In the search of a cause of Crohn's disease

Govind K. Makharia · Urvashi B. Singh

While the exact cause of Crohn's disease (CD) is not known, it is thought that chronic inflammation in CD results from an inappropriate and chronic activation of the innate and adaptive mucosal immune systems in a genetically susceptible host, and that enteric microflorae play a pivotal role in the initiation and maintenance of the disease.¹ Organisms such as *Pseudomonas maltophilia*, *Mycobacterium kansasii*, *Chlamydia trachomatis*, *Bacteroides fragilis*, *Listeria monocytogenes*, *Escherichia coli* and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) have been implicated and identified in the intestines of patients with CD; however, no other putative pathogenic organism except MAP causes a chronic granulomatous inflammation of the intestines in every other species in which it is present.² Moreover, because of remarkable clinical, morphological and epidemiological similarity between intestinal tuberculosis (caused by *Mycobacterium tuberculosis*) and CD in humans and Johne's disease (caused by *Mycobacterium avium paratuberculosis*) in cattle, a mycobacterial pathogen is thought to be a causative organism of CD.^{3–7} An association and causality of *Helicobacter pylori* with peptic ulcer disease revolutionized the treatment of peptic ulcers; similar implication of MAP as a cause of CD has raised hopes amongst physicians, microbiologists and basic scientists for the cure of this disease.⁸

In the first report almost 25 years back, Chiodini and colleagues isolated cell-wall deficient cells, called spheroplasts,

from tissue samples after several months of incubation from patients with CD. These spheroplasts were sub-cultured and they later developed a cell wall that stained positively with Ziehl-Neelson staining; they were classified as mycobacterial like organisms.^{9–11} These isolates were later confirmed as *M. avium paratuberculosis* by DNA hybridization. The unusual nature of the cell wall-deficient MAP and the challenges in culture of MAP with its fastidious and slow growing requiring months to years to culture and multiple studies reporting failure to culture MAP from patients with CD dampened the initial zeal.¹² There was then a period of silence in the 1990s. With availability of better culture techniques and use of molecular techniques to identify MAP in 2000s, there was resurgence of interest of many laboratories in this field. There has been a flurry of publications since then relating CD and MAP.^{13–25}

MAP infection in humans is difficult to detect. The organisms are intracellular and minimize their own immune recognition. They are therefore difficult to isolate and propagate in culture and are relatively resistant to chemical and enzymatic lysis. Reliable access to their DNA is only achieved during sample processing by combining exposure to stringent lysis buffers with an additional optimised mechanical disruption step. Freezing samples and tissue extracts especially at -20°C substantially reduces the PCR detection rate of their GC-rich DNA. The identification of MAP in patients with CD can be done by several different techniques such as culture of MAP from the intestines and blood; PCR amplification of blood or intestinal biopsies using IS900 DNA, a sequence specific for MAP; in-situ hybridization in tissues using IS900 sequence; serological tests using MAP specific antigens and direct visualization of MAP by light microscopy.^{4,26} According to a meta-analysis, compared with individuals free of IBD, the pooled odds ratio (OR) of those with CD having MAP was 7.01 (95% CI 3.95–12.4) using PCR in tissue and 1.72 (1.02–2.90) in studies using ELISA in the serum.²⁷ According to another recent meta-analysis, MAP was detected more frequently from patients with CD compared with those with ulcerative colitis (risk difference 0.19, 95% CI, 0.10–0.28).²⁸

Govind K. Makharia¹ · Urvashi B. Singh²

Departments of ¹Gastroenterology and Human Nutrition, and

²Microbiology, All India Institute of Medical Sciences, New Delhi 110 029, India

G Makharia (✉)

e-mail: govindmakharia@gmail.com;

govindmakharia@aiims.ac.in

© Indian Society of Gastroenterology 2009

In the present issue of the *Journal*, Sasikala *et al.*²⁹ have shown that none of their 81 patients with CD and 85 controls had MAP-DNA in intestinal biopsies using MAP-specific IS900. Similar results have also been reported from another center.³⁰

