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of the Food Safety Authority of Ireland

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Mycobacterium avium subsp. *paratuberculosis* and its links to Crohn's Disease

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Abbreviations

°C	degrees Celsius
CD	Crohn's disease
DAFM	Department of Agriculture, Food and the Marine
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
et al.	abbreviation for *et alia*: and others
EU	European Union
FAO	Food and Agriculture Organization
FSAI	Food Safety Authority of Ireland
IBD	inflammatory bowel disease
IJCP	Irish Johne's Control Programme
IMRL	Irish Mycobacterial Reference Laboratory
JD	Johne's disease
km	kilometer
L	liter
MAC	*Mycobacterium avium* complex
Map	*Mycobacterium avium* subsp. *paratuberculosis*
MsC	Masters of Sciences
PCR	polymerase chain reaction
PhD	Doctor of Philosophy
PMS	peptide-mediated magnetic separation
qPCR	quantitative PCR
s	second
S.I.	Statutory Instrument
subsp.	subspecies
UK	United Kingdom
US	United States of America
WGS	whole genome sequencing
WHO	World Health Organization

1. Executive summary

Mycobacterium avium subsp*. paratuberculosis* (*Map*) is the causative agent of paratuberculosis or Johne's disease in cattle (JD). Similarities between JD in cattle and Crohn's Disease (CD), a type of inflammatory bowel disease (IBD), in humans have prompted speculation on a possible role for *Map* in the pathogenesis of CD.

In 2000, the Microbiology Subcommittee of the Food Safety Authority of Ireland (FSAI) reviewed the evidence of causality between *Map* and CD (FSAI, 2000). The principal conclusion of that report was that the available data were inconclusive and a direct link between *Map* and CD could not be established. However, the report recommended that the Committee keep the issue under review. In 2008, the FSAI Scientific Committee conducted a more formal review of the research and published an opinion on the topic. Fifty-six key publications were identified between 2000 and 2008. Based on a review of those papers, the Committee concluded that the balance of available evidence did not support a causal relationship between *Map* and the incidence of CD (FSAI, 2009).

In 2020, an *ad-hoc* subcommittee of the Scientific Committee of the FSAI reviewed primary, peer-reviewed papers published in the scientific literature between 2009 and 2019 which reference the putative link between *Map* and CD. Numerous studies published during that period provide evidence of an association between the presence of *Map* or human exposure to *Map* and the occurrence of CD. However, no new evidence has been published to substantiate the suggestion that this association is causal.

The subcommittee also reviewed recently published papers (2009-2019) on the efficacy of thermal pasteurisation at inactivating *Map*. This review suggests that viable *Map* is unlikely to be found in milk that has been pasteurised at a time-temperature combination of at least 75°C for 20 seconds.

Finally, the Committee considered current gaps in knowledge which impact on the ability to assess the risk that *Map* poses to human health and the risk that humans could be exposed to *Map* in food; further studies to address these knowledge gaps are suggested.

2. Background

Mycobacterium avium subsp*. paratuberculosis* (*Map*), the causative agent of paratuberculosis or Johne's disease (JD) in cattle, was first identified in Germany in 1906, with the disease subsequently diagnosed in cattle across Europe, Asia and the US *Map* infection is more frequently found on dairy farms than on beef farms and in larger cattle herds. Transmission is mainly faecal-oral, with infection of cattle most usually occurring when they are calves. JD occurs in adult cattle as a chronic wasting disease – a protein losing enteropathy associated with intractable diarrhoea. It is a granulomatous enteritis, and the inflammatory changes are usually confined to the mucosal layer of the ileum. *Map* can also infect small ruminants and deer and can cause JD in those species. The disease has also been reported in non-ruminants such as wild rabbits, foxes and stoats. There are various direct and indirect test methods used to detect *Map* and antibodies to *Map*, respectively.

Similarities between JD in cattle and Crohn's Disease (CD), a type of inflammatory bowel disease (IBD), in humans have prompted speculation on a possible role for *Map* in the pathogenesis of CD. However, there are also significant differences between both disease entities. CD lesions tend to occur along the gastro-intestinal tract from the mouth to the rectum and are usually transmural. While there is an unequivocal causal association between *Map* infection and the occurrence of JD in cattle, there is conflicting evidence and opinion on the association between human infection with, or exposure to, *Map* and CD. Numerous studies have reported the detection of viable *Map* and *Map* DNA in intestinal biopsies, faeces and blood of CD patients. Because of the chronic inflammation and disruption of the mucosal barrier, the intestinal permeability may be higher/defective facilitating the entry of *Map* into the systemic circulatory systems of the host (Garvey, 2018). Furthermore, there is no evidence that people with occupational exposure to *Map* such as farmers and veterinarians are at any greater risk of developing CD than the general population. In addition, anti-mycobacterial treatments (effective against *Map*) do not provide a long-term cure for CD patients, as might be expected if *Map* were the cause of disease (Agrawal *et al.*, 2020; McNees *et al*., 2015).

In 2000, the Microbiology Subcommittee of the FSAI reviewed the evidence of causality between *Map* and CD (FSAI, 2000). The principal conclusion of that report was that the available data were inconclusive and a direct link between *Map* and CD could not be established. However, the report recommended that the Committee keep the issue under review. In 2008, the FSAI Scientific Committee conducted a more formal review of the research and opinions published in the years since the original report in 2000. Fifty-six key publications were identified between 2000 and 2008. Based on a review of those papers the Committee concluded that the balance of available evidence did not support a causal relationship between *Map* and the incidence of CD (FSAI, 2009).

Two considerations, explicitly referenced in the 2000 report, may have informed the views of both expert panels. The 2000 report states that "*The current incidence of Johne's disease in Irish cattle is low*" and that "*The sale of unpasteurised cows' milk for direct human consumption has been banned since 1 August 1997*". Neither of these statements currently hold true because of changes in the intervening years.

Firstly, there is evidence of an increase in the prevalence of *Map* infection in Irish cattle over the past two decades. The opening of the single market in 1992 with free movement of animals (and importation of large numbers of breeding cattle) is considered to have been a significant factor. A seroprevalence study conducted in 2005 estimated that 18% and 32% of Irish beef and dairy herds, respectively, have been exposed to *Map* (Good *et al.*, 2009). More recently, McAloon *et al.* (2016) using 2013-2014 data, estimated that 28% of Irish dairy herds contain at least one truly positive animal. In addition, the expansion of the Irish dairy herd following the removal of quota restrictions on milk production in 2015 is likely to favour further transmission of *Map* (McMahon *et al*., 2015). The increased sales of breeding animals and increased stocking density that accompany expansion is likely to favour both between-herd and within-herd transmission. To address these concerns, the Irish Johne's Control Programme (IJCP) was established in 2013. The IJCP is an industry-led national voluntary programme whose objectives include (1) to enhance the ability of participating farmers to keep their herds clear of JD, (2) to assist participating farmers to reduce the level of infection in their herds, where present, (3) to provide additional reassurance to the marketplace in relation to Ireland's efforts to control JD and (4) to improve calf health and farm biosecurity in participating farms. At the end of 2020, 1,740 dairy herds (and 10 beef herds) were participating in the IJCP, representing 11% of the dairy herds in Ireland (Animal Health Ireland, 2020).

