Association of *Mycobacterium paratuberculosis* in Crohn's disease and Johne's disease: A possible zoonotic threat

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***Mycobacterium paratuberculosis* is the well-known etiologic agent of Johne's disease (JD), of the ruminants. In recent years, this organism has drawn attention due to its possible role in Crohn's disease (CD), a chronic inflammatory bowel disease of the human gastrointestinal tract. But the growing concern over the zoonotic potential of *M. paratuberculosis* is still not well established. The possible association of *M. paratuberculosis* with CD and its zoonotic significance has been reviewed in the light of isolation studies, experimental disease production, histopathological and immunological evidences, besides DNA sequence analysis. Food-borne exposure, the possible source of human infection, has also been discussed.**

THE inflammatory bowel disease is also known as Crohn's disease (CD). It primarily affects the ileum of human beings. The inflammatory response in the ileum bears considerable similarity to the histopathological lesions of Johne's disease (JD) of ruminants caused by *Mycobacterium paratuberculosis*[1]. Historically, JD was first described in 1895 by Johne and Frothingham as a 'peculiar case of tuberculosis (TB)' in a cow with chronic enteritis, marked by thickening and corrugation of the intestinal mucosa, and the presence of acid-fast bacilli[2]. In 1906, this disease was named as JD or pseudotuberculosis and it was recognized that the disease was not TB[3]. Subsequently, the term paratuberculosis was coined and the bacillus was cultured and characterized[4,5]. The organism which was originally named *Mycobacterium enteritidis chronicae pseudotuberculosae bovis johne*, has since been named *M. johnei*, *M. paratuberculosis* and the most recent nomenclature is *M. avium* subspecies *paratuberculosis*. Although exact etiology of CD is still debatable, the recent data using improved cultural, molecular and serological techniques as well as epidemiological evidences have strengthened the association of *M. paratuberculosis* with CD[6]. It has also been suggested that exposure of genetically susceptible individuals during childhood to some unknown microbial or chemical factors leads to development of CD[7,8]. In 1984, *M. paratuberculosis* was isolated from a 15-year-old girl (strain Linda), a 12-year-old boy (strain Dominic) and a 78-year-old man (strain Ben) having CD[9]. Subsequent studies confirmed that these isolates were able to produce JD in goats on experimental infection by the oral route. These strains were also genetically identical to strains of *M. paratuberculosis* originating from cattle[10]. Recently, isolates of *M. paratuberculosis* from more number of human cases with CD have led to the hypothesis that CD may indeed be due to a mycobacterial infection possibly from *M. paratuberculosis*[11]. The histopathological lesion of JD in ruminants has been described to be of two distinct types: multibacillary lepromatous type being associated with the presence of mycobacteria and paucibacillary tuberculoid type with apparent absence of acid-fast mycobacterium. It is uncertain whether these two distinct histological forms of advanced lesions represent sequential or divergent stages of paratuberculosis[12]. Similar to ovine paratuberculosis, there is a dual clinical presentation in CD, which renders it analogous to other mycobacterial diseases, e.g. leprosy that also has dual clinical manifestations, tuberculoid and uncontrolled lepromatous forms.

Histopathological evidence

Pathological similarities between JD and CD were noted way back in 1932 and still earlier[13,14] in 1913. The report of Thomas Dalziel[14], a Glasgow surgeon operating on a colleague with chronic inflammation of the intestine, described that the histopathological character of the disease was similar to that of JD. He recognized that it was a different disease from intestinal TB, but belived that it was so similar to that disease and to paratuberculosis in cows that it must be a mycobacterial disease. JD affects the young stocks, and animals become infected early in life, but the typical clinical disease develops between 2 and 5 years of age. During this long subclinical stage, bacterial shedding from infected animals in

