Application of multiple laboratory tests for *Mycobacterium avium* ssp. *paratuberculosis* detection in Crohn's disease patient specimens

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SUMMARY

The difficulties involved in detecting and enumerating *Mycobacterium avium* subsp. *paratuberculosis* (MAP) as a pathogen potentially involved in Crohn's disease (CD) are well known. This study aimed to improve this situation through the application of multiple laboratory diagnostic tests to detect and isolate this bacterium from different specimens collected from CD-patients and non-CD subjects as controls.

A total of 120 samples (terminal ileum and colon biopsies, blood and stool) were obtained from 19 CD-patients and from 11 individuals who did not have a clinicopathological diagnosis of CD (non-CD controls) attending for ileocolonoscopy. All samples were processed by staining techniques, culture on both solid and liquid media, and Insertion Sequence 900/F57 real-time PCR.

The MAP frequency in CD-patients was found in a significantly greater proportion than in non-CD subjects; the most positive samples were biopsies from CD-patients tested by real-time PCR. MAP detection in biopsies, and in the other samples, by applying multiple and validated laboratory diagnostic tests, could be a marker of active infection, supporting MAP involvement in CD.

KEY WORDS: Crohn's disease, *Mycobacterium avium* subsp. *paratuberculosis*, Staining techniques, Culture, IS900/F57, Real-time PCR, Biopsies, Stool.

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INTRODUCTION

Crohn's disease (CD) is an idiopathic, relapsing, chronic, inflammatory disorder of the gastrointestinal tract. Although the etiology of CD is unknown, the pathogenesis is thought to be multifactorial, including microbial and environmental factors, immune dysfunction, and host susceptibility determined by genetic predisposition (Chamberlin *et al.,* 2001; Parrish *et al.,* 2009; Rosenfeld and Bressler, 2010; Lee *et al.,* 2011; Salem *et al.,* 2013a; Xia *et al.,* 2014). Given the similarity of clinical signs, pathology, and epidemiology between Johne's disease (JD) in ruminant animals, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and CD, coupled with positive diagnostic tests for MAP, some investigators have proposed the "MAP hypothesis" or rather the hypothesis that MAP, an obligate intracellular mycobacterium, is the etiologic agent of CD as an environmental factor (Collins *et al.,* 2000; Rosenfeld and Bressler, 2010; Tuci *et al.,* 2011; Agrawal *et al.,* 2014). In recent years, the question of the role of MAP in CD has aroused considerable controversy within the scientific and medical communities (Chiodini *et al.,* 1984; Sechi *et al.,* 2005; Rowe

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and Grant, 2006; Lee *et al.,* 2011). The list of autoimmune disease in which MAP has been implicated in a causal role is growing: not specific to MAP but mycobacteria in general has been found in several autoimmune diseases (Dow, 2012; Hruska and Pavlik, 2014). Moreover several gene defects are associated with mycobacterial infection and autoimmune diseases such as sarcoidosis, rheumatoid arthritis and multiple sclerosis (Dow, 2012; Dubaniewicz *et al.,* 2013; Hruska and Pavlik, 2014).

Characteristics distinguishing MAP from other *Mycobacterium* spp. include: its extremely slow growth, inability to produce mycobactin, possession of the IS900 genetic insertion and the F57 sequences (Schwartz *et al.*, 2000; O'Mahony and Hill, 2004; Herthenek and Bölske, 2006; Mendoza *et al.,* 2009; Sidoti *et al.,* 2011). IS900 occurs as 14-18 copies within the MAP genome but several publications report the presence of IS900-like sequences in other closely related environmental mycobacterial species (Schwartz *et al*., 2000; Herthenek and Bölske, 2006). On the other hand, the F57 gene is present in a single copy in the MAP genome and it has no known similarities to genes on other related organisms (Herthenek and Bölske, 2006; Sidoti *et al.,* 2011). Starting from these observations and with improvements in microbiologic and genetic techniques, the renewed interest in the possible relationship between MAP and CD is due to new cultural approach and to MAP DNA sequences discovery (Chamberlin *et al.,* 2001; Parrish *et al.,* 2009; Rosenfeld and Bressler, 2010; Sidoti *et al.,* 2011).