Secondly, the 1996 Irish legislation banning the sale of unpasteurised milk was superseded by an EU directive in 2006. Raw milk is a ready to eat product with well documented potential for contamination by pathogens (EFSA, 2015). Following the introduction of Statutory Instrument (S.I.) 306 of 2015, the European Communities (Food and Feed Hygiene) Regulations 2020 (S.I. 22 of 2020) now apply to primary producers supplying in excess of 30 L of raw milk per week, for direct human consumption, or supplying any quantity in excess of a 20 km radius from their establishment. Such producers must register with the Department of Agriculture, Food and the Marine (DAFM) Milk Hygiene Division. A niche market exists for raw milk and guidelines have been published for raw milk producers (Raw Milk Ireland, 2020).

Another significant change informing risk assessment is the application of whole genome sequencing (WGS) to *Map* isolates from human and animals. WGS provides for greater resolution of genetic differences between *Map* isolates than previous techniques allowed. The WGS

approach has revealed close genetic relationships between *Map* isolated from humans and animals in the same country (Bryant *et al*., 2016). Increasing use of WGS on *Map* isolates promises to provide new insight into pathogen transmission dynamics.

Previous expert groups who reviewed this topic, have advised that it should be kept under review. Therefore, over 10 years on from the last review by the FSAI Scientific Committee and with the publication of further research, with the above changes impacting on risk profile and with the advent of new technologies, it is time to revisit this question.

Consequently, the FSAI issued a request for advice (Appendix 1) to the Scientific Committee posing three questions:

1. Does the balance of the available scientific literature support a link between *Map* and Crohn's disease?
2. Does *Map* survive pasteurisation treatments applied by the dairy industry to milk and dairy products and if so, what if any pasteurisation time/temperature combination would be sufficient to inactivate *Map*?
3. Are there areas of research necessary to reduce uncertainty in the risk assessment?

The Scientific Committee convened a multidisciplinary *ad hoc* subcommittee to consider these questions and to provide a report which was discussed and adopted in December 2020.

3. Methodology

A traditional (narrative) literature review was conducted with the objective of collating the most relevant publications for consideration on the putative link between *Map* and CD and the efficacy of thermal pasteurisation at inactivating *Map*. Prior to database searching, a systematic methodology was designed, with a view to minimising bias, and to identify all studies meeting pre-defined eligibility criteria (Table 1). Additional references from 2008 which were not included in the 2009 FSAI report were also included for completeness.

Table 1 Literature review methodology

Exclusion criteria	Search strategy
▪ Non-English Publications ▪ Patent ▪ MSc/PhD Theses ▪ Editorials ▪ Book Chapters ▪ Conference Proceedings	▪ Search conducted using electronic databases: Web of Science, PubMed and Google Scholar. ▪ Search terms: (mycobacterium avium subsp. paratuberculosis) and (Crohn's); (mycobacterium avium subsp. paratuberculosis) and (pasteurization); (mycobacterium avium subsp. paratuberculosis) and (milk); (mycobacterium avium subsp. paratuberculosis) and (inactivation); (mycobacterium avium subsp. paratuberculosis) and (heat treatment) ▪ Performed searches to include abbreviations [e.g. (MAP), (CD)], synonyms [e.g. (thermal treatment)] and spelling variants [e.g. (Crohns), (Crohn*)] ▪ Searches to be limited to studies published from January 2009 to June 2019. ▪ From performed searches, application of exclusion criteria. Of the resulting searches, full articles to be retrieved and added to an internal database for application of inclusion criteria. ▪ Duplicate entries to the database to be removed. ▪ Search results not to be excluded based on publication status (accepted/in press).
Inclusion criteria	
▪ Peer-reviewed papers ▪ Primary research/Reviews ▪ Non declared conflicts of interest ▪ Topics: *Map* detection in humans, *Map* transmission to humans, genetics of CD, treatment of CD, immunology, *Map* thermal inactivation in milk.	

To establish if similar risk assessments have been undertaken in other EU member states in recent years, the EFSA focal point network was used to request an exchange of information. This network is an interface for collaboration between EU Member States, Iceland, Norway and observers from Switzerland and EU candidate countries. During the consultation period the Czech Republic forwarded the information received in relation to a similar but broader question posed in January 2019. The German authorities reported that they had published a literature review in 2003

which did not find strong evidence for an association between non-tuberculous mycobacteria[1] and CD; and the Dutch authorities provided a review of the literature and a case-control study they had published in 2004 and 2005, respectively. Other responses cited published studies on pigs and cattle, pneumonia, autoimmune diseases, drinking water and pasteurisation. Relevant papers that fell within the reference period (Jan 2009 – June 2019) and that had not already been captured during the initial search were considered for selection.

This search strategy resulted in a total of 83 papers (Figure 1).

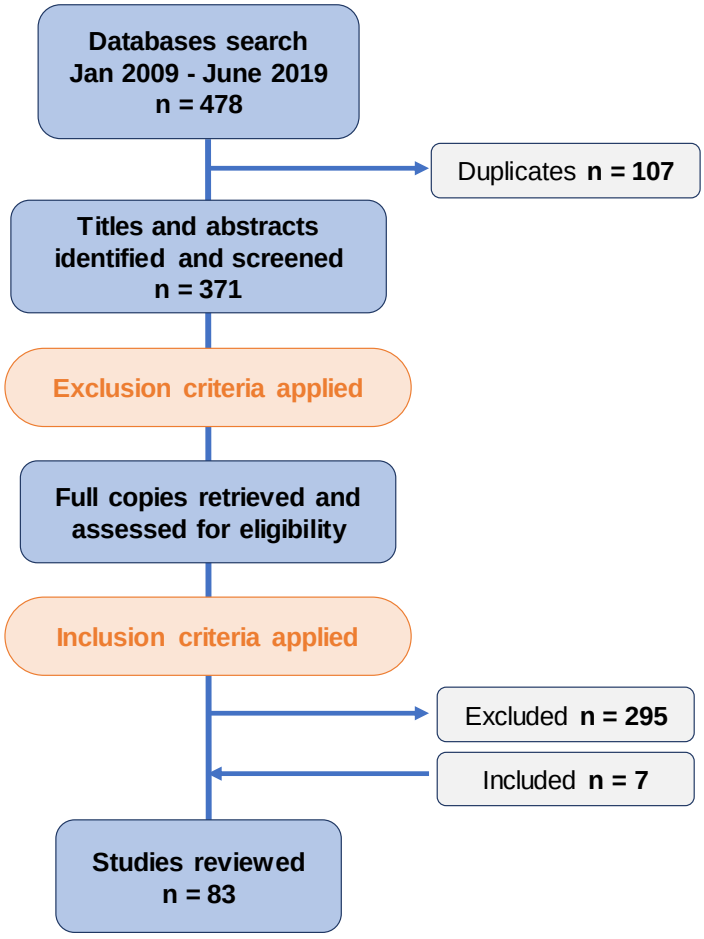

Figure 1: Flow chart of study selection process

[1] Nontuberculous mycobacteria or atypical mycobacteria are mycobacteria other than *M. tuberculosis* (the cause of human tuberculosis) and *M. leprae* (the cause of human leprosy).