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their faeces and milk is of epidemiological concern in spreading the disease to susceptible individuals[15]. The same bacterium has been implicated as a possible source of infection in susceptible human population. Both the diseases are chronic, debilitating inflammatory diseases of the intestine and have similar histopathological lesions[16]. In CD, pathological changes are mostly confined to the small intestine but may also extend to the large intestine, even up to the anus. It is also not unusual to detect pathological lesions at the upper alimentary tract, muscle, bone and skin. Due to oedematous inflammation of submucosa, the lesions in the intestines are segmented with a thickening of the intestinal wall. Histopathologically, the granulomatous lesions are non-caseous and are made up of loose aggregates of epitheloid cells, Langerhan's giant cells with lymphocytic infiltration extending from the mucosa up to the serosa. Lesions may also affect lymph nodes and lymphatic vessels. The inflammatory changes cause intestinal fibrosis that leads to bowel stenosis and ulcers that may aggravate to form fistulae[17]. Although controversial, there are evidences indicating two distinct clinical manifestations of CD, viz. 'perforating' -perforating' forms. The perforating form is the most drastic form having abscesses and/or free perforation with a higher reoperation rate. The non-perforating form has a slow clinical course and is associated with obstruction and bleeding[18]. Intestinal TB may be mistaken as CD in respect to intestinal ulceration and fibrosis. But the presence of tubercle with caseation and acid-fast organisms in intestinal TB usually enables the pathologists to distinguish it from CD. Non-caseating granuloma and lack of identifiable acid-fast bacilli from CD lesions are differentiating characters from intestinal TB. Nevertheless, CD cases may sometimes be difficult to distinguish clinically and pathologically from ulcerative colitis (UC), another form of inflammatory bowel disease (IBD). However, UC is principally a disease of the mucosa, with a diffuse mononuclear cell infiltration in the lamina propria with goblet cell depletion and crypt gland inflammation. The pathological lesions of CD have similarities with the tuberculoid paucibacillary form of JD, in respect of chronic, non-caseous granulomatous changes affecting the intestinal wall and regional lymphoid system. In contrast to CD, ulceration and fistulation are not typical to JD, however ulceration has been reported in some experimental infection studies[10]. Cell wall deficient, non acid-fast spheroplasts have been detected in intestinal biopsies of CD patients[19], as also is the case with paucibacillary intestinal lesions of cattle with subclinical cases of JD[20].

Bacterial isolation

Isolation and detection of *M. paratuberculosis* from CD tissues by cultivating the organism in the laboratory medium is the limiting factor for confirmative diagnosis. Isolation of the organism is most often attempted from biopsies and surgical resection from CD and UC lesions besides other gastrointestinal disorders and diseases, including infectious colitis, lymphocytic colitis, polyps and cystic fibrosis. The success of the isolation relies upon the choice of the decontaminating agent as well as bacteriological medium formulation. Nevertheless, no consensus has emerged on the best overall method. Similarly, for the diagnosis of JD, the faecal samples are the main source of isolation as the infected animals usually shed the bacteria in their faeces during the subclinical and clinical course of the disease[15]. Unlike the animals, there is no report of bacterial shedding through faeces in case of CD patients. The presently used BACTEC-12 radiometric culture method having a combination of selective antibiotic and a radioisotope-based growth indicator system, permits detection of as few as three *M. paratuberculosis* organisms per gram of the clinical sample[21]. Isolation of *M. paratuberculosis* from the surgical samples of regional enteritis has been reported[22] since 1952 and subsequently several workers have isolated the bacilli from the lesion of CD patients[9,19,23]. Cell wall deficient, non acid-fast spheroplasts have also been detected from CD intestinal biopsies[19,24]. In case of JD, *M. paratuberculosis* has been successfully isolated from the faecal samples as well as from the milk; it has been unequivocally proved that the causative agent for the disease is *M. paratuberculosis*[25–27]. Isolation of *M. paratuberculosis* with some pathological features of CD, in a colony of non-human primates (stumptail macaques) has raised concern regarding the belief that *M. paratuberculosis* is innocuous to humans[28]. The clinical and pathological features of paratuberculosis in these species were comparable to those reported for paratuberculosis in ruminants. Intestinal tissues from animals dying of this disease contained up to 10^9 colony forming units of *M. paratuberculosis* per gram of tissue. At necropsy, 13 animals aged 2 to 19 years were found to have granulomatous enteritis and regional lymphadenitis. Occasional lesions or acid-fast organisms were also seen in the renal pelvis, spleen and bone marrow. Possible association between *M. paratuberculosis* and CD in man has been strengthened by isolation of *M. paratuberculosis* from the tissues of those CD patients identical with pathogenic strains in ruminants[29].