In this context, the present study was carried out to define the potential prevalence of MAP in people with CD by assaying their different clinical samples at the same time: fresh mucosal biopsies, blood and stool. To accomplish this goal we applied multiple laboratory diagnostic tests for MAP, such as staining techniques (Kinyoun and phenolic acridine orange stains), isolation on both cultural solid American Type Culture Collection (ATCC) medium and liquid Mycobacterial Growth Indicator Tube (MGIT) medium and validate (Sidoti *et al.,* 2011), sensitive and reliable two independent real-time Polymerase Chain Reaction (PCR) assays targeting both the standard marker IS900 and specific target F57.

MATERIALS AND METHODS

Study design

The study was designed according to the principles of good clinical practice and to the Declaration of Helsinki principles.

A total of 76 samples were obtained from 19 CD-patients (19 pairs of endoscopic mucosal biopsies from terminal ileum and colon, 19 blood and 19 stool specimens) and 44 samples from 11 individuals without a clinicopathological diagnosis of CD, assigned to the non-CD controls (11 pairs of endoscopic mucosal biopsies from terminal ileum and colon, 11 blood and 11 stool specimens), all attending for ileocolonoscopy at the Gastroenterology Division of Mauriziano Hospital, Turin, Italy, for a total of 120 samples. The CD diagnosis, established on standard clinical, endoscopic, histological, and radiographic criteria, was categorized in remission, mild or moderate-to-severe activity, according to commonly accepted criteria (ECCO guidelines) (Van Assche *et al.,* 2010) at least 6 months before index colonoscopy. Consecutive patients were eligible if they gave their written consent to the study before colonoscopy and if active disease was within the reach of colonoscopy. Exclusion criteria were known tubercular infection or latent tuberculosis status, active infections with positive stool cultures, absence of consent to the procedure. A medication history was completed for each participant. Active immunosuppressive therapy (azathioprine and/or anti-TNF agents such as Infliximab or Adalimumab) was administered; none of the patients received previous or concomitant anti-tubercular treatment. The ethical approval for this study was given by the Local Ethics Committee.

Pairs of endoscopic mucosal biopsies were taken from the terminal ileum and colon both from visibly inflamed regions in CD-patients and from several regions throughout the intestine in non-CD controls.

Tissue and stool samples were placed in Portagerm flacons (bioMerieux Italia S.p.A., Rome, Italy). Blood samples were drawn into two sterile K_2-EDTA Vacutainer tubes (Becton Dickinson, Franklin Lake, NJ, USA). All clinical specimens obtained from every participant were coded to conceal the patient's identity and the diagnosis,

to ensure a blinded study, transported immediately to Bacteriology and Mycology laboratory of Department of Public Health and Pediatrics, University of Torino, and processed blinded within 1 hour in a class II biosafety hood. Here, each tissue sample, specifically colon and ileum biopsies, was cut into three parts: the first part was divided and placed on two microscope slides for the two staining techniques; the second one was divided and inoculated in the two different cultural media; the third part was used for DNA extraction and then subjected to real-time PCR (IS900 and F57).

Staining method for microscopy

Smears were prepared by placing endoscopic mucosal biopsies, blood or stool specimens on a microscope slide. The smears were heat-fixed and stained using either the Kinyoun acid-fast stain, targeting MAP cell wall, to detect mycobacterial acid fast bacilli or the phenolic acridine orange, targeting MAP nucleic acids, to detect spheroplasts by fluorescence. Samples were considered positive when mycobacteria were present in at least 5 oil-immersion fields per sample.