The inconsistency of findings across different studies, using methods that are prone to technical variability, continues to be a major impediment to a definitive assessment of the role of MAP in CD. It is worth noting that in-house PCR in the tuberculosis laboratories are prone to error, in part because of the many methodological variances between laboratories such as DNA extraction protocols, adequate removal of tissue inhibitors, variable PCR conditions (direct PCR vs. nested PCR, differing number of cycles and extension times, etc.) and amplicon detection methods.³¹ There are two other reasons which can hamper the sensitivity of PCR for MAP-related infections: (i) the cell wall is very thick and hard to lyse, and (ii) the organism is buoyant and therefore resists concentration by centrifugation.^{3,32}

The strength of the association as shown by two meta-analyses and consistent detection of MAP-DNA by many groups does not prove causality, and may arise from prior ingestion of nonviable organisms in water or milk. However, the detection of MAP-DNA in blood of patients with CD may suggest that a viable form of the organism is present in them.^{33,34} The presence of MAP in blood may also be a reflection of an increase in intestinal permeability which is seen in 40% of patients with CD.³⁵ However, the more frequent detection of MAP in CD compared with UC argues against increased gut permeability as the sole explanation.²⁷ Before we say that MAP is the cause of CD, MAP has to meet the Koch's postulates for microbial causation.³⁶ There are some preliminary data that suggest that MAP has partially fulfilled the Koch's postulates. There are also evidences that we are exposed to MAP through our environment. MAP has been isolated from pasteurized milk infant formula made from pasteurized milk, breast milk from women with CD, surface water, soil, cow manure "lagoons" that can leach into surface water, and municipal tap water, providing multiple routes of transmission to humans.^{4,37-39}

While there are many protagonists who believe that MAP may be a cause of CD, there are many who argue against the association. First, farmers and rural dwellers should be at increased risk of a livestock-associated pathogens, yet there is no evidence that they have increased rates of CD.⁴⁰ Secondly, environmental conditions, such as poor sanitation and overcrowding which should favour transmission of an infection, actually appear to protect against CD. Thirdly, immunosuppressive drugs and anti-tumor necrosis factor- α should be associated with increased rates and severity of mycobacterial diseases, rather than improvement in the disease.⁴¹

The protagonists defend objections in many ways. We are not aware of the best staining technique for MAP. The organism that causes cat scratch disease, *Bartonella henselae*,

was not seen under the microscope until researchers finally thought to try the Warthin-Starry silver stain on diseased lymph nodes almost 30 years after the disease was first described.⁴² While now "easily" seen by the usual Hematoxylin & Eosin stain, *Helicobacter pylori* were visualized by the Warthin-Starry silver stain in Warren and Marshall's first report.⁴³ All the available histochemical stains have not been tried to stain MAP in histological specimens of patients with CD. Most studies have used mucosal biopsies from the lesions or blood for showing MAP using various techniques described above. Mucosal biopsies generally are superficial and surrounded by necrosis and exudation; MAP may not be present in sufficient numbers in the superficial layers of intestine and may thus skip detection. It is possible that MAP is more often present in deeper layers of the intestine, and the fistulous tracts. One of the very important pathological features of CD is creeping fat in the mesentery which is not seen in other inflammatory conditions. The mesenteric fat may also be a good site to look for MAP.⁴⁴ To say that MAP is somehow "harmless to man" we will have to prove that and they are present in human as a bystander, once it is well known that MAP causes chronic inflammation of the intestines in many cattle, and even in subhuman primates.

The present study has included relatively large number of patients with CD. A negative association between CD and MAP as reported by the authors raises a question that in a tuberculosis endemic country, is MAP less prominent? Does *M. tuberculosis* or any other intestinal infection inhibit MAP? Before we believe this, such data needs to be replicated at other centers. There is another concern related with the duration of the disease included in this study. Most patients with CD reporting to tertiary care centers have a disease spanning over a long period of time,^{45,46} the duration of symptoms in this study is relatively short (mean 8 months). It may not be wise to conclude, as the authors of this study have concluded, that MAP is not the cause of CD in India, especially once there are evidences from other parts of the world. If MAP is the cause or not the cause of CD, it will not vary according to country or even continent.

