The etiology of *Mycobacterium avium subspecies paratuberculosis* in Crohn's disease

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ABSTRACT

The etiology of Crohn's disease (CD) has drawn heated controversy in the literature. Compelling evidence in the literature has accumulated lately that could incriminate the *Mycobacterium avium subspecies paratuberculosis* (MAP), the well-known agent of John's disease in cattle. The evidence is isolation of the organism or its DNA and RNA, detection of the anti-MAP antibodies in Crohn's patients, increasing incidents of CD in areas close to the cattle pastures, and the possibility of treating the disease with the antibiotics. The group that favors the immune dysregulation theory considered this evidence circumstantial due to the variations in these reports. The treatment of CD with humanized anti-tumor necrosis factor-alpha antibodies is considered great endorsement to the immune dysregulation theory. The endless debate could jeopardize public health rather than bring a final solution. Reconciliation between the 2 theories appears inevitable in view of possible classification of this disease as a zoonotic.

Saudi Med J 2007; Vol. 28 (10): 1479-1484

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Crohn's disease (CD) is one of the fastest growing diseases in the world, particularly in westernized countries. Population studies indicate that the incidents of CD range between 3.1 and 14.6 cases per 100,000 people. The overall prevalence rate is between 26-198.5 cases per year. The number of CD patients in the United States is approximately one million and in the United Kingdom, 13 per 100,000 person.[1,2] The onset of the disease is usually between the age of 20-30 years. In the Kingdom of Saudi Arabia (KSA), reports refer to an increase in the incidence of the disease. A population study on the CD incidence in KSA over the last 20 years has shown that the mean annual incidence in the first 10 years was 0.32:100,000 and 1.66:100,000 over the last 10 years with the total mean annual incidence of 0.94:100,000 over the last 20 years.[3]

Crohn's disease is a recurrent idiopathic chronic inflammatory bowel disease. Although the disease is restricted to the intestine, recent observations found that mouth, larynx, esophagus, stomach, colon, skin, muscle, synovial tissue, and bone could be involved. The most characteristic pathological features of CD are the transmural inflammation of the gut with non-caseating granulomas, microfistula, and ulcerative fissures. The main histological markers are non-caseating tuberculoid granulomas and overwhelming lymphocytic infiltrations of submucosa. Crohn's disease can manifest in 2 distinct forms, perforating and non-perforating.[1,2] The CD patient manifests pain and tenderness in the lower right quadrant, intestinal stenosis that might lead to the observation of lower abdominal destination, constipation, occasional vomiting and diarrhea due to granulomatous and ulcerative lesions. The CD patient could suffer from lifelong disturbances and uncomfort as a result of recurrent episodes. Children with CD could suffer growth retardation due to malnutrition and disturbance in their sexual maturity as a result of hormonal imbalance.[1,2]

Extensive investigation indicated that certain genetic mutations predispose to CD. Although, the real nature of the genetic predisposition is not fully explored, population studies showed that certain HLA haplotypes could render the related individual vulnerable for CD. Furthermore, genome screening of susceptible individuals has shown a frameshift mutation on nucleotide oligomerizaton domain 2 (NOD2)/caspase recruitment domain 15 (CARD15), a gene on chromosome 16. The NOD2/CARD15 encodes one of the intracellular nucleotide-binding site-leucine rich repeat protein receptor family, which is involved in the activation of nuclear factor κB (NF-κβ), a proinflammatory pathway, as a result of its interaction