Mycobacterial culture

Tissue and stool samples for mycobacterial culture were decontaminated using BBL MycoPrep according to the manufacturer's recommendations (Becton Dickinson, Pittsburgh, PA, USA) and re-suspended in 2 ml of phosphate-buffered saline (pH 6.8). Volumes of 0.2 and 0.5 ml of all decontaminated samples were used to inoculate one tube of ATCC medium and one of MGIT (Becton Dickinson) medium, respectively. The ATCC Medium was prepared as previously described in detail by our research group (Sidoti *et al.,* 2011), whereas the MGIT medium was supplemented with OADC (oleic acid, bovine albumin, dextrose, catalase), mycobactin J (2 mg/l, Allied Monitor) and PANTA antibiotic mixture as described by the manufacturer (Becton Dickinson). All inoculated media were incubated at 37°C, checked once a week. In the meantime, a positive control inoculated with MAP strain ATCC 43015 and a negative control was performed as we previously described (Sidoti *et al.,* 2011). The ATCC medium tubes were screened for the appearance of colonies. The MGIT tubes were visually examined for visible turbidity and fluorescence-quenching activity with a 365-nm UV-illuminator. A MGIT culture was classified as negative or positive based on the fluorescence intensity and turbidity observed in each tube relative to the inoculated positive and un-inoculated negative control. All negative cultures were incubated for at least 1 year before being discarded.

DNA extraction and IS900/F57 Real-Time PCR

DNA from original samples (biopsies, fresh stool and fresh blood) was extracted using DNeasy® Blood & Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The samples were homogenized with TissueRuptor (Quiagen) with the intent to release more nucleic acid before extraction. As we previously described in detail (Sidoti *et al.,* 2011), each DNA extract was subjected to real-time TaqMan PCR analysis for the presence of the IS900 and F57 amplified fragments. MAP-DNA was quantified. Appropriate controls were used including a DNA template from MAP strain ATCC 43015 as positive control.

MAP positive samples confirmation

Upon termination, all MGIT positive cultures were subsequently confirmed: they were concentrated by centrifugation, stained either with Kinyoun or phenolic acridine orange, and used for MAP DNA extraction and IS900/F57 real-time PCR as described above.

Statistical analysis

All results of staining, culture and double real-time PCR between CD and non-CD subjects were evaluated by the Fisher's exact test (two tailed) run on a GraphPad Prism 6 version (GraphPad Software, Inc., La Jolla, CA 92037, USA) and both P values ($P<0.05$) and odds ratios (OR) with 95% confidence intervals were indicated.

RESULTS

The code for concealment of participant's identities and diagnoses was broken and data were tabulated after the conclusion of all experi-

TABLE 1 - *Demographic information on Crohn's disease (CD) and non-CD patients.*

	CD patients	*Non-CD patients*
Total number of subjects	19	11
Age at sampling, years (range)	42.6 (23-78)	58.9 (32-75)
Gender, female no. (%)	11 (58%)	6 (55%)
Disease duration, years (range)	10 (1.8-18)	NA
Ileal location, no. (%)	12 (63%)	NA
Clinically active disease, no. (%)	9 (47%)	NA
Endoscopically active disease, no. (%)	7 (37%)	NA
Active immunosuppressive or biological treatment, no. (%)	10 (53%)	NA
C-reactive protein, mg/l (range)	7.5 (0-20)	NA

Age, disease duration and C-reactive protein levels are expressed as median and range; NA: not applicable.

ments. The demographics information of CD and non-CD subjects is presented in Table 1. As expected, ileal location of disease, alone or most frequently combined with right colonic involvement, was the most prevalent disease location; almost half of the patients underwent colonoscopy on an active flare of disease. Notably a large proportion of the patients (53%) were on active immunosuppressive treatment, as the result of a selected population of a tertiary referral center.