4. Question 1 - Does the balance of the available scientific literature support a link between *Map* and Crohn's disease?

4.1 Introduction

A link was made between paratuberculosis or Johne's Disease (JD) in cattle and Crohn's Disease (CD) in humans after both disease entities were initially described. This was because of the superficial similarities between the appearance of the two diseases – both diseases featuring a granulomatous enteritis. However, the distribution of inflammatory change along the gastro-intestinal tract and across the intestinal wall differs in humans and in cattle. Consequently, ever since *Mycobacterium avium* subsp*. paratuberculosis* (*Map*) was identified as the definitive aetiological agent of JD, some scientists have speculated that it could also be involved in the aetiology of CD. Occasional studies continue to appear in the peer-reviewed scientific literature which purport to provide evidence substantiating this view. Clearly this is evidence of "a link" being made between *Map* and CD but a more pertinent and specific question to ask is whether there is evidence of a causal relationship between *Map* and CD, and the strength of that evidence.

Koch's postulates are four microbiological criteria that were designed in the late 19th century to establish a causative relationship between a microbe and a disease. These postulates were generated prior to the clarification of many of the modern concepts of microbial pathogenesis. Consequently, they have been replaced by epidemiological principles or guidelines for causal association, such as the Bradford Hill Criteria. Lowe *et al.* (2008) highlighted the challenge posed by the abundance and diversity of the microflora in the gastrointestinal tract (GIT) in bridging the gap between an epidemiological association and one of causality for GIT diseases. They applied both Koch's postulates and the Bradford Hill criteria in respect of a few chronic idiopathic intestinal diseases, including CD, and in the case of CD considered two candidate microbes as cause – *Map* and adherent-invasive *Escherichia coli*. A review by Belkaid and Hand (2014) reports that adherent-invasive *E. coli*, *Yersinia* and *Clostridium difficile* are much more common in patients suffering from CD than healthy individuals and in some mouse models have been shown to be key contributors to IBD.

4.2 Results

On both the direct detection of *Map* in patients with CD and human immunological responses to *Map*, there was a lot of variability in study design and in the methodologies used and complete

information on design or method was not always available in published papers. The search identified a small number of papers on transmission of *Map* to humans, of which four were deemed relevant, and a few papers on treatment of CD. Small study size was a concern with most of the published studies. In addition, some of these studies, although referring to a causal link between CD and *Map,* did not provide any new evidence in support of this.

Direct detection of Map in Crohn's disease – case-control studies 2009–2019

Fourteen case-control studies which attempted to directly detect *Map* in clinical specimens from CD patients were identified (Appendix 2). All of these studies compare the percentage of CD cases in which *Map* was detected (by PCR and/or culture and/or direct staining) with the percentage detection rate in specimens collected from patients with ulcerative colitis and/or other IBD and/or in specimens from non-IBD or control patients. The number of cases and controls, the type of specimen(s) examined, the type of detection method(s) used and any evidence of a statistically significant difference between cases and controls are outlined in Appendix 2. Large differences in study design precluded much meaningful comparative analysis. However, PCR was the predominant methodology (used in 13 of the 14 studies) with six of these studies reporting a statistically significant difference in the detection rate of *Map* DNA between CD cases and other cases or controls.

Indirect detection of exposure to Map (a specific host immune response to Map) in CD – case-control studies 2008–2019

Nine case-control studies which attempted to demonstrate a specific host immune response to *Map* in clinical specimens from CD patients were identified (Appendix 3). All these studies compare the percentage of CD patients in which a specific response was detected (by antibody assay and/or lymphocyte/cytokine assays and/or T-cell proliferation assay) with that of patients with other IBD and/or control patients. The number of cases and controls, the type of specimen(s) examined, the type of assay used and any evidence of a statistically significant difference between cases and controls are outlined in Appendix 3. Different study designs, and in some cases the small numbers of cases and controls studies, preclude much meaningful comparative analysis. Eight studies report a statistically significant difference between CD cases and other cases or controls in some aspect of the immune response that is specific to *Map*.

Relatedness of Map isolates – genomic studies of isolates from humans and animals

Bryant *et al.* (2016) provided a global overview of *Map* genomics comparing isolates of *Map* obtained from humans and animals worldwide. The study showed no obvious geographical signature in the *Map* data. However, isolates were selected to maximise geographical spread. Human isolates that were sequenced as part of their study, from the US and the Netherlands,

clustered together in the phylogenetic tree; and while they were therefore genetically similar, they could not be epidemiologically linked. Furthermore, human and animal isolates from the same country clustered closely together; this may suggest a common source of infection.

Occupational exposure to Map and the occurrence of CD

A cross-sectional survey conducted by Qual *et al.* (2010) found no statistically significant association between exposure to cattle infected with *Map* and the development of CD among 774 veterinarians and 702 cattle producers from Michigan, Wisconsin, and Iowa in the US However, the authors indicated that the small number of CD cases included in the study limited its statistical power. On the contrary, Singh *et al*. (2011), albeit with an even smaller sample size (98 individuals) reported that the risk of developing gastro-enteric symptoms indistinguishable from those of non-specific IBD was higher for those who were in contact with goat herds in which JD was endemic.

4.3 Discussion

Although the pathogenesis of CD has not been fully elucidated and there are wide-ranging opinions and disagreements as to its aetiology, the prevailing medical opinion, substantiated by many and varied studies over the past decade, is that it is multifactorial (Rosenfeld and Bressler, 2010; Lee *et al.*, 2011; Baumgart and Sandborn, 2012; Salem *et al.*, 2013; Xia *et al.*, 2014; Banche *et al.*, 2015). A clinically focused review on CD by Baumgart and Sandborn (2012) concluded that CD is the result of susceptibility loci that, triggered by environmental factors, result in a disturbed innate and adaptive immune response towards a diminished diversity of commensal microbiota that is normally in a state of symbiotic mutualism with the human host.

Genetics and/or environmental factors are likely to be contributing factors as often with CD more than one member of a family is affected. The intestinal microbiome and diet are thought to be important environmental factors which trigger the onset of CD in susceptible individuals. Several studies have demonstrated that IBD, including CD, are associated with reduced intestinal microbiota diversity (Willing *et al.*, 2010; Joossens *et al.*, 2011; Andoh *et al.*, 2012; Loh and Blaut, 2012) whereas genome-wide association studies have identified at least 71 genes associated with CD (Franke *et al.*, 2010).

