Original Research

Absence of *Mycobacterium avium* subspecies *paratuberculosis* Components from Crohn's Disease Intestinal Biopsy Tissues

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ABSTRACT

BACKGROUND

Crohn's disease is a chronic human intestinal inflammatory disorder for which an etiologic agent has not been identified. Johne's disease is a similar chronic enteric granulomatous disease of ruminant species and has been used as a model of Crohn's disease. Johne's disease has been proven to be caused by *Mycobacterium avium* subspecies *paratuberculosis* (*M. avium* ss *paratuberculosis*). It has been proposed that *M. avium* ss *paratuberculosis* may also cause Crohn's disease. This is of particular concern because the organism may be spread to humans through inadequately pasteurized dairy products.

OBJECTIVE

We sought to determine whether *M. avium* ss *paratuberculosis* could be detected using identical techniques in paraffin-embedded tissue samples of bovine Johne's disease and human Crohn's, ulcerative colitis and diverticular diseases. Samples were obtained for analysis from national tissue banks.

DESIGN

Cross-species and cross-disease sample comparisons by multiple detection techniques.

METHODS

Histology, immunocytochemistry and polymerase chain reaction (PCR) were utilized to test and compare the presence of *M. avium* ss *paratuberculosis* components. Insertion sequence IS900, present in multiple copies and found only in *M. avium* ss *paratuberculosis*, was utilized in both PCR and immunocytochemical analyses.

RESULTS

The IS900 sequence was demonstrable in all samples of confirmed positive Johne's disease tissue. The sequence was not identified in the 35 Crohn's, 36 ulcerative colitis, and 21 diverticular disease samples.

CONCLUSION

M. avium ss *paratuberculosis* was not associated with the lesions in these Crohn's disease samples, using these methods.

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INTRODUCTION

Crohn's disease is a chronic inflammatory disorder of the human intestine characterized by protein losing enteropathy, general malabsorption and steatorrhea. Lesions that begin as mucosal erosions and neutrophil infiltrates within crypts and crypt abscesses, progress to transmural lymphogranulomatous enteritis with lymphocyte populations being predominantly T_H1 cells.¹⁻³ In approximately 50% of the cases, lymphoid aggregates contain noncaseating granulomas composed of epithelioid histiocytes and multinucleate giant cells of the Langhan's type. Ulcers both discrete and serpiginous, are hallmarks of Crohn's lesions.⁴ Neutrophils and eosinophils are prominent in many lesions, as are plasmacytes bearing IgG, IgM, IgA or IgD. These inflammatory processes underlie the characteristic cobblestone appearance of the mucosal surface.

The cause and some critical aspects in the pathogenesis of human Crohn's disease are not known. Suggestions that the etiological agent involves mycobacteria^{5,6} have been based on the detection of mycobacterial species in intestinal biopsy tissue from Crohn's disease patients including: *Mycobacterium avium-intracellulare*, *Mycobacterium chelonii*, *Mycobacterium fortuitum*, *Mycobacterium kansasii*, *Mycobacterium avium* subspecies *paratuberculosis* (*M. avium* ss *paratuberculosis*).⁵

Like human Crohn's disease, Johne's disease of cattle is a chronic, progressive lymphogranulomatous process with preference for the terminal ileum and ileocecal valve. Unlike Crohn's disease, acid-fast intracellular bacilli are always present in lesions, there is no ulceration and the causal bacterium, *M. avium* ss *paratuberculosis*, can be isolated. Early in the disease process there is marked edema and lymphangitis at the edge of the expanding enteric lesion. Enteric lesions in Johne's disease are infiltrated with *M. avium* ss *paratuberculosis* infected macrophages. Lesions spread and grow by accumulation, reinfection and expansion of macro-phage populations in the intestinal wall. *M. avium* ss *paratuberculosis* disseminate from infected to non-infected macro-phages, allowing the bacterium to avoid humoral and cellular mechanisms of immunity. Transmission of *M. avium* ss *paratuberculosis* among cattle is by ingestion from milk or feces and by placental infection. Chronic infiltration of macrophages in the lamina propria throughout the lower intestine results in villous atrophy and malabsorption. Although infection is often restricted to the intestine, bacteria can spread via monocytes from the primary site of infection in the intestine to liver, spleen, kidney, uterus and mammary through hematogenous or lymphatic routes.