The microbiological results are presented in Tables 2-3 and Supplemental Tables S1-S2. The initial Kinyoun stain smears obtained from all samples, obtained from 19 CD and 11 non-CD patients, revealed the presence of acid-fast bacilli in 3/76 CD and 0/44 non-CD samples in a no statistical significant way (P=1.000). Meanwhile, no positivity for cell wall deficient forms was detected for samples stained by phenolic acridine orange in either CD or non-CD samples.

TABLE 2 - *Screening of biopsies, blood and stool by Kinyoun stain, culture and real-time PCR for the presence of Mycobacterium avium subspecies paratuberculosis in Crohn's disease (CD) and non-CD patients.*

	Positive samples/total samples and %					
	Phenolic acridine orange stain	*Kinyoun stain*	*ATCC medium*	*MGIT medium*	*IS900 real-time PCR*	*F57 real-time PCR*
CD samples	0/76 (0%)	3/76 (3.95%)	0/76 (0%)	13/76 (17.11%)	40/76 (52.63%)	40/76 (52.63%)
Non-CD samples	0/44 (0%)	0/44 (0%)	0/44 (0%)	2/44 (4.55%)	4/44 (9.09%)	4/44 (9.09%)
P value based on Fischer's exact test	*P*=1.000	*P*=0.2975	*P*=1.000	*P*=0.0497*	*P*<0.0001*	*P*<0.0001*
Odd Ratio	OR=0.5817 (0.01133-29.86)	OR=4.238 (0.2137-84.04)	OR=0.5817 (0.01133-29.86)	OR=4.333 (0.9295-20.20)	OR=11.11 (3.617-34.14)	OR=11.11 (3.617-34.14)

**P* value <0.05 was judged to be significant. ATCC: American Type Culture Collection; MGIT: Mycobacterial Growth Indicator Tube; IS: Insertion Sequence; PCR: Polymerase Chain Reaction.

TABLE 3 - *Global evaluation by site of Mycobacterium avium subspecies paratuberculosis detection in Crohn's disease (CD) and non-CD patients by IS900/F57 real-time PCR.*

	Positive samples/total sample type and %			
	Ileum biopsy	*Colon biopsy*	*Blood*	*Stool*
CD samples	17/19 (89.47%)	17/19 (89.47%)	5/19 (26.32%)	1/19 (5.26%)
Non-CD samples	0/11 (0%)	0/11 (0%)	3/11 (27.27%)	1/11 (9.09%)
P value based on Fischer's exact test	*P*<0.0001*	*P*<0.0001*	*P*=1.000	*P*=1.000
Odd Ratio	OR=161.0 (7.030-3672)	OR=161.0 (7.030-3672)	OR=0.9524 (0.1784-5.083)	OR=0.5556 (0.03124-9.880)

**P* value <0.05 was judged to be significant.

TABLE S1 - *Mycobacterium avium subspecies paratuberculosis detection in non-Crohn's disease (non-CD) subjects by stain, cultures and real-time PCRs.*

		Diagnostic techniques					
Non-CD subjects	*Clinical samples*	*Kinyoun stain*	*Phenolic acridine orange stain*	*ATCC medium*	*MGIT medium*	*IS900 real-time PCR*	*F57 real-time PCR*
1	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
2	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	+	+
3	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	+	+	+
	Blood	-	-	-	-	-	-
4	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
5	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
6	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	+	+
7	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
8	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	+	-	-
	Blood	-	-	-	-	-	-
9	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
10	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
11	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	+	+

ATCC: American Type Culture Collection; MGIT: Mycobacterial Growth Indicator Tube; IS: Insertion Sequence; PCR: Polymerase Chain Reaction.