Among genes associated with CD, the strongest linkages are with those involved in the recognition by the immune system of intestinal bacteria, bacterial clearance and response to intracellular infections. These genes include the ‘nucleotide-binding oligomerization domain-containing 2’ gene (*NOD2*), previously referred to as *CARD15* gene, which is part of the ancestral innate immune system that recognizes bacteria peptidoglycan by recognizing the muramyl dipeptide; the ‘toll-like

receptor 4' gene (*TLR4*) which induces the expression of cytokines and co-stimulatory molecules on antigen-presenting cells; the 'autophagy-related 16-like 1' gene (*ATG16L1*), which is part of a large protein complex that is necessary for autophagy; the 'immunity-related GTPase family M' (*IRGM*), which plays a role in the innate immune response by regulating autophagy formation in response to intracellular pathogens and the 'solute carrier 11a1' gene (*SLC11A11*) whose polymorphisms may impair phagosome acidification yielding a permissive environment for the persistence of intracellular bacteria (Franke *et al.*, 2010). It has been reported that some mutant gene alleles associated with CD are also associated with leprosy (i.e. *CARD15* and *LACC1*), tuberculosis and susceptibility to other mycobacterial infections (Sechi and Dow, 2015), suggesting that these genetic mutations in CD patients may provide opportunity for microbial agents, including *Map*, to replicate and survive within the host (Agrawal *et al.*, 2020).

Multiple studies, including those listed in Appendix 2, have detected the presence of *Map* in CD patients. However, many studies have also reported the detection of *Map* in intestinal tissues, faeces and blood samples from healthy controls, although usually at lower frequencies. Furthermore, *Map* has been detected in patients with intestinal diseases other than CD such as ulcerative colitis and diverticulitis and intralesional infectious agents other than *Map* have been sought and found in biopsy specimens of CD patients (Knösel *et al.*, 2009). Therefore, it is entirely possible that the presence of *Map* in tissues of CD patients and other IBD could be a secondary phenomenon due to increased intestinal permeability (Biet *et al.*, 2011; Lefrançois *et al.*, 2011; Garvey, 2018) and/or the inability of macrophages from CD patients to kill *Map* (Lefrançois *et al.*, 2011).

It has been suggested that the variation in *Map* detection rates in different studies could be due to differences in tissue processing, extraction methods, or PCR assays. While culture methods are still "the gold standard" for *Map* detection, they are time-consuming due to the slow growth of the organism and its pleomorphic nature. PCR is the most widely used method despite the difficulties in extracting *Map* DNA and removing PCR inhibitors from clinical specimens. The PCR methods generally used to identify and detect *Map* are based on the IS900 sequence. *Map* genome contains 15-17 copies of IS900, making this a good target for molecular diagnosis purposes. However, some studies have reported the presence of IS900-like sequences in other closely related environmental mycobacterial species. An alternative sequence is F57 which is present in a single copy in the *Map* genome and it has no known similarities to genes on other related organisms. As for detection by direct staining and microscopy, in human tissues *Map* exists primarily in an intracellular and cell-wall deficient form which is difficult or often impossible to identify microscopically while acid-fast staining methods such as Ziehl-Neelsen will not detect this wall-deficient cells (Timms *et al.*, 2011; McNees *et al*., 2015).

Studies of specific immune response to *Map* in CD patients yield quite varied results. Chronic inflammation and increased permeability of the gut wall is likely to result in increased antigen presentation for any microbial peptides present in the lumen of the gut. Müller *et al.* (2008) evaluated serological responses to several ubiquitous mycobacteria. They found an association between the presence of anti-mycobacterial antibodies in CD patients and the presence of anti-*Saccharomyces cerevisiae* mannan antibodies (ASCA, which has been used as a biomarker of CD) that they attributed to a predisposition towards increased immune reactivity to various ubiquitous antigens and specifically to mannosylated antigens. While there is a high frequency of anti-*Map* antibodies in CD patients (and in patients with coeliac disease) relative to patients with ulcerative colitis and healthy controls, this does not prove a role for *Map* in CD pathogenesis (Biet *et al.*, 2011).

In addition to the case-control studies listed in Appendix 2 and 3, systematic reviews and meta-analyses of the scientific literature have established a link between the presence of *Map* and the occurrence of CD but none have concluded that this is a causal association (Feller *et al.*, 2007; Abubakar *et al*., 2008; Waddell *et al.*, 2008; Waddell *et al.*, 2015). It is not known if *Map* has any causal effect, as either a primary trigger initiating inflammation or a secondary contributor to exacerbating inflammatory pathology, or if it is simply a bystander. The clinical response of CD patients to different treatment regimens might also be taken to weaken the argument of a causative role for *Map* in the pathogenesis of CD. In recent times, *Map* has been associated with other chronic human diseases including granulomatous inflammatory diseases such as Blau syndrome and sarcoidosis, and autoimmune diseases such as type 1 diabetes, Hashimoto's thyroiditis and multiple sclerosis. The association between *Map* and these diseases tends to be weaker than that shown for CD and again there is little by way of substantial evidence to support causality (Sechi and Dow, 2015; Waddell *et al.*, 2015). Furthermore, if *Map* was a causal factor in the pathogenesis of CD it might be expected that anti-*Map* (antimicrobial) treatment in CD patients would be more effective than it is. Outcomes from the US phase 3 clinical trial for a novel anti-MAP therapy (RHB-104) in adults with CD are awaited (US National Institute of Health, 2020). In addition, immunosuppressive therapies such as TNFα (tumour necrosis factor alpha) inhibitors which are effective in the treatment of CD, would instead facilitate *Map* replication and an exacerbation of disease symptoms as it is seen with *Mycobacterium tuberculosis* (Agrawal *et al*., 2020).

Reviews of *Map* and CD can draw on the same data and reach entirely different conclusions (Gitlin *et al*., 2012; Alhagamhmad *et al*., 2016). Given the extent of disagreement and difference of opinion in the medical and scientific literature about the role of *Map* in the aetiology and pathogenesis of CD, it is particularly important to carefully scrutinise whatever scientific data is

available. The provenance of papers, potential bias of the authors and the calibre of journal should be considered when reviewing scientific articles. Waddell *et al.* (2009) critiqued the methodological soundness of literature reviews addressing different potential zoonotic public health issues, including the putative role of *Map* in causing CD. They found that most reviews lacked structure and transparent methodology and argued that reviews should adhere to more rigorous structured scientific principles like those used in papers describing primary research. The application of knowledge synthesis methods such as systematic review and meta-analysis is now well-established in the public health field (Young *et al.*, 2014) and a number of systematic reviews and meta-analyses focused on the link between *Map* and CD have already been published (Feller *et al.*, 2007; Abubakar *et al*., 2008; Waddell *et al.,* 2008; Waddell *et al.,* 2015).

