

Epidemiological evidence for *Mycobacterium avium* subspecies *paratuberculosis* as a cause of Crohn's disease

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SUMMARY

Mycobacterium avium subspecies *paratuberculosis* is the causative agent of Johne's disease, a chronic enteritis in ruminants including cattle, sheep, goats, and farmed deer. Recently, this bacterium has received an increasingly wide interest because of a rapidly growing body of scientific evidence which suggests that human infection with this microorganism may be causing some, and possibly all, cases of Crohn's disease. Recent studies have shown that a high percentage of people with Crohn's disease are infected with *M. avium* subsp. *paratuberculosis*; whether the association of this bacterium and Crohn's disease is causal or coincidental is not known. Crohn's disease is a gastrointestinal disease in humans with similar histopathological findings to those observed in the paucibacillary form of Johne's disease in cattle. The search for risk factors in Crohn's disease has been frustrating. However, epidemiologists have gathered enough information that points to an association between *M. avium* subsp. *paratuberculosis* and Crohn's disease. This paper reviews epidemiological models of disease causation, the major philosophical doctrines about causation, the established epidemiological criteria for causation, and the currently known epidemiological evidence of *M. avium* subsp. *paratuberculosis* as a possible cause of Crohn's disease.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* is a pathogenic bacteria in the genus *Mycobacteria*. It is often abbreviated as *M. paratuberculosis*, *M. avium* subsp. *paratuberculosis* or MAP. MAP causes paratuberculosis or Johne's disease, a chronic granulomatous gastroenteritis in ruminants [1]. Johne's disease occurs worldwide and is primarily a disease of domesticated ruminants, including cattle (both beef and dairy), sheep, goats, and farmed deer [2,3]. The host range for Johne's disease has been reported to include wild ruminant species, such as deer [4–7], as well as non-ruminants, such as wild

rabbits [8,9], their predators, including foxes and stoats [10], and primates, such as mandrills and macaques [11,12]. The disease is characterized by profuse and intractable diarrhoea, severe weight loss and diagnostic changes in the lining of the small intestine [13,14].

Crohn's disease is a chronic inflammatory disease of the intestines in humans [15]. The disease primarily causes ulcerations of the small and large intestines, although it can affect the digestive system anywhere from the mouth to the anus. Common symptoms of Crohn's disease include severe bouts of watery or bloody diarrhoea, cramping, abdominal pain, fever, weight loss, and bloating [15].

Morphological changes in Crohn's disease include chronic inflammation involving all layers of the intestinal wall (transmural involvement), thickening of involved segments, with narrowing of lumen, linear ulceration of the mucosa, submucosa oedema with elevation of the surviving mucosa, producing a characteristic cobblestone appearance. Crohn's disease in humans has long been suspected of having a mycobacterial cause [1, 16–18]. This proposition was first advanced by Dalziel [19]. According to Clarke [20], the histopathology of Johne's disease ranges from the more common pluribacillary or lepromatous form to the less common paucibacillary or paucimicrobial tuberculoid form like leprosy in humans. Due to the histopathological features of Crohn's disease closely resembling those found in animals with the paucibacillary form of Johne's disease, it has been suggested that the two diseases shared the same aetiology [13,14,21,22]. The objectives of this paper were: (i) to review the epidemiological evidence involving the potential association of MAP with Crohn's disease in humans, and (ii) to determine if causation of Crohn's disease can be inferred based upon the evidence reviewed.