TABLE S2 - *Mycobacterium avium subspecies paratuberculosis detection in Crohn's disease (CD) patients by stain, cultures and real-time PCRs.*

		Diagnostic techniques					
CD patients	*Clinical samples*	*Kinyoun stain*	*Phenolic acridine orange stain*	*ATCC medium*	*MGIT medium*	*IS900 real-time PCR*	*F57 real-time PCR*
1	Ileum biopsy	-	-	-	+	+	+
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	+	+	+
2	Ileum biopsy	-	-	-	+	+	+
	Colon biopsy	-	-	-	+	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
3	Ileum biopsy	-	-	-	+	+	+
	Colon biopsy	-	-	-	+	+	+
	Stool	-	-	-	+	+	+
	Blood	-	-	-	-	+	+
4	Ileum biopsy	-	-	-	-	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	+	+
5	Ileum biopsy	-	-	-	-	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
6	Ileum biopsy	-	-	-	+	+	+
	Colon biopsy	-	-	-	+	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
7	Ileum biopsy	-	-	-	-	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
8	Ileum biopsy	+	-	-	-	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
9	Ileum biopsy	-	-	-	-	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
10	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	-	-
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
11	Ileum biopsy	-	-	-	-	+	+
	Colon biopsy	+	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	+	+

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		Diagnostic techniques					
CD patients	*Clinical samples*	*Kinyoun stain*	*Phenolic acridine orange stain*	*ATCC medium*	*MGIT medium*	*IS900 real-time PCR*	*F57 real-time PCR*
12	Ileum biopsy	-	-	-	-	-	-
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
13	Ileum biopsy	-	-	-	-	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
14	Ileum biopsy	-	-	-	-	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
15	Ileum biopsy	-	-	-	-	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
16	Ileum biopsy	+	-	-	+	+	+
	Colon biopsy	-	-	-	+	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
17	Ileum biopsy	-	-	-	-	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-
18	Ileum biopsy	-	-	-	-	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	+	+	+
19	Ileum biopsy	-	-	-	+	+	+
	Colon biopsy	-	-	-	-	+	+
	Stool	-	-	-	-	-	-
	Blood	-	-	-	-	-	-

ATCC: American Type Culture Collection; MGIT: Mycobacterial Growth Indicator Tube; IS: Insertion Sequence; PCR: Polymerase Chain Reaction.

The positivity for MAP by culture on both ATCC and MGIT media was detected after 1 year of incubation. No MAP growth was observed for samples from both 19 CD and 11 non-CD subjects on the solid ATCC medium. On the contrary, MAP growth in MGIT was shown in 13/76 CD *versus* 2/44 non-CD samples (P=0.0497). All cultures were negative for bacterial contamination or evidence of other microbial flora. All negative and positive cultural media control setup during sample processing were confirmed to be negative and positive, respectively. All 15 MGIT cultures from both CD and non-CD individuals in which a possible mycobacterial growth was detected were assessed for the presence of MAP either by staining techniques or IS900/F57 real-time PCR. MAP positive cultures of samples, negative by staining techniques during the early weeks of culture incubation, were positively by Kinyoun stain

or phenolic acridine orange after 14 weeks for acid-fast bacilli and occasional presence of deformed cells containing remaining cell wall components.
For CD samples assayed by real-time PCR, 17/19 biopsies both from ileum and colon, 5/19 blood and 1/19 stools were positive for MAP IS900 and F57 sequences. For non-CD samples only 3/11 blood and 1/11stools were positive. All real-time mastermix and extraction reagent negative controls were negative and all real-time PCR MAP DNA positive controls were positive.

