remission, *CDR-IFX or UC-IFX* CD or UC patients in remission, under infliximab treatment

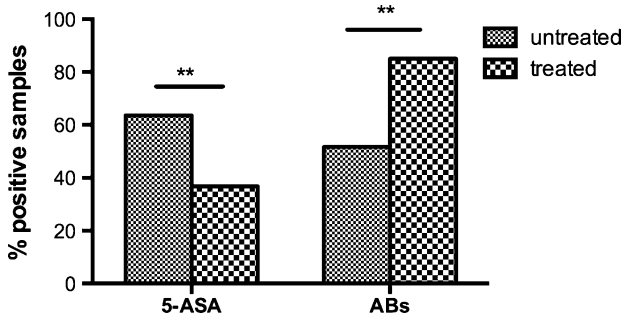

Fig. 4 Percentage of IS900-positive samples (peripheral blood mononuclear cells) in CD patients treated or untreated with 5-aminosalicylate (5-ASA) or antibiotics (AB). The antibiotic used was one of the following: ciprofloxacin, metronidazole or ceftriaxone. *$p < 0.050$; **$p < 0.010$

lymph node tissues [57] and stool cultures [56, 58]. AIEC strains possessing enhanced invasive ability [33] have also been detected and isolated from ileal mucosa of CD patients [59] expressing specific receptors [40]. In each of these studies, the prevalence was high and concordant with disease but none investigated both MAP and AIEC and thus did not address the possibility that coinfection may provide a joint contribution to CD development.

In this report, we have explored this possibility by investigating prevalence, in peripheral blood, of MAP DNA and EC DNA in patients at different periods of IBD. We compared patients with active disease and patients in remission for more than 12 months. We further studied possible influences of IBD therapies and compared against two different control groups including CIR patients with evidence of a compromised intestinal barrier and a normal healthy group.

To ensure good sensitivity and specificity, we used nested PCR and species-specific primers including IS*900* for MAP [60, 61] and *mal*B for EC [62] detection. AIEC presence was evaluated using nested PCR for *fimH*, an adhesive subunit of type 1 fimbriae of EC, which was sequenced for a panel of specific mutations associated with increased mucosal adherence in IBD tissue [63, 64], antibiotic resistance and nosocomial dissemination [65].

CIR patients had high individual and coinfection rates (75 % MAP, 88 % EC and 67 % MAP and EC) whilst HC controls had lower MAP prevalence (38 %) and EC was undetectable in this control group. Indeed, CIR patients carry a high proportion of *Enterobacteriaceae* in the gut [66]. EC is the most commonly isolated organism from CIR patients because this disease facilitates bacteria reaching mesenteric lymph nodes and persisting in the systemic circulation due to abnormalities in hepatic clearance [67].

MAP was detected in nearly all subject groups studied, with the exception being UCA-IFX. When comparing CD and UC patients, we found a trend toward higher MAP detection in CD patients in each of the relative groups (active, remission, IFX treated) possibly reflecting the lower ability of CD macrophages to eliminate intracellular bacteria, although a significant difference was only measurable in active disease patients under infliximab treatment (CDA-IFX vs. UCA-IFX). A trend toward higher MAP detection in active disease IBD patients was also observed, although this was only significant when comparing CD to the HC group. This finding may be related to higher intestinal permeability, since CIR patients with evidence of a compromised epithelial barrier (i.e., the presence of ascites) also showed high frequency of both MAP and EC DNA. Infliximab treatment, which is known to ameliorate intestinal barrier function [51], resulted in decreased MAP DNA frequency of detection (most evident in the UCA-IFX group), further suggesting that higher intestinal permeability might

facilitate bacterial internal access in addition to the existing invasive mechanisms. Previous work has shown that some CD patients show enhanced gut permeability independent of inflammation [52], not antagonized by anti-TNF treatment. The observed decrease in MAP detection may, as we have previously suggested [68], thus additionally be a result of infliximab treatment inducing dormant MAP phenotypes which persist at very low loads but do not proliferate, thus making them much more difficult to detect.

The EC detection rate was high in CIR patients but also more clearly associated with CD than UC. EC DNA prevalence was lower in UC than in CD patients and undetectable in the control group, emphasizing the previously described polarity of EC in active IBD. We did not find a correlation between remission and decreased detection, as CDA patients showed lower EC DNA frequency than CDR patients. A possible explanation could be that the host inflammatory status during disease activity effects sufficient immune activation for partial EC clearance, but fails to affect the more intracellularly adapted MAP. Eighty percent (80 %) of selected samples from CD patients carried the *fimH30* allele, corresponding to an R166H amino acid change, strongly associated with AIEC [40]. The concomitant presence of both EC and MAP DNA was higher in all CD patient groups as compared to the equivalent UC groups, suggesting that both bacteria may contribute to the perpetuation of inflammation in CD, whilst MAP alone may contribute to UC immunopathology.

The considerable MAP prevalence in blood of the HC group (38 %) is consistent with previous studies [22] and may relate to the ubiquitous presence of MAP in the environment, milk and water reservoirs [6, 15]. It is also supportive of the proven MAP ability to adhere, cross normal human gut epithelium and persist in a range of both activated and naïve immune cell populations. Unsurprisingly, EC DNA was not detected in peripheral blood samples from HC, confirming the requirement for development of susceptible gut mucosal conditions before EC invasion can occur. The genomic homogeneity of human MAP strains isolated from IBD and non-IBD patients [21] and their high similarities with virulent bovine isolates excludes the possibility that specific virulence factors present in MAP strains are involved in CD development. Instead, it requires presentation, infection, invasion and chronic intracellular persistence combined, most possibly, with individual host susceptibility traits and perhaps the concomitant presence of AIEC, for MAP to participate in CD exacerbation. This is in line with thinking that MAP colonization may not be deleterious in itself to humans lacking susceptibility.

In accordance with previous studies [22], we found that CD patients on therapies including anti-inflammatory agents with potential anti-mycobacterial activity such as 5-ASA, presented with lower MAP DNA prevalence. Conversely, treatment with antibiotics resulted in higher MAP DNA prevalence. Reasons for this effect are unclear but could be related to antibiotic induced gut dysbiosis combined with inherent MAP antibiotic resistance that would possibly favor MAP persistence. Importantly, no correlation was found between any therapeutic regimen and EC DNA prevalence possibly because antibiotic treatment would not be expected to alter the expression of critical adherence receptors needed for EC involvement.

In conclusion, although increased intestinal permeability could influence MAP and EC colonization, EC was clearly more associated with CD alone, which is in favor of a major role of this pathobiont in CD. Nevertheless, the concomitant colonization of CD patients with both MAP and EC (the majority of which being suggestive of the AIEC phenotype) may be an important contributing factor in perpetuating inflammation, with the significant decrease in MAP DNA prevalence after anti-inflammatory therapy (associated with disease amelioration) supporting this hypothesis.

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Conflict of interest The authors declare that they have no competing interests.

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