Epidemiology of Johne's disease

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a member of the *M. avium* complex [23]. *M. avium* strains are widely distributed in the environment as well as in birds, animals, and humans [24–26]. *M. avium* strains do not usually cause disease unless the host is debilitated or immunocompromised. By contrast MAP is a specific pathogen with the ability to cause chronic inflammation of the intestine (Johne's disease) in many species [27–30]. MAP is a well recognized cause of disease and economic loss in dairy herds, and most control programmes have been designed for the dairy industry [31–33]. It is estimated that nearly 40% of United States dairy herds are infected with MAP and that losses to the dairy industry may exceed \$1·5 billion per year [34,35]. MAP is most commonly transmitted via the faecal–oral route [36,37]. However, it can also be transmitted in the semen of bulls, in milk (or colostrum), and *in utero* across the placenta to the newborn calf [2]. Moreover, it has been suggested that MAP can exist within the tissues of animals for years without causing clinical disease [38]. Subclinically or clinically infected animals shed MAP in faeces and milk, enabling dissemination to susceptible calves, the environment, and in retail milk [39]. MAP in milk may survive pasteurization [39]. In the United Kingdom, the United States, and the Czech Republic, MAP has been cultured from 1·6% to 2·8% of units of retail pasteurized cow's milk [39–42], and it has been suggested that live organisms might be transmitted to humans by this route.

Epidemiology of Crohn's disease

Crohn's disease occurs throughout the world, with a prevalence of 161–319 cases/100 000 people in Canada [43]. It is most prevalent in Europe and North America [44]. The disease affects between 400 000 and 600 000 people in North America alone [45]. Prevalence estimates for Northern Europe have ranged from 27–48/100 000 [43]. The incidence of Crohn's disease in North America has been estimated at 6/100 000 per year, and is thought to be similar in Europe, but lower in Asia and Africa [46,47]. The incidence of Crohn's disease in industrialized parts of the world has been reported to be

increasing [48–51]. The disorder occurs most frequently among people of European origin, is 3–8 times more common among Jews than among non-Jews [52]. However, this excess risk is not evident in the Jewish population of Israel [53]. Although the disorder can begin at any age, its onset most often occurs between 15 and 30 years of age [54–57].

Satsangi *et al.* [58] reported that parents, siblings or children of people with Crohn's disease were 3–20 times more likely to develop the disease than the general population. Twin studies show a concordance of greater than 55% for Crohn's disease [59–61]. Mutations in a gene called *NOD2/CARD15* are associated with Crohn's disease [62–64], and with susceptibility to certain phenotypes of disease location and activity [65]. The *NOD2/CARD15* susceptibility does not apply to Chinese [66], Japanese [67], Korean [68], Tunisian [69] or Turkish [70] patients with Crohn's disease. A susceptibility locus for Crohn's disease has been mapped to chromosome 16 [71]. Three independent studies reported that mutations within the *NOD2/CARD15* gene were strongly linked to Crohn's disease in Europeans [62,71,72]. However, Greenstein [21] reported that the presence of a gene that is associated with an increased susceptibility to Crohn's disease does not preclude the possibility that the disease may be caused by an infectious agent. Another study [67], suggested the possibility of genetically identifiable subpopulations having different tendencies to develop Crohn's disease when exposed to the same infectious agent. Recent studies have identified an association between inflammatory bowel disease (IBD) and mutations in yet another gene termed *NRAMP1* (also known as *SLC11A1*) [73]. This gene has been reported to be associated with both Crohn's disease and ulcerative colitis.

Epidemiological models for causation

Epidemiology is the scientific inquiry into the causation of disease; it is the search for the risk factors that cause the effect or the disease [74]. In this search, various models or theories for causation have been developed over the years in an attempt to explain the interaction of risk factors and their effect on disease; Models are purposely simplified representations of that interaction [75]. The various models of causation include: epidemiological triad/triangle [76,77], web of causation [78], wheel of causation [79] and Rothman's causal pie [75].

Epidemiological triangle/triad This model makes the agent a component of causation along with the host and environment (Fig. 1). The model implies that all components are equally important in disease causation and that a change in any one of them would change the frequency of disease. The model applies to both infectious or non-infectious diseases. For instance, in Johnne's disease the agent would be the bacterium, MAP; host factors include non-immune, weakened resistance, poor nutrition, age, gender; and environmental factors include animal stocking density, poor environmental conditions (such as temperature, humidity, wind velocity, precipitation, poor housing as in crowded conditions, poor ventilation, and bad sanitation).