with the peptidoglycan motif of bacterial pathogens.[1,2] The frameshift mutation in NOD2/CARD15 is caused by cytosine insertion and other nucleotides polymorphism, which will result in considerable disturbance and interference with the ability of innate immunity to detect and prevent bacterial infection. The etiology of CD is still a stimulating overwhelming controversy in the scientific community. However, the overall picture of the disease, favors the dysregulation of the mucosal immune system of the gastrointestinal tract. The dysregulation leads to the domination of the helper cell type 1 (Th1) immune responses and infiltration of the intestinal flora.[4] Two major theories summarize the rivalry on the etiology of CD. The immune dysregulation, and infection by the *Mycobacterium avium subspecies paratuberculosis* (MAP), the causative pathogen of John's disease in cattle and other ruminant and non ruminant animals. The implication of MAP in CD has stimulated heated debate, which lead to extensive research. The advocates of the MAP theory presented extensive evidence in the last decade that emphasized on the incrimination of this pathogen as the causative agent of CD. However, the evidence was dismissed by the pro immune dysregulation theory by considering them far beyond convincing. The theme of this review is based on presenting the evidence of the MAP theory, and the arguments presented by the anti-MAP theory advocates. The main evidence presented by the MAP theory and will be reviewed here are: 1. Close identity of MAP DNA strains to that isolated from CD patients. 2. Isolation of cell wall deficient (CWD) MAP from CD patient. 3. Detection of MAP DNA in CD patients using different techniques, polymerase chain reaction (PCR) and in situ-hybridization. 4. Detection of anti-MAP IgG in CD patients. 5. Evidence of promising antibiotics treatment of CD patients.

The MAP and John's disease. The first organism was identified in 1895 and it was classified as one of the Mycobacterial species.[5] Currently the organism is classified as MAP.[6] It is an acid fast bacillus of 0.51-1.5 μm with the slow tendency to grow on solid media supplied with mycobactin (Herrold's egg yolk medium).[7] The MAP genome is the single circular chromosome of 4,829,781 base pairs.[8] The size is approximately the same with *Mycobacterium tuberculosis*, but it is larger than that of *Mycobacterium leprae*.[9] The MAP immunoantigens are complex and diverse. Several heat shock proteins, GroEd and 65 KDa antigens and alkyl hydroperoxide reductases C and D (AhpC and AhpD). Several other species-specific antigens were identified, and its function is under investigation.[9] The MAP causes John's disease in domestic and wild ruminants such as cattle, sheep, goats, deer, antelope, and bison.[5] In KSA, John's disease was reported in sheep, goat, and camel[10,11] and most recently in dairy cattle.[12] The infection usually occurs during the early months of life. It is of chronic nature due to the long incubation period, but the onset of the disease is associated with the development of contagious, chronic progressive, and fatal enteric granuloma.[13,14] John's disease is characterized by a long subclinical period, 2-3 years, in which the infected animals express no distinct symptoms. The most important clinical signs of John's disease are weight loss and continuous diarrhea. The ingested organisms find their way to the small intestine particularly ileal peyer's patches through M-cells. The immune responses to MAP in ruminants are of a paradoxical nature. In general, the early stage of the infection is dominated by the cell mediated immune (CMI) responses, whereas in the late stages humoral immune responses prevail.[7] At the early stages of the infection, Th1 dominates the immune responses with copious production of interferon-γ (IFN-γ). Most of T-lymphocytes at the subclinical stage that express T-cell receptor (TCR) γδ are of the CD^{4+}/CD^{8+} phenotype. The T-cells with γδ TCR play a crucial role in restricting MAP infection through the production of cytokines, particularly IFN-γ and recognition of antigen with no major histocompatibility complex class II restriction.[14,15] However, the predominance of the CD8$^+$ T cells or CD^{4+} T cells in the early stages of infection is a matter of controversy. The strategies that MAP employs to evade immune responses were extensively examined. The phagocytosis mechanism was severely hindered by MAP due to the abolition of phagosome-lysosome fusion and abortion of oxygen radical production.[16] The T lymphocytes appear less capable of presenting antigen with distinct reduction in IFN-γ, tumor necrosis factor-alpha (TNF-α) and interlukin-2 (IL-2).[14]