Due to the clinical symptoms of Crohn's disease closely mimicking those found in animals with Johne's disease, it was proposed almost 90 years ago that the two diseases shared the same etiology.^{7,8} Specifically, *M. avium* ss *paratuberculosis* continues to be suspected as the causative agent of both disorders. However, reviews of Johne's disease and its connection to human Crohn's have not established any connection.^{6,9-12}

There is no sound epidemiological evidence that links exposure to *M. avium* ss *paratuberculosis* to an increased incidence of Crohn's disease, even though cows with clinical paratuberculosis do shed viable organisms in their milk at low levels (50 CFU/50 ml milk).¹³ In Great Britain it has been demonstrated that *M. avium* ss *paratuberculosis* DNA is present in milk samples obtained from retail markets.¹⁴ Viable *M. avium* ss *paratuberculosis* have been cultured from the pasteurized retail milk samples. These results suggest that current pasteurization techniques may not be adequate to kill *M. avium* ss *paratuberculosis* in raw milk, or that milk may become contaminated post pasteurization. Because of the potential for spreading *M. avium* ss *paratuberculosis* through widely consumed dairy products, it has become crucial to definitively establish whether or not this organism is actually involved in the etiology of Crohn's diseases.

In recent serologic studies, up to 83% of Crohn's patients showed evidence of serum antibodies to *M. avium* ss *paratuberculosis*.¹⁵⁻²⁰ Recently it has been demonstrated that lactating mothers with Crohn's disease shed *M. avium* ss *paratuberculosis* in their breast milk.²¹ However, unlike the case for Johne's disease, reports are mixed as to the presence or absence of *M. avium* ss *paratuberculosis* at the site of the intestinal lesions.

Studies that used genetic primers in polymerase chain reaction (PCR) analyses to detect the organism in Crohn's patient tissues have yielded the most provocative data.²²⁻²⁸ The most widely used primers are for insertion sequence 900 (IS900), a genetic element proven to be quite specific for *M. avium* ss *paratuberculosis*.²⁹⁻³² In those studies, from 13% to 100% of Crohn's patients tested positive for *M. avium* ss *paratuberculosis* by IS900 DNA/PCR.³³⁻⁴⁴ Other studies, however, have been unable to demonstrate *M. avium* ss *paratuberculosis* DNA in Crohn's disease tissue.^{5,11,12,45-50} One potential problem with these results is that some primers designed from IS900 can cross-react with closely related IS901 and IS902.⁵¹⁻⁵⁵

It is therefore crucial to use primers that are very sensitive and specific to the *M. avium* ss *paratuberculosis* organism. In this study we used the specific IS900 primers in PCR analyses to search for evidence of *M. avium* ss *paratuberculosis*-infection in formalin-fixed, paraffin-embedded tissue sections from bovine Johne's disease and human Crohn's disease, ulcerative colitis and diverticular disease. For comparison the more widely used immunohistochemistry was applied. This was compared to the PCR analyses of a DNA insertion sequence that allows for specific identification of DNA from *M. avium* ss *paratuberculosis*. The IS900 sequence was demonstrable in all samples of confirmed positives of Johne's disease tissue. The sequence was not identified in the 35 Crohn's, 36 ulcerative colitis, and 21 diverticular disease samples. The evidence does not support the involvement of *M. avium* ss *paratuberculosis* in the etiology of Crohn's disease.

METHODS

Tissue samples

A retrospective sample from a large individual collection of human Crohn's, ulcerative colitis and diverticular disease, was obtained from the Department of Pathology at Mayo Clinic. Additionally, samples of confirmed bovine Johne's disease from the United States Department of Agriculture were obtained and examined. Tissue sections were formalin-fixed, paraffin-embedded and processed by standard procedures via standard operating protocols from both the Department of Pathology, Mayo Clinic and the National Veterinary Services Laboratory, Animal and Plant Health Inspection Service, United States Department of Agriculture.^{56,57} Samples were screened for colonic lesions that showed clear evidence of granulomas. These samples were then analyzed by immunohistochemistry and transmission electron microscopy for cytologic characteristics of affected macrophages, and lymphoid and vascular cells at the margins of the progressive granulomatous lesions.