Given the continuing uncertainty and diverging opinion about the putative role of *Map* in the pathogenesis of CD, it is likely that some measures will continue to be taken to safeguard human health by minimising human exposure to *Map* until such time as we have definitive evidence supporting or refuting a causative link between *Map* and CD. The widespread nature of the *M. avium* complex (*Map* being a member of this complex) implies that humans face persistent exposure to *Map* and related mycobacteria. A survey of professionals that specialise in *Map*, concluded that humans are exposed to *Map* directly via ruminants, consumption of dairy products, drinking water and other food (Waddell *et al.*, 2016).

Direct shedding of *Map* into the udder or milk of infected ruminants and widespread dissemination of *Map* into other tissues (meat) is only likely to occur in advanced cases of clinical disease and clinically affected animals are very unlikely to be lactating. However, at earlier, pre-clinical stages, infected cattle and sheep can shed relatively large amounts of *Map* in faeces, thereby contaminating the environment and potentially also contaminating food and drinking water. Working from first principles, mitigation is therefore based on: (1) reducing the burden of infection in ruminants through herd health programmes – this may involve a combination of sampling, testing and culling of infected cattle and a range of biosecurity precautions to reduce the spread of *Map* (and prevent its introduction into uninfected herds), (2) good husbandry and hygienic precautions to prevent or reduce faecal contamination of udders and ultimately of milk, and (3) heat treatment of potentially contaminated food to inactivate any *Map* present, including pasteurisation of milk.

4.4 Conclusion

Currently available scientific data remains inconclusive and does not indicate a causal relationship between human exposure to and/or infection with *Map* and the occurrence of Crohn’s disease.

4.5 Recommendations

- The question of the putative link between *Map* and CD should be revisited as further primary research findings are published – see Q3.
- On the next occasion on which the FSAI Scientific Committee decides to review the literature on this subject, a systematic review and meta-analysis should be considered.

5. Question 2 - Does *Map* survive pasteurisation treatments applied by the dairy industry to milk and dairy products and if so, what if any pasteurisation time/temperature combination would be sufficient to inactivate *Map*?

5.1 Background

Pasteurisation[2] has been used for heat treatment of milk to reduce microbial load since the 19th century, and in the dairy factories today it is commonly carried out using a heat exchanger. Modern milk production practices include strict hygiene regimes at farm level, however, even with the best practices in place, contamination of raw milk with microorganisms can still occur from the surface of the udder. A useful definition of pasteurisation is given in the FAO/WHO Codex Alimentarius Commission's Code of hygienic practice for milk and milk products (CAC/RCP 57-2004), i.e. "Pasteurisation is a microbiocidal heat treatment aimed at reducing the number of any pathogenic microorganisms in milk and liquid milk products, if present, to a level at which they do not constitute a significant health hazard" (FAO/WHO, 2004). Pasteurisation parameters are effectively designed to kill the micro-organisms, *Mycobacterium tuberculosis* and *Coxiella burnetii* (FAO/WHO, 2004). The time-temperature combinations recognised by FAO/WHO 2004 are (i) 63 °C for 30 min for a batch system or (ii) 72 °C for 15 s for a continuous system with turbulent flow. European Regulation (EC) No 853/2004 on the specific hygiene rules for food of animal origin also refers to milk pasteurisation as any other combination of time-temperature conditions to obtain an equivalent effect to the aforementioned heat treatments, such that the products show, where applicable, a negative reaction to an alkaline phosphatase test immediately after such treatment.

Important considerations for pasteurisation are equipment design (direct/indirect heating), time-temperature combination and the physical properties of the liquid medium such as viscosity, pH and heat transfer characteristics. In the latter case, turbulent flow is required to ensure effective heat transfer, and consequently this makes the pasteurisation process difficult to replicate in the laboratory, requiring a large pilot plant or commercial scale facilities to provide reliable results.

[2] For the purposes of this report, pasteurisation refers to thermal pasteurisation.

5.2 FSAI report 2009

The 2009 report published by the FSAI on *Map* and the possible links to CD also addressed the ability of *Map* to survive in milk during pasteurisation. That report cited studies in which low levels of *Map* were detected in pasteurised milk, from both laboratory-based and commercial pasteurisation, but not when using controlled pilot plant conditions. Studies in the UK (Grant *et al.*, 2002), US (Ellingson *et al*., 2005) and the Czech Republic (Ayele *et al.*, 2005) reported viable cells present in 1.8, 2.8 and 1.6% of milk samples tested, respectively. This contrasts with studies in Ireland (O'Reilly *et al.*, 2004) and Canada (Gao *et al.*, 2005) where no viable *Map* cells were detected in commercially pasteurised milk samples. Details of parameters used for heat treatment and downstream processing (i.e., filling and packing) are not given, however, in the case of the UK survey, it is stated that the conditions which were used met or exceeded EU minimum requirements. O'Reilly *et al.* (2004) reported that, based on processing records, 90% of commercial samples were subjected to temperatures in excess of 75 °C and that 62% of samples were treated at holding times in excess of 25 s. Pilot scale studies in Ireland (Lynch *et al.*, 2007) and New Zealand (Pearse *et al.*, 2001), where milk was inoculated with *Map*, demonstrated a 100% kill on pasteurisation using 72.5 °C for 27 s. For the most part in the studies described here, molecular methods (DNA based) were used as confirmatory tests in addition to conventional culture techniques. It is important to note that while *Map* DNA may be present in a sample after pasteurisation, this does not confirm the presence of viable cells. Overall, the evidence was conflicting, and some laboratory-based studies showed survival was dependent on initial microbial loading.

5.3 Current position: Does *Map* survive pasteurisation and what is the optimal time – temperature combination?

The possibility of *Map* surviving pasteurisation is the subject of a number of critical reviews including recent publications by Robertson *et al*. (2017) and Mullan (2019). Mullan (2019) reported that levels of *Map* in raw milk, based on studies from Europe, UK and US, were unlikely to exceed 10^2 cells/mL, except for one study by Ricchi *et al*. (2016) in Italy which found levels >10^3 cells/mL. Ricchi *et al*. (2016) also discuss the lack of consensus on the heat tolerance of *Map*, stating that a range of z-values from 6.3 to 9.76 °C have been reported, making it difficult to calculate equivalent time-temperature combinations for inactivation during thermal processing. Gerrard *et al*. (2018) proposed that the major source of *Map* infection comes from the polymorphonuclear leucocytes, macrophages, lymphocytes and mammary epithelial cells in the mammary gland of infected cows rather than faecal contamination. Regardless of the source of infection, the dilution effect from 'un-

infected milk' can explain the low numbers detected in commercial milk. Although Robertson *et al*. (2017) recognised that measurement of *Map* is challenging, they still concluded that the evidence indicates commercial pasteurisation inactivates *Map* in milk.