The failure of the anti-MAP drug trial (clarithromycin, rifabutin, and cefazolin) from Australia beyond sixteen weeks may not be taken as the final evidence against an association between MAP and CD.^{47,48} We do not exactly know the anti-MAP drugs, the best combination regimen, and the duration of the therapy.⁴⁹ It is just a beginning of the process, the evidences either for or against have started pouring in. We will have to work and connect evidences into a complete story.

The remarkable work by Barry Marshall and Robin Warren led to a change in the concept and more importantly treatment of peptic ulcers. Despite many arguments against it, MAP stands tall as a candidate pathogen for CD. Gastroenterologists, microbiologists, pathologists, surgeons, and

basic scientists should join hands and try find out the exact cause of this crippling disease.

References

1. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 2006;3:390–407.
2. Eckburg PB, Relman DA. The role of microbes in Crohn's disease. *Clin Infect Dis* 2007;44:256–62.
3. Cocito C, Gilot P, Coene M, de Kesel M, Poupart P, Van-nuffel P. Paratuberculosis. *Clin Microbiol Rev* 1994;7:328–45.
4. Greenstein RJ. Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. *Lancet Infect Dis* 2003;3:507–14.
5. Sartor RB. Does *Mycobacterium avium* subspecies paratuberculosis cause Crohn's disease? *Gut* 2005;54:896–8.
6. Chacon O, Bermudez LE, Barletta RG. Johne's disease, inflammatory bowel disease, and *Mycobacterium paratuberculosis*. *Annu Rev Microbiol* 2004;58:329–63.
7. Greenstein RJ, Collins MT. Emerging pathogens: is *Mycobacterium avium* subspecies paratuberculosis zoonotic? *Lancet* 2004;364:396–7.
8. Ford A, Delaney B, Forman D, Moayyedi P. Eradication therapy for peptic ulcer disease in *Helicobacter pylori* positive patients. *Cochrane Database Syst Rev* 2004;4:CD003840.
9. Chiodini RJ, Van Kruiningen HJ, Thayer WR, Merkal RS, Coutu JA. Possible role of mycobacteria in inflammatory bowel disease. I. An unclassified *Mycobacterium* species isolated from patients with Crohn's disease. *Dig Dis Sci* 1984;29:1073–9.
10. Chiodini RJ, Van Kruiningen HJ, Thayer WR, Coutu JA, Merkal RS. In vitro antimicrobial susceptibility of a *Mycobacterium* sp. isolated from patients with Crohn's disease. *Antimicrob Agents Chemother* 1984;26:930–2.
11. Chiodini RJ, Van Kruiningen HJ, Merkal RS, Thayer WR Jr, Coutu JA. Characteristics of an unclassified *Mycobacterium* species isolated from patients with Crohn's disease. *J Clin Microbiol* 1984;20:966–71.
12. Graham DY, Markesich DC, Yoshimura HH. Mycobacteria and inflammatory bowel disease. Results of culture. *Gastroenterology* 1987;92:436–42.
13. Naser SA, Schwartz D, Shafran I. Isolation of *Mycobacterium avium* subsp paratuberculosis from breast milk of Crohn's disease patients. *Am J Gastroenterol* 2000;95:1094–5.
14. Bull TJ, Hermon-Taylor J, Pavlik I, El-Zaatari F, Tizard M. Characterization of IS900 loci in *Mycobacterium avium* subsp. Paratuberculosis and development of multiplex PCR typing. *Microbiology* 2000;146:2185–97.
15. Whittington RJ, Hope AF, Marshall DJ, Taragel CA, Marsh I. Molecular epidemiology of *Mycobacterium avium* subsp. paratuberculosis: IS900 restriction fragment length polymorphism and IS1311 polymorphism analyses of isolates from animals and a human in Australia. *J Clin Microbiol* 2000;38:3240–8.