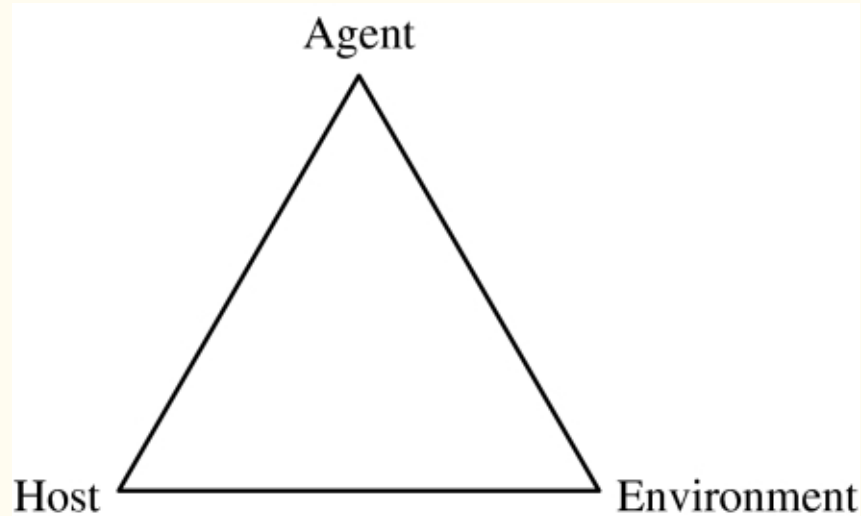


Fig. 1

Epidemiological triangle/triad [77].

Wheel of causation The wheel model places genetic factors in the core of the wheel and varies the size of the host and environmental components depending on their influence in the particular disease process [78]. Surrounding the host is the total environment divided into the biological, physical, and social environments (Fig. 2). These divisions, of course, are not true divisions – there are considerable interactions among the environment types. Although it is a general model, the wheel of causation does illustrate the multiple aetiological factors of human infectious diseases [79]. According to Jantchou *et al.* [80], many environmental factors for IBD have been investigated, including infectious agents, diet, drugs, stress and social status. Among these factors, MAP, oral contraceptives and antibiotics could play a role in Crohn's disease [80–84]. Sicilia *et al.* [81] reported that the pathogenesis of IBD probably involves an interaction between genetic and environmental factors: cigarette smoking, appendectomy and oral contraceptives are the factors most frequently linked to its aetiology.

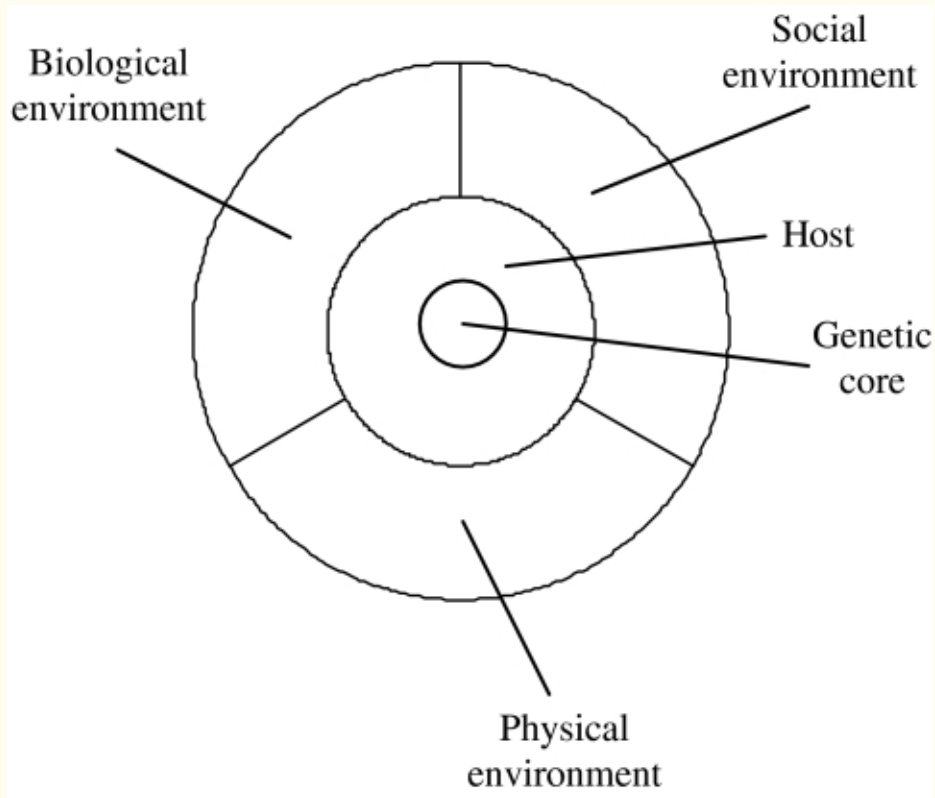


Fig. 2

Wheel of causation [79].