Is Crohn's disease a MAP infection? In the last decade, the advocates of the MAP theory in CD seem to have present overwhelming evidence that categorically incriminates MAP as the causative agent of CD. The major evidence can be categorized in 3 groups: 1. Isolation of MAP from the CD patients. 2. Detection of the MAP IS900 DNA and RNA in the CD patients. 3. Detection of the MAP DNA by in situ-hybridization. The major breakthrough was achieved when MAP was isolated from 50% of the blood samples,[17] intestinal tissue (42%),[18] and breast milk of the CD patients.[19] The isolation of MAP was made possible by the development of media that met the fastidious need of MAP growth requirements. The mycobacterial growth indicator tube is a culture medium produced by Becton Dickinson USA that supports the growth of MAP following 10-12 weeks or more of incubation.[20] The peculiar property of these viable isolates from the tissue of CD patients was shown to be CWD. Several investigators reported the isolation of CWD or spheroplast organisms since 1970.[21-24]

The association of MAP to CD was further investigated using the polymerase chain reaction (PCR) technique that was based on detection the system of the IS900 DNA and RNA. Detection of MAP IS900 was made possible as a result of optimizing the quantity and quality of nucleic acid isolation from fresh clinical tissue and enhancing the PCR amplification by designing high precision primers to minimize any secondary structure formation. Following these stringent procedures, several studies reported the detection of MAP DNA by PCR.[17,24-28] Isolation of MAP RNA was considered vital to link the organism to the pathological changes in CD patients. As Ziehl-Neelsen stain is impractical to detect the MAP isolate in the CD patients due to its CWD nature, the in-situ hybridization approach was seen essentially in providing evidence of MAP implication in CD. Numerous studies reported the detection of MAP DNA by in-situ hybridization in tissue of CD patients.[19,26,29-31] However, the ardent skeptics of the MAP etiology theory dismissed this evidence. Isolation of the viable MAP from inflamed tissue and detection of its genetic materials was questioned as they lack the requirements to substantiate the following: 1. Evidence should indicate beyond doubt that MAP is the prime factor in initiating the CD. 2. The presence of MAP in the genetically susceptible patient is the major player in priming the disease. 3. Contrary to what is known, the incidence of CD in underdeveloped countries is less correlated with the endemic status of MAP.

The most unsustainable paradigm that is made by the critics of this theory is reflected by Shanahan et al[32] that apparently renders the presented evidence flawed and untenable "Detection of MAP in CD is neither disease-specific nor bacterial-specific. Detection of bacterial DNA in the granulomas of intestinal CD is not specific to MAP; other forms of bacterial DNA are also present. This may reflect disturbed host-flora interactions in patients with CD and is consistent with other observations of increased mucosal bacteria in CD." Whether MAP is the prime causative agent of CD or not, it is a dilemma in both groups.[33-35] This is clearly stated in the recommendations of the panel conducted by the National Institute of Health.[36] The interesting recommendation is "there is insufficient evidence to prove or disprove that MAP is a human pathogen or that it is the cause of Crohn's disease. Considerable controversy continues to exist in the scientific community on this point". Hence, the absence or presence of MAP in CD patients is becoming a less critical issue in comparison with the pressing need to elucidate which of the following steps are triggering the CD, intestinal permeability imbalance as a result of immune dysregulation or presence of pathogenic