Quantitative PCR (qPCR) is a more sensitive and rapid detection method than traditional culture methods. However, a limitation of PCR assays when used after heat treatments such as pasteurisation is that they are unable to differentiate between dead and living cells (Cocolin *et al*., 2011; Timms *et al*., 2011). Consequently, phage amplification methodologies have been developed for enumeration of viable *Map* in heated foods such as dairy matrices. Butot *et al*. (2019) evaluated three analytical methods: (i) Culture based; (ii) qPCR; and (iii) a peptide-mediated magnetic separation (PMS) phage-based assay for *Map* detection in raw, heat-treated and powdered milk. The authors showed that the PMS-phage assay technique worked in pure liquid, however its performance as a quantification method in dairy matrices was lower than either culture based or qPCR. Therefore, the authors stated that culture based and qPCR were more suitable for detection of *Map* in milk products. However, they emphasised that qPCR is only appropriate if cell viability does not have to be assessed, highlighting that culture is required to establish if any viable cells are present post-pasteurisation.

Studies on pasteurisation that have been published in the scientific literature were categorised as: (i) laboratory-based; (ii) pilot plant pasteurisation; (iii) commercial pasteurisation; (iv) pasteurisation of milk-based beverages; and (v) review articles on pasteurisation. Consideration is given to the type of study performed and recommendations are made based on findings from the previous report in 2009 and those reported in studies from the literature over an eleven year period. When evaluating suitable time-temperature combinations for pasteurisation to inactivate *Map*, consideration is given to factors such as bacterial load, type of heat exchanger and turbulence of milk flow through the exchanger. Information on bacterial load is key for the purpose of advising on *Map* inactivation, i.e. concentration of *Map* that is likely to be present in raw milk obtained from infected herds/cows. The level of *Map* contamination in milk is primarily dependent on the stage of infection. Direct shedding of *Map* into the udder is unlikely to occur until the later (clinical) stages of infection but faecal contamination can result in the presence of *Map* in milk at an earlier stage. These are all considerations for testing and the load that the heat exchanger will need to inactivate during pasteurisation.

Recent studies on *Map* include country specific surveys on finished product, which, like the studies reported on in the 2009 FSAI report, vary in their conclusions. Carvalho *et al*. (2012) reported that one out of 37 commercially pasteurised milk samples tested was suspected to have *Map* present. Using a phage-PCR methodology, Gerrad *et al*. (2018) stated that pasteurisation is not capable of removing human exposure to viable *Map* and reported that 10.3% of the total milk samples

evaluated in their study contained *Map*. In contrast, no viable *Map* cells (using traditional culture techniques) were found in a study by Serraino *et al*. (2017), whereby 160 one-liter bottles of pasteurised milk from two dairy plants located in two different regions were analysed. The authors suggested that the combination of good hygiene practices at farm level and commercial pasteurisation resulted in very low or undetectable levels of *Map* in pasteurised milk. They suggested that based on the dairies investigated, pasteurised milk is not a source of human exposure to *Map*.

The phage-PCR methodology was also used by Botsaris *et al.* (2016) who reported the presence of viable *Map* in reconstituted infant formula powder. However, as stated above, concerns have been expressed about the suitability of phage amplification assays as a technique for detection of *Map* in milk-based products. Butot *et al.* (2019) reported that test sensitivity (including trueness; i.e., correctly assigned positive and negative results to the reference value) for a PMS-phage method was lower than either IS900 qPCR or culture methods. In addition, given the number of sequential heat treatments used during infant formula manufacture, it is highly unlikely that *Map* would survive the overall manufacturing process. This is supported by the study of Acharya *et al*. (2017) in which a low level of *Map* contamination was detected in infant formula powder using qPCR methods, but no viable cells were present. Such findings (detection of *Map* DNA but not viable *Map*) are much more likely given the temperatures used at the batch make-up, evaporation and drying stages of infant formula manufacture.

Over the last decade few controlled studies have been carried out on plate heat exchangers to simulate commercial units, however, Peterz *et al*. (2016) investigated the effectiveness of heating, using a pilot plant direct steam injection system at 105 °C for 3 s to inactivate *Map*. The authors reported that no viable *Map* were detected (confirmed by qPCR) and concluded that >7 $\log_{10}$ reduction in *Map* can be achieved using direct steam injection. A different approach was taken in a study by Hammer *et al.* (2014), who considered the effect of bacterial clumping, as *Map* that has been cultivated in vitro and *Map* present in faeces is known to occur in clumps. They tested the ability of homogenisation to enhance the effect of heat treatment by breaking up clumps and found that a pasteurisation temperature of 72 °C for a time of 27–28 s reduced *Map* numbers by a mean of 5.5 $\log_{10}$ (ranging from 3.7–6.9 $\log_{10}$). However, the inclusion of a homogenisation step, wherever positioned in the process (upstream, in-hold, or downstream), did not increase the efficacy of heat treatment over that achieved by pasteurisation alone. While it has been suggested that clumps may provide protection from heat, several studies have shown the impact on heat transfer is negligible (Davey, 1990; Cerf *et al*., 2007). It is noteworthy to mention that in-process failures in relation to design safety margins of pasteurisers are not within the scope of this report; however, a study by Chandrakash and Davey (2017) adds to existing knowledge by simulating

Map survival after milk pasteurisation and suggests process intervention strategies to mitigate a potential reduction in process lethality.

Mullan (2019) provided times and temperatures used to pasteurise milk in a range of approved plants in Northern Ireland in 2018 (supplied by Milk Inspectorate – Agri-food Inspection Branch, Department of Agriculture, Environment and Rural Affairs) and found the lowest temperature used was 74 °C (for 27 s) with temperatures of 77 ºC (for 24 or 31 s) and 78 ºC (for 26 or 27 s) reported. The author stated that Northern Ireland, Republic of Ireland, New Zealand and Australia have increased the severity of the heat treatment for additional protection against *Map* in recent years.

Based on the evidence from past studies in Ireland (O'Reilly *et al*., 2004; Lynch *et al*., 2007), the literature since 2009 (including experimental design and testing methodologies used), it is suggested that a time-temperature combination of 75 °C for 20 s should be sufficiently effective to inactivate *Map* at the levels of contamination likely to be found in raw milk under typical farming and processing conditions in Ireland. It is important to note that even with good hygiene practices at milking, it is not possible to rule out faecal contamination of raw milk, such that pasteurisation remains an important critical control point in dairy product manufacture. It should also be noted that dairy products such as powdered infant formula are subjected to additional heat treatments during the manufacturing process ensuring that the probability of *Map* survival in the finished product is negligible.

5.4 Conclusion

Considering the levels of *Map* that are likely to be found in raw milk produced under typical Irish dairy farming conditions, it is considered unlikely that viable *Map* would be found in processed Irish milk that has been pasteurised commercially at a time-temperature combination of 75 °C for 20 s.

5.5 Recommendation

- A study is recommended to develop a qPCR methodology which can distinguish between ‘live and dead’ cells for determination of viable numbers of *Map* after heat treatment for use in dairy product testing.

6. Question 3 - Are there areas of research necessary to reduce uncertainty in the risk assessment?

Yes, further applied research and development studies are indicated to:

- Establish the presence/prevalence of *Map* in tissues of Irish CD patients and the extent to which the Irish human population has been exposed to *Map*.
- Elucidate the genetic relatedness of human, animal and environmental isolates of *Map* to clarify infection dynamics.
- Explore any causal association between the gut microbiome and either *Map* infection in humans and/or the occurrence of CD.
- Quantify viable *Map* in milk to measure the efficacy of heat treatments.