Web of causation This model refers to the 'web' of interconnected factors which lead to disease. The web of causation merely reflects the fact that there is a complex mixture or a 'web' of factors that can cause disease [78]. Many aetiological factors for Crohn's disease have been suggested, including autoimmune, genetic, dietary components plus various infectious agents including MAP [82–84].

Causal pie model The main causal model used by epidemiologists today is Rothman's 'pies' [75]. The idea is that a sufficient causal complex (a pie) is represented by the combination of several component causes (slices of the pie) (Fig. 3). A set of component causes occurring together may complete the 'pie', creating a sufficient cause and thus initiating the disease process. Rothman & Greenland [75] define both 'necessary' cause and 'sufficient' cause. A 'sufficient' cause is one that always results in disease [75], while a 'necessary' cause is one that must be present but might not be the cause of a disease to develop. In other words, a necessary cause is a component cause that is a member of every sufficient cause [85–91].

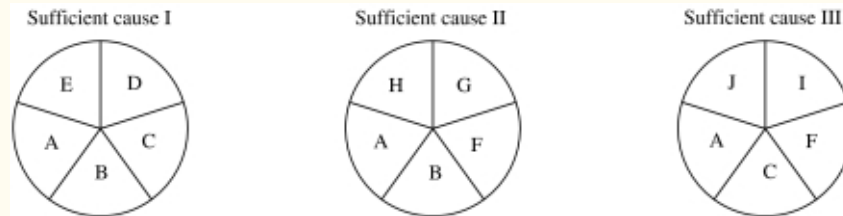


Fig. 3

Causal pie model [92]. This illustration shows a disease that has three sufficient causal complexes, each having five component causes. A is a necessary cause since it appears as a member of each sufficient cause. B, C, and F are not necessary since they fail to appear in all three sufficient causes.

Rothman [93] defines ‘a cause of a disease event as an event, condition, or characteristic that preceded the disease event and without which the disease event either would not have occurred at all or would not have occurred until a later time’. If disease does not develop without the factor being present, then the causative factor is termed ‘necessary’. If the disease always results from the factor, then the causative factor is termed ‘sufficient’. In reference to Rothman's causal pie model, the possibility exists that MAP is a ‘necessary’ but not a ‘sufficient’ cause of Crohn's disease. As a necessary cause, MAP is required to be present to trigger the inflammatory reaction seen in Crohn's disease. However, not being a sufficient cause means that MAP cannot cause Crohn's disease alone but acts in concert with immune dysfunction and genetic susceptibility in order for Crohn's disease to occur [82,84]. Therefore, not every one with the presence of MAP in the intestine would suffer from Crohn's disease. Moreover, the failure to detect MAP in some cases of Crohn's disease may not necessarily indicate that MAP is absent. Low specificity and sensitivity of the test, among other factors, may explain the inability to detect MAP in some patients [94]. It has been reported that the sensitivity of nucleic-acid based tests is influenced by the bacterial burden, as exemplified by the compromised sensitivity of PCR-based assays for sputum smear-negative tuberculosis [95–97]. By extension, assays that reliably detect abundant MAP organisms in livestock with Johne's disease may not provide sufficient sensitivity to study human Crohn's disease [94].

The major philosophical doctrines about causation

The two major philosophical doctrines that have influenced modern science include inductivism and refutationism.