Immunohistochemistry

An immunohistochemical technique to specifically detect *M. avium* ss *paratuberculosis* has been developed to detect antigens of *M. avium* ss *paratuberculosis* in formalin-fixed, paraffin-embedded tissue sections.⁵⁸ The probe detected *M. avium* ss *paratuberculosis* in infected bovine tissues. Tissue sections from animals infected with *M. avium* ss *avium* were negative. Thus, differentiation between *M. avium* ss *paratuberculosis* and *M. avium* ss *avium* in formalin-fixed, paraffin-embedded bovine tissues was possible.⁵⁸

Identification of mycobacteria

Procedures used to cut paraffin-embedded tissue sections and prepare crude DNA extracts have been previously described.^{56,57} Ten microliter aliquots of each crude extract were tested with each set of PCR primers (table 1). Samples that failed to react with any primer were retested using purified DNA. The DNA purification procedure was modified slightly from the original.^{47,56} The 10 µl aliquots were used for all PCR amplifications.

PCR

Each sample was examined with a PCR primer set derived from IS900, as previously described.⁵⁶ IS900 characteristically is present in multiple copies and is considered specific for *M. avium* ss *paratuberculosis*.^{56,62,71}

All amplification reactions were conducted with a "Hot Start" procedure that is based on heat-activated polymerase, as previously described. The concentration of reactants (50 µl final volume) was as follows: 20 pM primers, 1.25 U DNA

polymerase, 0.2 mM nucleotides, and 2.5 mM Mg⁺⁺. Each set of test samples included a negative control tube in which water was substituted for a test sample. A complete mastermix was prepared for the IS900 primer set. The 40 µL aliquots were introduced into the reaction tubes which were held at -70°C until use. Positive control DNA (10 ng) was obtained from bacterial culture extracts and from sections cut from paraffin-embedded tissue of confirmed Johne's disease.

Amplification conditions for the IS900 primers were as follows: 10 minutes at 94°C, then 1 minute at 94°C, 15 seconds at 65°C, and 2 minutes at 72°C for 50 cycles, and a final 10 minute extension at 72°C.

RESULTS

Histology and immunohistochemistry

Macrophages and multinucleate giant cells in granulomas in Crohn's biopsies did not contain bacilli; there was no evidence of other bacteria, fungi or viruses in tissues stained using acid-fast Stain (auramine-rhodamine)⁵⁹ and gram stains. There was no ultrastructural evidence of intracellular bacterial replication; e.g., cells lacked bacteriophorous vacuoles, well-developed Golgi complexes and vacuolar transport systems. In three cases of Crohn's disease, acid-fast material was present, but by electron microscopy this material was shown to be cellular debris, not bacilli. No acid-fast bacilli were detected within enteric lesions of tissues from ulcerative colitis or from diverticular disease.

Intestines of Johne's disease were composed of three hallmark lesions: transmural masses of relatively uniform populations of large macrophages bearing many small, acid-fast bacilli; lymphangitis with dilation of lymphatic vessels in lamina propria of the villus, muscularis mucosa and submucosa; and marginal zones of lymphocyte-monocyte/macrophage infiltrates at the advancing edge of the submucosal lesion (figure 1).

The latter was composed of interstitial edema, lymphangitis, lymphoplasmacytic perivascularitis, and lymphoid cells, many of which were associated with infected macrophages. Lymphocytes with close apposition to infected macrophages had chromatin aggregates, ruffled surfaces and pale cytoplasm typical of apoptosis (figure 2). Bacilli in Johne's disease lesions stained strongly with auramine, acridine orange and by electron microscopy, had ultrastructural characteristics of *Mycobacterium* spp. Ultrastructurally, macrophages in the granulomatous response had high cytoplasmic-nuclear volume ratios, with a great part of the cytoplasm mass being bacteriophorous vacuoles and intact bacilli.

Table 1. IS900 oligonucleotide primers used for PCR amplification of *M. avium* ss *paratuberculosis*.

Organism	Primer	PCR Product (bp)	Sequence
<i>M. avium</i> ss. <i>paratuberculosis</i>	IS900	229	CCGCTAATTGAGAGATGCGATTGG AATCAACTCCAGCAGCGCGCCTCG

Figure 1. Transmurular granulomatous lesion in the intestine, cow, Johne's disease. Villi of the ileal mucosa are atrophic and fused, and masses of macrophages separate glands, efface the muscularis mucosa and infiltrate the submucosa. There is marked dilation of submucosal lymphatic vessels (see arrows).