DISCUSSION

Until recently, the difficulty in detecting MAP in affected tissues left open the question of the role MAP might play in the pathogenesis of CD. Improvements in isolation and detection methods have now revealed that MAP can be detected in affected tissues as well as in peripheral blood, stool and breast milk from patients with CD (Naser *et al.,* 2004; Parrish *et al.,* 2009; Allen *et al.,* 2011).
Within the limitations of the present study due to the small number of patients, to the best of our knowledge this is the first paper reporting data on MAP detection from different human clinical samples (biopsies, blood and stool samples) collected from both CD and controls at one and at the same time by different multiple diagnostic tests such as staining techniques, culture on ATCC and MGIT media, and IS900/F57 real-time PCR.
Different staining techniques to detect MAP have been used for a long time, "classic" ones such as Ziehl Neelsen or Kinyoun stain, targeting the MAP cell wall, or those who target its nucleic acids by fluorescence (mainly auramine rhodamine or phenolic acridine stain), and recently *in situ* hybridization. Literature data on their sensitivity and specificity are still conflicting (Sechi *et al.,* 2001; Naser *et al.,* 2004; Sechi *et al.,* 2005; Jeyanathan *et al.,* 2006; Jeyanathan *et al.,* 2007; Mendoza *et al.,* 2009). In fact, "classic" techniques are not helpful for spheroplast detection, and other stains, such as auramine rhodamine, could generate positive signals in controls, indicating the nonspecificity of these assays. In addition, *in situ* hybridization is superior in sensitivity and specificity to common staining approaches, but is quite difficult to achieve as a routine methodology. In this scenario, MAP stain results should be interpreted with caution: in fact MAP negativity by Kinyoun stain in CD-patients is compatible with the theory that MAP in man is present in spheroplasts frequently reported in the literature (Singh *et al.,* 2008). Our stain results (Table 2) showed only 3/76 (3.95%) samples, from 19 CD-patients positive to Kinyoun stain, and no CD sample positive for phenolic acridine stain, confirming the literature data (Naser *et al.,* 2004; Sechi *et al.,* 2005; Mendoza *et al.,* 2009). The initial smears from fresh biopsies were negative by staining indicating that staining usually underestimates MAP presence and cannot correctly identify the microorganisms: hence it does not seem to be as accurate as routine techniques except as a confirming protocol after culture growth.
According to the literature, the gold standard for MAP detection is based on its isolation using culture methods (Mendoza *et al.,* 2009; Pozzato *et al.,* 2011). However, MAP recovery in culture is complicated by the requirement for special nutritional components and long incubation periods since culture for months, or even years, has been necessary before conversion of the cell wall-deficient form to acid fast bacilli (El-Zaatari *et al.,* 2001; Singh *et al.,* 2008). We chose two different cultural media according to other authors who reported the superiority of the liquid medium for its higher analytical sensitivity and faster results than the solid one (Mendoza *et al.,* 2009; Pozzato *et al.,* 2011). From our results (Table 2) MGIT liquid culture was significantly more sensitive than conventional culture on the solid ATCC medium ($P<0.0001$; OR=0.02824), confirming the literature. MAP positive cultures of CD samples, negative by staining techniques during the early weeks of culture incubation, were positive by acridine orange after 14 weeks for acid-fast bacilli pre-spheroplasts (Naser *et al.,* 2004; Sechi *et al.,* 2005; Mendoza *et al.,* 2009).
Although conventional culturing remains the gold standard for detection of MAP, nowadays serological methods and PCR detection are widely-used alternatives (Schwartz *et al.,* 2000; O'Mahony and Hill, 2004; Feller *et al.,* 2007)

to discern the two MAP forms: cell wall-defective and cell wall-intact bacteria. In our study (Tables 2, 3) a significantly different positivity among CD samples compared to that among controls was detected by applying the two specific and sensitive real-time assays targeting the IS900 and F57 for MAP DNA detection.