Experimental disease production

Early attempts for experimental production of the disease in the laboratory animals have given unconvincing results. One has to accept that Koch's postulates have yet not been met for leprosy[30]. Nevertheless, an isolate with characteristics of *M. paratuberculosis* from a clini-

cal case of CD was used to infect goats, which produced lesions typical of ruminant paratuberculosis[10]. Many laboratory animal species, including rats, hamsters, gerbils, guinea pigs, voles and even pigeons have also been investigated as experimental models for *M. paratuberculosis*[15]. Oral and parenteral infection of rabbits produced typical lesions in the intestine and other tissues[31]. Recently, confirmation of *M. paratuberculosis* infection in wild rabbits with typical lepromatous lesions, has been reported[32]. Experimental oral infection in immunodeficient mice produced focal granulomatous lesions, containing acid-fast organisms in the intestine[33]. Parenteral inoculation in mice produced wide-spread granulomatous lesions, including early lesions in Peyer's patches[34].

Immunological evidence

Immunological evidence for asssociation of *M. paratuberculosis* with CD needs more confirmation due to lack of species-specific antigens used in the test proper. Antibody binding to 38 kDa protein band of *M. paratuberculosis* was demonstrated by the Western blot technique in 57% of CD patients[35]. Antibody response directed towards the native protein antigens of *M. paratuberculosis* has been evidenced in clinical and experimental cases of JD in small ruminants[36,37]. Adsorbed ELISA using *M. paratuberculosis* protoplasmic protein antigen has shown significant positive results in sera of CD patients, suggesting that this organism may play some role in the pathogenesis of CD[38]. Similarly, improvement in the immunogical detection system for JD has been carried out by using adsorbed ELISA as well as incorporating affinity purified antigens in the detection system[39,40]. It has been confirmed that the sera from CD patients reacted to *M. paratuberculosis* recombinant protein p36 with same frequency as the sera from patients who were exposed to mycobacterial antigen, further supports the hypothesis of mycobacterial etiology of CD patients[6]. Evidence of cell-mediated immune (CMI) response against *M. paratuberculosis* antigens has been well documented with JD[15,41,42], whereas insignificant T-cell response of CD patients towards mycobacterial antigens has raised some doubt to support a mycobacterial etiopathology of CD[43]. Using *M. paratuberculosis* species-specific recombinant polypeptide (a362) it has been revealed that 36% of patients with CD had an anti-a362 IgA level higher than the control, that suggested *M. paratuberculosis* as an etiological agent for CD[44]. Increased sensitivity in ELISA has been observed with the same recombinant polypeptide in case of JD in sheep, whereas no significant elevation of mean IgG or IgA titers of CD patients over controls was recorded against a protease-resistant *M. paratuberculosis*-specific 18 kDa protein antigen. These findings make it unlikely that *M. paratuberculosis* is of primary pathogenic importance in CD[45]. Preabsorption of the sera with *M. phlei* sonicate markedly reduced the nonspecific serological cross-reactions, while the 32–42 kDa lipoarabinomannan (non-protein carbohydrate type moiety) antigens appeared to be specific for detection of JD in cattle[46]. While evaluating the diagnostic tests for JD in young cattle, McDonald and coworkers have categorically stated that detection of *M. paratuberculosis* continues to be difficult using current tests, viz. absorbed enzyme immunoassay, IFN-γ enzyme-immunoassay and faecal culture[47]. By using long-linear and logistic modelling approaches to investigate the dependence among diagnostic tests, it has been concluded that for bovine paratuberculosis, both faecal-based tests and ELISA tests are necessary in serial and parallel testing schemes[48].