Studies to establish the presence/prevalence of *Map* in tissues of Irish CD patients and the extent to which the Irish human population has been exposed to *Map*

Direct detection of Map *in clinical specimens from human patients*

The Irish Mycobacterial Reference Laboratory (IMRL) has an extensive database of human mycobacterial isolates but no record of an Irish human clinical isolate of *Map*. One possibility is that any *Map* isolates may simply have been classified as belonging to the *Mycobacterium avium* complex (MAC), to which *Map* is a member. DAFM has provided IMRL with Irish bovine *Map* isolates to assess whether current diagnostic test-kits and protocols can detect *Map* and differentiate it from the wider MAC family. IMRL is increasingly, although not yet routinely, sequencing mycobacterial isolates. A move towards routine sequencing of all human mycobacterial isolates would ensure that any *Map* present in future submissions would be identified as such.

Seroprevalence studies to determine the exposure of the Irish human population to Map

A study of new recruits into the US army involved screening them for antibodies against a range of microbial peptides and subsequent follow up over more than a decade. There was a significant difference in the antibody responses of those who subsequently developed CD and those who did not – with antibodies to some of these microbial peptides being detected up to 10 years in advance of the development of CD (Torres *et al*., 2020). A similar seroprevalence study might be considered as part of any large cohort studies in Ireland. For example, the TILDA (The Irish Longitudinal Study on Ageing) study led by Trinity College Dublin looks at the health of older adults (50+) in Ireland and the researchers are open to ideas as to what screening tests might be used on this population to provide useful information for the future.

While serological methods such as ELISA (Enzyme-Linked ImmunoSorbent Assay) are widely used to screen ruminants for antibodies to *Map*, the antigens used are usually a crude protein extract such that cross reactions can occur. The low diagnostic specificity of these ELISAs can make it difficult to interpret the results. Therefore, before undertaking any major serological studies in the human population it may be worthwhile to look at key antigens that are immunodominant in humans and would be most appropriate as the basis for serological studies.

Studies to elucidate the genetic relatedness of human, animal and environmental isolates of *Map* to clarify infection dynamics

There is relatively little information on genomic analysis of *Map* populations in the scientific literature. A DAFM-funded, University College Dublin-led project is currently sequencing *Map* isolates from Irish cattle. The development and validation of shotgun metagenomic sequencing of *Map* would allow *Map* genomes to be analysed without prior isolation and cultivation, making it faster and easier to assemble and compare genomic data from clinical specimens (human and animal) and the environment.

Studies to explore any causal association between the gut microbiome and either *Map* infection in humans and/or the occurrence of CD

Lowe *et al.* (2008) concluded their paper with a suggestion that further studies of CD should extend beyond the search for a particular agent of interest to consider the potential interaction of different microbes, while emphasising that such a synergistic process might be more challenging to formally prove than causation by a single pathogen. Consequently, the characterisation and comparative analysis (quantification, speciation, etc.) of the gut microbiome in CD patients, in patients with other chronic IBD and in healthy controls may help to elucidate predisposing factors contributing to the complex pathogenesis of CD and ultimately support a more holistic approach to its prevention.

Studies to quantify viable *Map* in milk to measure the efficacy of heat treatments

Further studies using more sensitive and rapid methodologies capable of distinguishing between live and dead cells (i.e., nucleic acid amplification methodologies such as qPCR) should be undertaken to measure the effectiveness of pasteurisation and other heat treatments at inactivating *Map*. There is also a requirement for new methods (which do not require bacteriological culture) that might be used to detect *Map* and to determine its viability in dairy products, including milk powders.

7. Overall conclusions and recommendations

Conclusions:

- Currently available scientific data remains inconclusive and does not indicate a causal relationship between human exposure to and/or infection with *Map* and the occurrence of Crohn's disease.
- Considering the levels of *Map* that are likely to be found in raw milk produced under typical Irish dairy farming conditions, it is considered unlikely that viable *Map* would be found in processed Irish milk that has been pasteurised commercially at a time-temperature combination of 75 °C for 20 s.
- Further applied research and development studies may:
    - Establish the presence/prevalence of *Map* in tissues of Irish CD patients and the extent to which the Irish human population has been exposed to *Map*.
    - Elucidate the genetic relatedness of human, animal and environmental isolates of *Map* to clarify infection dynamics.
    - Explore any causal association between the gut microbiome and either *Map* infection in humans and/or the occurrence of CD.
    - Quantify viable *Map* in milk to measure the efficacy of heat treatments.

Recommendations:

- The question of the putative link between *Map* and CD should be revisited as further primary research findings are published.
- On the next occasion on which the FSAI Scientific Committee decides to review the literature on this subject, a systematic review and meta-analysis should be considered.
- A study is recommended to develop a qPCR methodology which can distinguish between 'live and dead' cells for determination of viable numbers of *Map* after heat treatment for use in dairy product testing.
- Further research to address knowledge gaps, which impact on the ability to assess the risk that *Map* poses to human health and the risk that humans could be exposed to *Map* in food, should be commissioned and funded.

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Appendix 1 Request for advice from the Scientific Committee

Topic title: *Mycobacterium avium* subsp*. paratuberculosis* and the possible links to Crohn's disease

Date Requested: 9 April 2019

Date Accepted: 9 April 2019

Target Deadline for Advice: Q2 2020

Form of Advice required: Written report

Background/Context

Crohn's disease (CD) is one of the inflammatory bowel diseases (Leach & Day, 2006). The cause or causes of CD are the subject of ongoing research and may be genetic, immunologic, environmental and microbial in origin (Alhagamhmad *et al.*, 2012). Disruption of the gut flora and consequential inflammation of the intestinal mucosa appear to play a part and further information can be found in a 2016 review by Alhagamhmad *et al.* The possibility of a link between *Map* and CD remains a subject of debate due to the similarities between the pathology of Johne's disease (JD) in cattle and that of human CD. The arguments for and against causality between *Map* and CD have continued in the literature (Gitlin *et al.*, 2012; Garvey, 2018).

In 2000 the Scientific Committee of the Food Safety Authority of Ireland reviewed the evidence of causality between *Map* and CD (FSAI 2000). The principal conclusion of that report was that the available data were inconclusive and a direct link between *Map* and CD could not be established. However, the report recommended that the Committee keep the issue under review. In 2008 the Microbiology Sub-committee conducted a more formal review of the research and opinion published in the years since the original report in 2000. Fifty six key publications were identified between 2000 and 2008. Based on a review of those papers the Committee concluded that the balance of available evidence did not support a causal relationship between *Map* and the incidence of Crohn's disease (FSAI, 2009).

Ten years on with the publication of further research and the advent of new technologies like whole genome sequencing and alternative viability tests to traditional plating of bacteria, it is time to revisit this question.