Other samples easier to collect such as blood and stool (Tuci *et al.,* 2011) were obtained from all participants besides the biopsies always considered irreplaceable samples showing the site of disease (Sechi *et al.,* 2005). Our results indicated that for the PCR positive CD-patients the majority were biopsies (17/19) consistent with an active infection with CD, confirming that biopsies remain the gold standard sample, followed by blood (5/19) and stool specimens [(1/19); Table 3; Supplemental Tables S1-S2]. MAP presence not only in biopsy but also in blood and stool samples from CD-patients should not be surprising if we consider that in animals with JD MAP multiplies in the intestinal mucosa, is shed in feces, and the infection is systemic (Singh *et al.,* 2008; Falkinham, 2009; Singh *et al.,* 2010; Dow, 2012). Naser and co-workers (2004) isolated MAP from CD-patients' blood and suggested that, as in animals, MAP infection in humans is systemic related to the inability of macrophages in CD to kill MAP (Mendoza *et al.,* 2009). Detection of viable MAP in stool samples could be attributable to the fact that in CD-patients multiple extra-intestinal manifestations occur (Das and Seril, 2012). It has to be emphasized however that from our results it emerges that among controls 3/11 blood and 1/11 stool samples were positive for MAP by both real-time PCRs (Table 3; Supplemental Tables S1-S2), suggesting that MAP is common in the environment with exposure through the food and water supply, consistent with a state of colonization or dormancy in the absence of CD (Naser *et al.,* 2004; Falkinham, 2009). Our results on stool samples are in contrast to those from Tuci and colleagues (2011) probably because of the different methodologies used (sample collection/processing and PCR assay) and the CD-patient characteristics. In addition, the similar higher MAP detection in stools, for both CD-patients and healthy subjects, obtained by Tuci, could indicate an overestimated presence of MAP in this sample type maybe due to its transient presence in gut normal flora. Indeed no other sample, such as biopsy or blood, was in parallel obtained and processed to confirm these data (Tuci *et al.,* 2011).

The global evaluation by site (Table 3) and patient groups (Supplemental Tables S1-S2*)* highlights a MAP frequency in CD-patients (18/19; 94.74%) significantly higher (P=0.0011; OR=31.50; 2.976-333.4) than in non-CD (4/11; 36.36%) confirming other studies from Italy (Chiodini *et al.,* 1984; Sechi *et al.,* 2005; Molicotti *et al.,* 2013) and worldwide (Collins *et al*., 2000; Naser *et al*., 2004; Singh *et al*., 2008; Mendoza *et al.,* 2009; Singh *et al.,* 2010; Lee *et al.,* 2011). Controversy over the "MAP hypothesis" in CD continues due to conflicting evidence and differences in laboratory techniques among the studies (Chiodini *et al.,* 2012; Gitlin *et al*., 2012; Salem *et al.,* 2013b).

Among the CD group, 53% received immunosuppressants (Table 1) that did not correlate with positive culture results, indicating no influence on viable MAP in specimens, as other authors have postulated (Mendoza *et al.,* 2009). Immune suppression promotes *M. tuberculosis* dissemination; however MAP itself is incapable of dissemination and surviving outside the human cells (Das and Seril, 2012). Therefore the role of immunosuppressive medication on MAP survival/dissemination requires further investigation. This is a small case-control study that attempted to correlate the presence of MAP with CD-patients. We can speculate that detection of MAP in biopsies, blood and stool, blinded to patient disease status, by applying multiple and validated diagnostic tests, could be a marker of active infection.

Among the diagnostic tests assayed, we can assume that real-time PCR is the most accurate approach for MAP detection followed by MGIT culture, even if it is not possible to sustain MAP viability by PCR, whereas it is possible by culture. Intriguingly, these data suggest that MAP was detected by PCR either when it had died, leaving behind its molecular footprint (IS900/F57 sequences), or viable but in a non-culturable state. The staining methods are not as accurate as routine techniques, except as a confirming protocol after culture growth. Our findings do not prove that MAP is a cause of CD, but they suggest that any form of MAP in

specimens of CD-patients would support its association with the disease even if CD pathology could require both microbial and host genetic factors. Investigation of the MAP role played in CD pathogenesis provides fruitful avenues for future research: further studies will be necessary to investigate immune response and genetic role on a larger number of CD and non-CD subjects.

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In memory of Raffaello Sostegni, he recently passed away..but his ideas still live with us.

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