Evidence from DNA sequence

Discovery of the genetic amplification technique called polymerase chain reaction (PCR) has revolutionized the research on paratuberculosis[49,50]. The classification of etiological agents of paratuberculosis is still incompletely resolved. Classification based on biochemical characterization has categorized this organism as a subspecies of *Mycobacterium avium* (*M. a. paratuberculosis*) closely related to the pathogens *M. a. silvaticum* and *M. avium* subspecies *avium*[51]. Recently, genomic DNA analysis has shown that insertion sequence IS900 is unique to *M. avium* subspecies *paratuberculosis* with 15 to 20 copies per organism[49,52]. A related insertion sequence IS901 is found in some pathogenic strains of *M. a. avium* and *M. a. silvaticum* but was not detected in *M. a. paratuberculosis*. This became the basis of recent classification[53]. The identification of this insertion sequence from the bacterial culture, faeces and tissues by PCR technique has made it possible for rapid and sensitive detection with accuracy[54–56]. IS900 sequence, specific for *M. a. paratuberculosis* has been detected up to a level of 72% in the intestinal tissues of CD patients. A large number of workers using PCR amplification and primer specific for IS900, have found that CD patients tested significantly positive, more often than did the controls[57,58]. A typical cervical lymphadenitis of a 7-year-old healthy boy, followed five years later by terminal ileitis similar to CD was found to be positive for IS900 PCR on the DNA extracted from the incised lymph node[59]. Reverse transcription polymerase chain reaction (RT-PCR) amplification to probe for the presence of IS900 sequences in the clinical samples from CD and UC has confirmed the presence of *M. paratuberculosis* RNA in the test samples[11]. Presence of *M. paratuberculosis* has been ascertained in the intestine of patients with CD and UC by RT-PCR followed by

Southern blot[58,60]. Another probe specific for *M. paratuberculosis,* is a recombinant clone F-57 that is currently being used in some laboratories for diagnosis of *M. paratuberculosis*[61]. Comparative studies of *M. paratuberculosis* strains isolated from CD and JD have favoured a common clonal origin for the 4 strains isolated from CD and for 8 of the 11 strains isolated from cattle and sheep with JD[62]. Although some studies had negative results regarding the identification of IS900 sequence from the clinical samples of CD patients, the consensus is that roughly one-half of CD patients were infected with *M. paratuberculosis*[63].