Questions to be addressed by the Scientific Committee

1. Does the balance of the available scientific literature support a link between *Map* and Crohns disease?
2. Does *Map* survive pasteurisation treatments applied by the dairy industry to milk and dairy products and if so, what if any pasteurisation time/temperature combination would be sufficient to inactivate *Map*?
3. Are there areas of research necessary to reduce uncertainty in the risk assessment?

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Appendix 2 Direct detection of *Map* in cases of Crohn's disease – case-control studies 2009-2019

CD (Positive samples/total samples)	UC (Positive samples/total samples)	non-IBD (i.e. healthy controls) (Positive samples/total samples)	Sample type	Direct stain	Culture	PCR	Statistical result	Reference
22/56	7/22	6/39	Intestinal biopsy	-	-	Nested PCR (IS900)	P<0.05 (CD *versus* non-IBD)	Kirkwood *et al.*, 2009
8/50	2/25	0/31	Blood	-	-		P<0.05 (CD *versus* non-IBD)	
4/56	-	0/20	Intestinal biopsy	-	-	Conventional PCR (IS900)	No statistical analysis	Knösel *et al.*, 2009
11/20	11/20	4/18	Blood	-	MGIT medium	Nested PCR (IS900)	P<0.009 (PCR; IBD *versus* non-IBD)	Naser *et al.*, 2009
0/130	-	1/130	Blood	-	MGIT medium	Conventional and nested PCR (IS900)	All tested negative (Culture)	Parrish *et al.*, 2009
0/81	-	0/85	Intestinal biopsy	-	-	Nested PCR (IS900)	All tested negative	Sasikala *et al.*, 2009
30/30	29/29	10/10	Blood	-	-	Nested PCR (IS9000	All tested positive	Mendoza *et al.*, 2010
30/30	1/29	0/10		Ziehl-Neelsen stain and phenolic acridine orange stain	MGIT medium	-	No stats analysis	
48/70	7/59	19/71	Blood	-	-	Nested PCR (IS900)	P<0.0001 (CD *versus* UC and CD *versus* non-IBD)	Di Sabatino *et al.*, 2011

CD (Positive samples/total samples)	UC (Positive samples/total samples)	non-IBD (i.e. healthy controls) (Positive samples/total samples)	Sample type	Direct stain	Culture	PCR	Statistical result	Reference
7/20	1/20	1/19	Intestinal biopsy (paediatric patients)	-	-	Nested PCR (IS900)	P<0.05 (CD versus no-IBD)	Lee *et al.*, 2011
21/31	13/20	11/23	Faeces	-	-	Conventional PCR (IS900)	P>0.05	Tuci *et al.*, 2011
0/28	-	0/17	Intestinal biopsy	Immunohistochemistry	-	-	All tested negative	Magin *et al.*, 2013
8/12	-	1/11	Oral Biopsy	-	-	Nested PCR (IS900)	No statistical analysis	Molicotti *et al.*, 2013
17/19	-	0/11	Intestinal biopsy	Kinyoun stain and Phenolic acridine orange stain	ATCC medium and MGIT medium	TaqMan PCR (IS900 and F57)	P<0.001 (PCR)	Banche *et al.*, 2015
5/19	-	3/11	Blood	Not detected			P=1 (PCR), P<0.001 (Culture)	
1/19	-	1/19	Faeces	Not detected			P=1 (PCR), P<0.001 (Culture)	
6/22	3/20	2/42	Intestinal biopsy	-	Middlebrook medium	Conventional PCR (IS900 and F57) and nested PCR (IS900)	P=0.02 (PCR; CD *versus* other groups)	Timms *et al.*, 2016
14/30	-	4/30	Intestinal biopsy	Zeihl-Neelsen stain	-	Conventional PCR (IS900)	No statistical analysis	Zarei-Kordshouli *et al.*, 2019

CD, Crohn's disease; UC, Ulcerative Colitis; non-IBD, non-Inflammatory Bowel Disease; PCR, Polymerase Chain Reaction; MGIT, Mycobacterial Growth Indicator Tube; Statistically significant differences were considered for P values ≤0.05.

Appendix 3 Indirect evidence of exposure to *Map* in cases of Crohn's disease – case-control studies 2008-2019

CD (Positive samples/total samples)	UC (Positive samples/total samples)	Non-IBD (Positive samples/total samples)	Sample type	Antibody assay	Lymphocyte / cytokine assay	T-cell proliferation assay	Conclusions and statistical result	Reference
31/105	6/45	2/35	Serum	Yes	-	-	No statistically significant differences in anti-*Map* serum IgG between CD and UC patients only	Müller *et al.*, 2008
ns/46	ns/30	ns/18	Blood	-	Yes	-	No statistically significant differences in cytokine secretion in blood cultures in subjects with IBD and controls	Ren *et al.*, 2008
ns/45	ns/22	ns/44	Peripheral blood mononuclear cells	-	-	Yes	When co-incubated with *Map*, significantly higher T-cell proliferation in blood cells isolated from CD patients than from UC patients or controls	Sibartie *et al.*, 2010
ns/48	-	ns/46	Serum	Yes	-	-	ELISA OD450nm of *Map* antigens was significantly different between CD patients and controls	Shin *et al.*, 2010
ns/20	-	ns/20	Serum	Yes	-	-	CD patient sera had significantly higher titer of antibodies against *Map*-derived PtpA and PknG compared to controls	Bach *et al.*, 2011
27/81	3/36	5/120	Serum	Yes	-	-	-	Biet *et al.*, 2011

CD (Positive samples/total samples)	UC (Positive samples/total samples)	Non-IBD (Positive samples/total samples)	Sample type	Antibody assay	Lymphocyte / cytokine assay	T-cell proliferation assay	Conclusions and statistical result	Reference
ns/8	-	ns/8	Serum	Yes	-	-	ELISA OD490nm of *Map* antigens for the CD group was significantly higher compared to that of the control group	Lefrançois *et al.*, 2011
5/5	-	2/4	Intestinal biopsy	-	Yes	Yes	The frequency of *Map* reactive T-cell clones in CD patients ranged from 0.17 to 1.63% while in UC patients ranged from 0 to 0.78%	Olsen *et al.*, 2013
ns/24 (adults) ns/47 (children)	ns/33 (children)	ns/45 (adults) ns/31 (children)	Serum; gut lavage fluids	Yes	-	-	In CD patients, IgG reactivity in gut lavage fluids was increased against the four *Map* antigens (3 non *Map*-specific and 1 *Map*-specific named L5P) but in sera it was only increased against L5P. By contrast, anti-L5P IgG were not increased in patients with UC.	Verdier *et al.*, 2013

CD, Crohn's disease; UC, Ulcerative Colitis; non-IBD, non-Inflammatory Bowel Disease; ns, not stated; IgG, Immunogloblulin G; ELISA, enzyme-linked immunosorbent assay; OD, optical density; nm, nanometer; PtpA, protein tyrosine phosphatase A; PknG, protein kinase G; L5P, *Map*-specific lipopentapeptide.

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