Inductivism This doctrine holds that science proceeds from observation to theory, beginning with observations derived from experiments, and extrapolating from these to general laws [76, 98–102]. Bacon's vision of the ‘true induction’ comprises three interrelated stages: (i) Observation and Experiment (ii) Classification and Concept Formation and (iii) Eliminative Induction and Causal Inference [102]. The traditional view of science is that induction – the formation of a hypothesis based on observation – is cardinal to the scientific method. However Hume [103], the deductivist and others [104] argued that a hypothesis that is derived by induction is flawed because it can be refuted by the first observation that proves an exception. Deduction refers to reasoning that proceeds from the general to the particular and relies on general theory to infer particular conclusions [76]. A century after Hume,

Mill [105] proposed a canon of five methods to infer causes from their effects incorporating some of the ideas that had been proposed earlier by Bacon [106]. The canons of Mill [107] have evolved into inferential criteria that are in use today.

Refutationism or falsificationism This theory is a rival account of the processes involved in scientific research to inductivism. While inductivism holds that science proceeds from observation to theory, beginning with observations derived from experiments, and extrapolating from these to general laws, falsificationism suggests that science proceeds in the opposite direction, beginning with scientific theories or ‘conjectures’, and then conducting experiments and eliminating those theories that are falsified by results [108–111]. Karl Popper, one of the most influential philosophers of science of the twentieth century [112], followed Hume in rejecting induction, claiming that it is always possible to produce a theory to fit any set of observations [113]. According to Karl Popper ‘Our belief in a hypothesis can have no stronger basis than our repeated unsuccessful critical attempts to refute it’ [114]. Popper and other scientists believed that causation is established through a process of conjecture and refutation, and that science advances only by disproofs [113,115,116]. Popper insisted strictly on deduction, allowing the sole capability of science to be the falsification of prior hypotheses (the so-called hypothetico-deductive method), rejecting any place for verification [108].

Relationship between association and causation

Association is an identifiable relationship between an exposure and disease. Association implies that exposure might cause disease [75,78]. Epidemiologists infer causation based upon the association and several other factors [80–82]. Causation implies that there is a true mechanism that leads from exposure to disease [77, 80, 82, 117–120]. However, the presence of an association does not necessarily mean that the relationship is causal [74,77,82].

Deriving causal inferences The variation among the viewpoints of epidemiologists with regard to causality is rooted in the variation among philosophical viewpoints. However, Hill's criteria provide interpretive guidelines for evaluating epidemiological evidence. Hill established the following classic operational causal criteria: strength of association, consistency, specificity, temporality, biological plausibility, dose–response effect, coherence, experimental evidence, and analogy [121–124]. According to Hill [121], not all of these guidelines will be applicable in all situations and that there may be times when we wish to conclude that a putative cause–effect relationship is real even when some of the criteria are not met. According to Rothman [125], only the criterion of temporality is a *sine qua non* for causality. If the putative cause did not precede the effect, that indeed is indisputable evidence that the observed association is not causal. Other than that one condition, there is no necessary or sufficient criterion for determining whether an observed association is causal [125]. This conclusion is in accordance with the view of Hume, Popper, and others that causal inferences cannot attain the certainty of logical deductions [103,110,111]. There is no explicit consensus about what constitutes sufficient evidence to establish causation from association.

Established epidemiological criteria for causation

The established epidemiological criteria for causation are meant to be guidelines in assisting judgement as to whether an association is causal or not. Criteria of causation refer to a set of criteria used to assess the strength of a relation between a cause and an effect, and provide a way of reaching judgements on the likelihood of an association being causal. The most widely cited list of causal criteria, originally posed as a list of standards, is attributed to Hill [121], who adapted them from the U.S. Surgeon

General's 1964 report on smoking and health [126]. Most of these lists stem from the canons of inference described by Mill [107] and the rules given by Hume [103]. The widely adopted criteria that have been refined by several scientists [77,78,109,127,128] include: (i) strength of association; (ii) consistency of effect; (iii) specificity of effect; (iv) temporality; (v) biological gradient or dose response; (vi) biological plausibility.