Possible mode of transmission to humans

Epidemiological evidences correlating exposure to *M. paratuberculosis* with incidence of CD is not readily available[64]. No detailed study has been made regarding the prevalence of CD in respect to geographical region, age, sex and occupation. A study in the Netherlands between 1979 and 1983 has indicated that the incidence of CD appeared to be 3.9 per 100,000 persons per year and was even higher in urban areas than rural areas[65]. Standard culture technique for mycobacteria proved inefficient in isolating *M. paratuberculosis,* as it often alters its phenotype to a cell wall deficient, unrecognizable non-bacillary form (spheroplasts). These studies have shown prevalence rate ranging from 40 to 75% in CD patients compared to 0 to 25% in the controls[66]. The prevalence of paratuberculosis among Michigan dairy cattle herd has indicated positive correlation with acidic soil and increased soil iron content[67]. On estimating the prevalence of paratuberculosis in Danish dairy herd by using bulk-tank milk ELISA, it was found to be technically unsuitable for surveillance[68]. However, if *M. paratuberculosis* is a matter of public health concern and of zoonotic importance, the most possible source of infection may be the infected dairy animals. Several sources of exposure are feasible, including milk, milk products, meat or even contaminated water. Excretion of *M. paratuberculosis* from infected animals occurs primarily in the faeces and to a lesser extent in the milk[69,70]. Excretion of the paratuberculosis organism through the faeces in sheep with multibacillary JD occured daily, proving that environmental contamination can be continuous on farms with endemic ovine paratuberculosis[71]. Moreover, this pathogen is chronically shed in the milk of asymptomatic animals[27], a condition favouring the possibility of oral transmission of the disease through milk. Therefore, two different routes of contamination may be possible, the 'direct route' in which *M. paratuberculosis* is shed directly into the milk and the 'indirect route' in which the milk is contaminated by the faeces. In the later course of the disease in animals, the organisms are disseminated to various internal organs and have been isolated from lymph nodes, kidney, spleen, uterus and epididymis, with a possible chance of both pre-harvest and post-harvest contamination of food products originating from the infected animals[63]. The liquid milk is the major food item that has been the focus of research as a potential source of infection. The modern technique of pasteurization of fluid milk is an efficient and effective method to kill the pathogenic microbes, but the laboratory report of Chiodini and Herman-Taylor has demonstrated that the thermal resistance of *M. paratuberculosis* is higher than *M. bovis,* one of the bacterial pathogens used to define pasteurization time and temperature standard[72]. Chiodini and Hermon-Taylor have reported that 10^4 CFU/ml *M. paratuberculosis* in raw milk survived exposure to 71.7°C for 15 s in a water bath which is the standard temperature and holding time in the pasteurization process. Subsequent studies have confirmed that *M. paratuberculosis* suspended in milk is more resistant to heat than *M. bovis* and thus could survive the high temperature short time (HTST) method of pasteurization[73]. A study conducted on commercially processed milk samples has shown that 22 out of 312 samples contained *M. paratuberculosis* as detected by PCR[74]. Out of these, only nine samples were culture positive for *M. paratuberculosis* on long-term incubation, indicating that at least some of the milk samples contained viable organisms that might have escaped the pasteurization process. A modelling approach study[75] in the Netherlands has indicated that the point estimate of human exposure would be about 0.5 CFU for every litre of milk consumed, where the prevalence of *M. paratuberculosis* in the dairy herd was assumed to be 20%. Unfortunately, no such detailed study and statistical prediction has been recorded in the Indian dairy herds and/or in milk processing units for improving the food safety. The unusually long incubation period required to detect the viable *M. paratuberculosis* bacilli from a food sample is the main limitation for quality control measures. Undoubtedly, the PCR technique is extremely quick and reliable but cannot distinguish between living and dead bacteria. Hence a sample found to be positive by gene probe for IS900 sequence may add confusion but not clarity. The margin of safety in the process of pasteurization is of prime concern and it should be sufficient to kill the viable bacilli before being released for human consumption. Out of the total milk produced in India, the bulk amount is marketed as raw milk without pasteurization, but in future the senario may be reversed due to high urbanization. Therefore, the food safety should not be dealt with casually. A matter of satisfaction is that the Indian community usually never consume either the pasteurized or the raw milk unless it is boiled (boiling temperature of milk is sufficient to kill the bacilli). From accumulated evidences, it has been hypothesized that CD may indeed be due to a mycobac-

terial infection, possibly from *M. paratuberculosis*. Many a times available literature has shown that paratuberculosis of animals and CD of humans shared similar clinical signs but that may not be enough to accept the causative bacilli as a definite potential zoonosis.

Conclusion

Uncertainty about the etiologic role of *M. paratuberculosis* in CD has led to many arguments for and against the disease. Despite years of investigation, a definite association between JD and CD has not been established. It has not yet been determined whether *M. paratuberculosis* is of primary etiological significance or is a secondary invader in the case of CD. Detection of *M. paratuberculosis* from the tissues of CD patients and from pasteurized milk, as well as isolation of the bacilli from the non-human primates has raised concern regarding susceptibility of human beings to this causative agent. Most of the emphasis about food-borne diseases has been focused on the immediate effects of acute infection, whereas *M. paratuberculosis* which takes a long period of incubation to exhibit its clinical symptoms should be of concern for long-term sequelae to food-borne diseases. Until *M. paratuberculosis* is declared as non pathogenic to human beings, it should be handled with caution. If this is true, JD deserves to be dealt with seriously as a potential source of human pathogen from food hygiene and public health point of view.

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