16. Sechi LA, Mura M, Tanda F, et al. Identification of *Mycobacterium avium* subsp. paratuberculosis in biopsy specimens from patients with Crohn's disease identified by in situ hybridization. *J Clin Microbiol* 2001;39:4514–7.
17. Roholl PJ, Herrewegh A, van Soolingen D. Positive IS900 in situ hybridization signals as evidence for role of *Mycobacterium avium* subsp. paratuberculosis in etiology of Crohn's disease. *J Clin Microbiol* 2002;40:3112.
18. Naser SA, Shafran I, Schwartz D, El-Zaatari F, Biggerstaff J. In situ identification of mycobacteria in Crohn's disease patient tissue using confocal scanning laser microscopy. *Mol Cell Probes* 2002;16:41–8.
19. Bull TJ, McMinn EJ, Sidi-Boumedine K, et al. Detection and verification of *Mycobacterium avium* subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *J Clin Microbiol* 2003;41:2915–23.
20. Naser SA, Ghobrial G, Romero C, Valentine JF. Culture of *Mycobacterium avium* subspecies paratuberculosis from the blood of patients with Crohn's disease. *Lancet* 2004;364:1039–44.
21. Sechi LA, Scanu AM, Mollicotti P, et al. Detection and Isolation of *Mycobacterium avium* subspecies paratuberculosis from intestinal mucosal biopsies of patients with and without Crohn's disease in Sardinia. *Am J Gastroenterol* 2005;100:1529–36.
22. Autschbach F, Eisold S, Hinz U, et al. High prevalence of *Mycobacterium avium* subspecies paratuberculosis IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut* 2005;54:944–9.
23. Romero C, Hamdi A, Valentine JF, Naser SA. Evaluation of surgical tissue from patients with Crohn's disease for the presence of *Mycobacterium avium* subspecies paratuberculosis DNA by in situ hybridization and nested polymerase chain reaction. *Inflamm Bowel Dis* 2005;11:116–25.
24. Bentley RW, Keenan JJ, Gearry RB, Kennedy MA, Barclay ML, Roberts RL. Incidence of *Mycobacterium avium* subspecies paratuberculosis in a population-based cohort of patients with Crohn's disease and control subjects. *Am J Gastroenterol* 2008;103:1168–72.
25. Singh AV, Singh SV, Makharia GK, Singh PK, Sohal JS. Presence and characterization of *Mycobacterium avium* subspecies paratuberculosis from clinical and suspected cases of Crohn's disease and in the healthy human population in India. *Int J Infect Dis* 2008;12:190–7.
26. Semret M, Turenne CY, Behr MA. Insertion sequence IS900 revisited. *J Clin Microbiol* 2006;44:1081–3.
27. Abubakar I, Myhill D, Aliyu SH, Hunter PR. Detection of *Mycobacterium avium* subspecies paratuberculosis from patients with Crohn's disease using nucleic acid-based techniques: a systematic review and meta-analysis. *Inflamm Bowel Dis* 2008;14:401–10.
28. Feller M, Huwiler K, Stephan R, et al. *Mycobacterium avium* subspecies paratuberculosis and Crohn's disease: a systematic review and meta-analysis. *Lancet Infect Dis* 2007;7:607–13.
29. Sasikala M, Reddy DN, Pratap N, et al. Absence of *Mycobacterium avium* ss paratuberculosis-specific IS900 sequence in intestinal biopsy tissues of Indian patients with Crohn's disease. *Indian J Gastroenterol* 2009;28:169–74.