Strength of association Strength of association refers to the extent to which a supposed cause and effect are related and should not be confused with statistical significance [109]. The most common measure of strength of association is relative risk or rate ratio [109]. Other measures of association in epidemiology include the odds ratio, a correlation coefficient and attributable risk [109]. According to Chamberlin *et al.* [129], technical advances have allowed the identification and/or isolation of MAP from a significantly higher proportion of Crohn's disease tissues than from controls. These methodologies include: (i) improved culture techniques; (ii) development of MAP-specific polymerase chain reaction assays; (iii) development of a novel *in situ* hybridization method; (iv) efficacy of macrolide and anti-mycobacterial drug therapies; and (v) discovery of Crohn's disease-specific seroreactivity against two specific MAP recombinant antigens [129]. Several studies [130,131] reported that 50% of Crohn's disease patients and 22% of ulcerative colitis patients were MAP positive and MAP was not cultured from the non-IBD patients. Some researchers suggest that all of IBD may be due to MAP [130,131]. Chiodini *et al.* [132], described the isolation of a slow-growing, mycobactin-dependent *Mycobacteria* species from the intestinal mucosa of Crohn's disease patients but not from control tissue.

Consistency of effect This epidemiological criteria refers to the fact that an association is found in many studies despite different circumstances, research designs, or time-periods [78]. Relationships that are demonstrated in multiple studies are more likely to be causal than those that are not. Several studies conducted at different times by different research methods have reported on the isolation of MAP from patients with Crohn's disease [129, 133–138]. MAP has been found in Crohn's disease patients by genetic probes (including both DNA, and RNA) [94]. The insertion element IS900, found at 14 to 18 copies per genome has been shown to be genomically specific for MAP [138] and, has been widely used as a target for PCR [129, 131, 139–146].

Specificity of effect Specificity describes the precision with which a factor will predict the occurrence of a specific disease; it adds plausibility to the causal claim but, if absent, does not detract from it [111]. Routine culture of MAP from Crohn's disease patients' tissues is difficult because when present MAP is commonly in spheroplast form (cell wall deficient), which does not thrive in standard culture conditions [129,143]. It has also proved difficult to detect MAP in Crohn's disease tissues by other methods: the mycobacterial cell wall Ziehl–Neelsen (ZN) staining techniques, first described in 1882 [147,148] have not shown MAP in humans because MAP exists in the cell-wall-deficient form [21]; serology studies have been beset by problems of non-specificity because of antigen cross reactivity [149], although more recent studies have reported a specific high immune reactivity to recombinant MAP antigens in Crohn's patients [150,151]. These difficulties reflect the fact that MAP microorganisms when present in Crohn's disease are few in number, relative to bovine cases of MAP infection (Johne's disease) [152]. An assay for MAP in Crohn's disease must be able to specifically detect small numbers of organisms with tissue, near or below the threshold of microscopic detection [94]. Molecular methods have been used to determine the prevalence of MAP in cases of Crohn's disease [153]. An important limitation of studies looking for novel pathogens is that information about the sensitivity and specificity of assays applied is generally lacking [94]. In separate studies, it has been

shown that IS900 element is genomically specific for MAP [139] and that IS900 sequences from a heterogenous collection of MAP are invariant [154]. According to Sechi *et al.* [138], MAP has been identified by *in situ* hybridization to the MAP-specific IS900 gene in tissue specimens of Crohn's disease. However, despite these favourable considerations, the IS900-based *in situ* probe was prone to non-specific hybridization, compromising the utility of IS900-based *in situ* hybridization and indirect *in situ* PCR [155]. Jeyanathan *et al.* [94], reported that the alternative means of increasing specificity and sensitivity involves the use of rRNA-specific oligonucleotide probe *in situ* hybridization. Probes targeting rRNA provided excellent specificity resulting in forms that were morphologically consistent with ZN-positive organisms on adjacent sections. Ryan *et al.* [146], reported the detection of MAP DNA in 40% of Crohn's cases where microdissected granulomas were examined. However, only half of the granuloma-positive cases had corresponding whole tissue sections that were positive for MAP. The greater detection rate of MAP in laser capture microdissection (LCM) isolated granulomas compared with whole tissue sections may have been attributable to better targeting of MAP DNA in granulomas – PCR may suffer loss of sensitivity because of the potential dilutional effect of the large quantities of non-target DNA found in whole tissue sections. Failure to detect MAP in some studies may have been attributable to inefficient amplification of long sequences (>250 bp) [156].