factor such as MAP that initiates the whole process in the genetically susceptible host. Although, most of the advocates of MAP etiology theory in CD admit that MAP is one of the heterogeneous factors of this syndrome, the exact mechanism by which MAP initiates CD needs to be tackled. It is generally accepted that the potential of developing CD in the genetically susceptible individuals requires an external factor that perturbs the intestinal permeability in a way that it leads to penetration of the microbial flora to the submucosal level. Hence, does MAP possess potential virulent arsenals that enable the organism to act as an external factor that initiates the intestinal immune dysregulation? The wide scale of species that are infected with MAP could imply the capability of MAP to cross the species barriers. The organism was isolated from unconventional hosts, mainly primates; such as macaques, baboons, cotton top tamarins, and gibbons.[36] In addition to the numerous reports of MAP isolation from CD patients like isolation of acid-fast bacilli from hemophilic patient, AIDS and from draining lymph nodes of 6 year-old boy with ileocecal CD.[37,38] Furthermore, analysis of MAP genome of isolates from different animals revealed close relation to that isolates from CD patients.[39] The MAP is a well-known pathogen with virulent factors that fuel the pathogenicity that induces severe intestinal inflammation.[9,13,15,16] Coussens[13] developed an interesting model that explains MAP strategies in establishing infection in the cattle intestine. The major bacterial membrane proteins were shown to act as important MAP virulent factors in colonization of residential macrophages in the intestine. Persistence of MAP in macrophages is achieved through the excessive production of interlukin-1alpha (IL-1α), which is considered as one of the major factors in intestinal tissue damage, by enhancing the macrophage survival through abolishing of the apoptosis program cell death. Rumsy et al[16] studied the mechanism of MAP survival in the peripheral blood mononuclear cells (PBMCs) of CD patients by comparing the survival of MAP, *Mycobacterium tuberculosis*, and *Escherichia coli* in human PBMCs. The phagolysosome fusion inhibition in human leukocytes was a potential factor that enables MAP in colonizing the intestinal tissue by disturbing the intestinal mucosal immunity.

Recently, epidemiological studies on the MAP endemic regions and the possible relation to the escalation of the CD attracted reasonable attention.[40,41] Pickup et al[40] suggested a link between the contamination of water with MAP in one of the rivers in Wales, United Kingdom that crosses through pastures grazed by livestock in which MAP is endemic with the significant elevation in the CD incidents in the population

residing close to this water resource. Furthermore, the incidents of CD among people who were in close contact to clinical cases of bovine Paratuberculosis were examined.[41] The study failed to detect a relation between CD and bovine paratuberculosis, however, it was speculated that children are more susceptible to the infection than adults. Hence, exposure to the infection during childhood was considered crucial for the subsequent development of CD. The MAP theory validity is continuously questioned by presuming the CD incidence in underdeveloped countries is low. It was assumed that the CD incident should be contrary to what is known, higher in the poor countries if MAP is the major factor in initiating the disease. The presumption that the CD incidents in under developed countries are lower than that in the westernized countries is merely a speculation. There are no dependable population studies in the under developed countries that could substantiate this.

Immune responses to MAP in CD patients: a myth or reality? The theory of MAP etiology in CD challenged to provide evidence of immunocompetent response to MAP in CD patients. In respect to the antibody response to MAP in CD patients, numerous studies reported the detection of anti-MAP antibodies.[42-46] The elicited antibodies in CD patients were shown to be against anti-IS900 product,[41] anti-p35 and P36 proteins,[44] and 14KDa.[46] The evidence of anti-MAP antibodies were dismissed by the dogma of the immune dysregulation theory. They considered these antibodies as either a consequence of exposure to MAP in the environment or normal intestinal commensals,[4] or a result of molecular mimicry between the intestine, normal commensals, and MAP antigens.[47] If mimicry plays a pivotal mechanism in priming the autoimmunity, this mimicry could be a further proof that MAP has a role in initiating the CD. Despite this controversy, the reality will continue to exist that these antibodies were detected in individuals with inflamed tissue and MAP were isolated from and they expressed a level of immunoreactivity to this pathogen. One of the obstacles continuously raise against the MAP theory is the absence of detectable CMI. Accumulating evidence refers to the strategies of MAP to induce T-cell anargy and survive phagocytosis.[15] Lack of CMI to MAP infection in CD patients could be identical to John's disease. In John's disease, CMI dominates the early stage of subclinical infection while antibody response prevails in the clinical stage.[15] *Mycobacterium avium subspecies paratuberculosis* infection therefore, follows the similar pattern in CD. Lack of a detectable level of CMI during the sever stage of the disease is probably due to a switch from CMI to humoral immunity as well as due to the sever suppression that MAP brought on the immune cellular system.[13]