30. Parrish NM, Radcliff RP, Brey BJ, *et al.* Absence of mycobacterium avium subsp. paratuberculosis in Crohn's patients. *Inflamm Bowel Dis* 2009;15:558–65.
31. Flores LL, Pai M, Colford JM Jr, Riley LW. In-house nucleic acid amplification tests for the detection of Mycobacterium tuberculosis in sputum specimens: meta-analysis and meta-regression. *BMC Microbiol* 2005;5:55.
32. Chiodini RJ. Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. *Clin Microbiol Rev* 1989;2:90–117.
33. Mendoza JL, Lana R, Díaz-Rubio M. Mycobacterium avium subspecies paratuberculosis and its relationship with Crohn's disease. *World J Gastroenterol* 2009;15:417–22.
34. Liu Y, van Kruiningen HJ, West AB, Cartun RW, Cortot A, Colombel JF. Immunocytochemical evidence of Listeria, Escherichia coli, and Streptococcus antigens in Crohn's disease. *Gastroenterology* 1995;108:1396–404.
35. Benjamin J, Makharia GK, Ahuja V, Kalaivani M, Joshi YK. Intestinal permeability and its association with the patient and disease characteristics in Crohn's disease. *World J Gastroenterol* 2008;14:1399–405.
36. Koch R. die Aetiologie der Tuberkulose. *Mitteilungen Kaiserlichen Gesundheitsamte* 1884;2:1–88.
37. Pierce ES. Possible transmission of Mycobacterium avium subspecies paratuberculosis through potable water: lessons from an urban cluster of Crohn's disease. *Gut Pathog* 2009;1:17.
38. Lund BM, Gould GW, Rampling AM. Pasteurization of milk and the heat resistance of Mycobacterium avium subsp. paratuberculosis: a critical review of the data. *Int J Food Microbiol* 2002;77:135–45.
39. Waddell LA, Rajia A, Sargeant J, *et al.* The zoonotic potential of Mycobacterium avium spp. paratuberculosis: a systematic review. *Can J Public Health* 2008;99:145–55.
40. Jones PH, Farver TB, Beaman B, Cetinkaya B, Morgan KL. Crohn's disease in people exposed to clinical cases of bovine paratuberculosis. *Epidemiol Infect* 2006;134:49–56.
41. Behr MA, Kapur V. The evidence for Mycobacterium paratuberculosis in Crohn's disease. *Curr Opin Gastroenterol* 2008;24:17–21.
42. Wear DJ, Margileth AM, Hadfield TL, Fischer GW, Schlagel CJ, King FM. Cat scratch disease: a bacterial infection. *Science* 1983;221:1403–5.
43. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984;i:1311–5.
44. Pierce ES. Where are all the Mycobacterium avium subspecies paratuberculosis in patients with Crohn's disease? *PLoS Pathog* 2009;5:e1000234.
45. Amarapurkar DN, Patel ND, Rane PS. Diagnosis of Crohn's disease in India where tuberculosis is widely prevalent. *World J Gastroenterol* 2008;14:741–6.
46. Das K, Ghoshal UC, Dhali GK, Benjamin J, Ahuja V, Makharia GK. Crohn's disease in India: a multicenter study from a country where tuberculosis is endemic. *Dig Dis Sci* 2009;54:1099–107.
47. Selby W, Pavli P, Crotty B, *et al.* Antibiotics in Crohn's Disease Study Group. Two-year combination antibiotic therapy with clarithromycin, rifabutin, and clobazimine for Crohn's disease. *Gastroenterology* 2007;132:2313–9.
48. Lipton JE, Barash DP. Flawed Australian CD study does not end MAP controversy. *Gastroenterology* 2007;133:1742.
49. Krishnan MY, Manning EJ, Collins MT. Comparison of three methods for susceptibility testing of Mycobacterium avium subsp. paratuberculosis to 11 antimicrobial drugs. *J Antimicrob Chemother* 2009;64:310–6.

Indian Journal of Gastroenterology J Mitra Memorial Award

The Indian Journal of Gastroenterology bestows this award for the best original scientific contribution published in the Journal during the year

This award carries a prize of Rs 20 000, and will be given to the department(s) submitting the selected paper.

The paper will be selected by a scientific committee appointed by the Editor, from among all the Original Articles published in the Journal during the year. In the event of a tie, the award will be distributed equally.

Terms for eligibility will apply.

The award has been made possible by a generous endowment from M/s J Mitra and Co Ltd,
New Delhi.