Temporality Temporality refers to the necessity that the cause precedes the effect in time [128].

Causation is not possible without the cause occurring before the effect [93]. Data exist that indicate that temporal sequence criteria have been fulfilled for the association between Crohn's disease and MAP [157,158]. In a study by Van Kruiningen *et al.* [157], a goat was infected with MAP organism taken from a human patient with Crohn's disease and showed progression to Johne's disease. A 1991 report found that 24-day-old specific pathogen-free Leghorn-Cochin chicks could be infected by multiple exposure routes using the same MAP strain ('Linda') [158].

Dose–response relationship A dose–response effect is present when the effect increases with the dose or level of exposure. In a study conducted by Schwartz *et al.* [159], the intestinal mucosal layer from patients with IBD had high numbers of bacteria compared with people without Crohn's disease, however, there was no correlation between the numbers of bacteria present and either the degree of inflammation or the use of anti-inflammatory agents or sulfasalazine compounds [159]. This study suggests that a demonstration of dose–response criterion may not be applicable to a relationship between MAP and Crohn's disease. The pivotal event that convinced a totally sceptical gastroenterological community to accept that *Helico bacter pylori* was the aetiological factor in peptic ulcers was the cure rate that was achieved when the putative *H. pylori* infection was treated with appropriate antibiotics [21]. Similarly, Greenstein [21] suggested that the failure to cure IBD with anti-MAP antibiotics is the main impediment to convincing a sceptical gastroenterological community that MAP is zoonotic. Possible reasons that could account for this inability to cure patients with Crohn's disease include, the use of the wrong antibiotics and lack of satisfactory performed studies that are prospective, randomized, double blinded and placebo controlled, that have been performed using acknowledged satisfactory anti-MAP antibiotics [21]. Recently, Greenstein *et al.* [160], demonstrated that methotrexate and 6-mercaptopurine inhibit MAP growth *in vitro*. However, the dosages of methotrexate and 6-mercaptopurine in clinical use have not been titrated according to standard antibiotic conventions [160].

Biological plausibility A hypothesized effect is biologically plausible if it makes sense in the context of current biological knowledge [75]. By the 1930s, Johne's disease was found to be caused by an odd bacteria named *Mycobacteria paratuberculosis*. This organism is from the same family of bacteria

which cause tuberculosis and leprosy. Current concepts regarding the cause of Crohn's disease emphasize a dysfunction of the immune system resulting in a prolonged and intense process of inflammation [161–165]. The damage to the bowel appears to be due to this inflammatory process [163–165]. MAP is thought to produce disease by over-stimulating the immune system. The bacterium lives inside the cells of the host, where it divides only once about every 2–12 h. By way of contrast, other bacteria in the gut such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., divide about once every 20 min. There are no toxins or poisons produced by MAP. Disease happens when the immune system recognizes the ‘foreign’ proteins of the bacteria, even inside a living cell and mounts a furious attack [161–163]. The immune ‘attack’ focuses on the infected cells in the mucosal layer of the digestive system and results in massive inflammation, as well as ulcers, diarrhoea and weight loss [159, 161–163].

CONCLUSION

This paper has attempted to highlight current scientific evidence in regard to fulfilling the epidemiological criteria for a causal association between MAP and Crohn's disease. We were able to demonstrate that data exist that show that the MAP Crohn's disease phenomenon has fulfilled at least four (strength of association, consistency of effect, temporality and biological plausibility) of the six epidemiological causal criteria outlined by Hill.

In summary, the current epidemiological evidence strongly supports the conjecture that Crohn's disease is caused by MAP especially for those who believe in the theory of inductivism. Several studies that demonstrated scientific evidence, including temporality, necessary to infer a causal association between MAP and Crohn's disease were highlighted. For the followers of Popper who believe in falsification/deductivism, whether enough observations or experiments have been conducted to falsify the MAP/Crohn's disease phenomenon is a matter of personal judgement. Moreover, there are people who believe that studies can falsify a theory only to a certain degree.

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DECLARATION OF INTEREST

None.

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