Does antibiotic therapy cure CD? The current treatment of CD is mainly based on immunosuppressive regimens that target the exacerbated immune responses. The immunosuppresents and anti-inflammatory agents such as budesenide and prednisolones are the common drugs that are prescribed to the CD patients. However, corticosteroids such as azathioprine, 6-mercaptopurine, and other drugs such as cyclosporine are recommended, too. Development of humanized anti-TNF-α antibody was considered the cornerstone therapy in the treatment of this disease.[48] The success of anti-TNF-α therapy in subsidizing the symptoms of CD patients raises fundamental controversy against the MAP etiology theory. It was concluded that if CD was an infectious disease, the anti-TNF-α therapy would certainly exacerbates the MAP growth. The fundamental idea in this notion, which posed as deadlock to the MAP theory is based, unfortunately on premature knowledge. First, the central role of TNF-α in the regulation of MAP infection in human is not fully illustrated. Second, the MAP strain that was expected to flourish, if it is existed is CWD MAP. The pathogenesis of CWD MAP could be based on strategies different from what is known by other Mycobacterial species. In addition, the notion that immunosuppressant therapy could not be envisaged for the treatment of MAP infection, contradicts the routine practice of treating severe tuberculosis and leprosy with immunosuppressants. It is considered a conventional therapeutical approach to facilitate the effective antibiotic therapy.[48,49] Although, anti-TNF-α therapy remains crucial in tackling the excessive immune responses in CD, antibiotic therapy continues to be the most important therapeutical alternatives in CD. In the last decade, antibiotic trials of CD revealed 2 important fundamental findings that were observed crucial in achieving successful antibiotic therapy. They are type, number of antibiotics and course of therapy. Antibiotic trials lasted for more than 6 months and had an incorporated combination of at least 2 antibiotics, such as clarythromycin, azithromycin rifabutin, or rifampin, and produced 60-80% remission of the CD.[50] Contradiction in the results of different antibiotic trials were most probably due to the variation in the type of antibiotics or course of therapy. The antibiotic trials for the elimination of MAP in CD patients were intensively evaluated in the last decade.[51,52]

Debate or reconciliation? Diligent research in the last few years on the MAP theory provided sentimental evidence that substantiated its validity more than ever, and has deepened its roots in the CD literature. The notion that CD could be a zoonotic disease is becoming more eminent. Thanks to the state of art research tools that broke the principle obstacles used to

question the legitimacy of the MAP theory. These major breakthroughs are isolation and growth of MAP, isolation and detection of its DNA and RNA in the inflamed tissue, and most importantly the evidence of detectable antibody responses to MAP in CD patients. Although the research in MAP theory sounds victorial in defeating its skeptics, fundamental questions remainto be tackled. One of these questions is the need for obvious evidence that links the infected animals to the patients that are exposed to them or their products. The answer should address beyond doubt the factors that interfere with the flourishing of CD among the workers of dairy farms endemic with MAP. Exploiting these factors is important in understanding the immune mechanism(s) involved in contemplating this infection. The numerous dark holes that exist in understanding the CD pathology were the most important reasons in fueling this debate on the MAP theory in CD. Further research on the role of MAP in CD should exploit beyond doubt whether CD is a multiform disease or it is merely induced by MAP infection. If either of these presumptions appeared to be valid, the research will change the course from dipolar theory (MAP and immune dysregulation theories) to research of a collaborative and reciprocal nature. However, the following enquires continue to hunt the researchers of MAP theory: 1. Is CD a human counter part of John's disease or it is an exploitation of another evolutionary state of MAP pathogenicity. 2. Does MAP theory explain one type of the multiform of CD? Or is MAP the only factor implicated in priming CD.

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