Figure 2. Submucosa from tissue adjacent to that of figure 1. Advancing edge of submucosal granulomatous lesions with evidence of edema, lymphatic dilation. Large activated monocytes, macrophage-lymphocyte interaction and lymphangitis. Lymphocyte closely apposed to infected macrophage has evidence of apoptosis.

PCR

The IS900 PCR analysis is purportedly specific for *M. avium* ss *paratuberculosis*. It is very sensitive and specific for *M. avium* ss *paratuberculosis*, displaying no cross-reactivity. The IS900 sequence was demonstrable in all samples of bovine Johne's disease tissue. The sequence was not identified in the Crohn's (35), ulcerative colitis (36), and diverticular disease (21) samples (example set, figure 3). However, three Crohn's disease reactions were suspicious, yielding abnormal sized PCR products (data not shown). These abnormal sized PCR products were very weak bands using purified DNA, and they failed to give a product upon reamplification. It was suspected that these bands may have been the result of minor contamination or nonspecific annealing products. Three ulcerative colitis samples yielded much larger sized bands than expected. However, based on our specific criteria they were considered negative. These samples were confirmed negative upon reamplification.

DISCUSSION

M. avium ss *paratuberculosis* is not associated with the lesions in these Crohn's disease samples using these methods. This study represents the first direct comparison of PCR analyses from both Crohn's and Johne's tissues. It utilizes specific primers from IS900. At this time we believe that Crohn's disease and Johne's disease have separate and distinct etiologies. Importantly, these results should also assuage concern over the spread of Crohn's disease to susceptible populations via the consumption of *M. avium* ss *paratuberculosis*-contaminated dairy products.⁶³⁻⁶⁸

Previous studies have reported the isolation of *M. avium* ss *paratuberculosis* from colonic tissue,^{38,40,48,60,61} the detection of *M. avium* ss *paratuberculosis* DNA by PCR of IS900

in suspensions of colonic tissues,^{33,41,62,69} in paraffin-embedded tissue sections^{35,37,45,70,71} and the presence of serum antibodies against *M. avium* ss *paratuberculosis* in Crohn's patients.¹⁵⁻²⁰ However, none of these studies purport to detect evidence of *M. avium* ss *paratuberculosis* in all Crohn's tissues sampled. In fact, the occurrence rate is often quite low. This contrasts sharply with the association of *M. avium* ss *paratuberculosis* with bovine Johne's disease.⁷⁰ The low incidence rates in Crohn's tissues may be more of a reflection of the widespread occurrence of mycobacterial organisms, than an indication that *M. avium* ss *paratuberculosis* is a causative agent in Crohn's disease.

IS900 has been considered to be the most selective primer set for *M. avium* ss *paratuberculosis*. Although some previously used IS900 PCR primer have exhibited cross-reactivity with IS901, a closely related *M. avium* ss *avium* specific insertion element, thus opening the possibility that some positive reports might also be attributed to *M. avium* subspecies other than *paratuberculosis*.

In contrast the IS900 specific primers used in this study have exhibited no cross-reactivity in extensive screenings with hundreds of organisms in the American Type Culture Collection (<http://www.atcc.org/>). It may thus be considered to be the most specific and sensitive primer set for *M. avium* ss *paratuberculosis* available to date.

Due to the specificity and sensitivity of the IS900 primer set and the dramatic difference seen between the presence of *M. avium* ss *paratuberculosis* in Johne's tissues and Crohn's tissues, we feel that this organism is not directly involved in the granulomatous responses of Crohn's disease.

Figure 3. IS900 specific PCR amplification of crude DNA extracts from paraffin-embedded tissue sections from human Crohn's, human ulcerative colitis, human diverticular disease, and animal culture positive cases of *M. avium* ss *paratuberculosis* infection. Lanes are as follows: Lane 1: molecular size markers; Lanes 2, 7, 16: reagent control (as template added); Lanes 3-6: bovine tissues; Lane 8: bison tissue; Lanes 9 and 10: human Crohn's; Lanes 11 and 12: human ulcerative colitis; Lane 13: purified positive *M. avium* ss *paratuberculosis* control DNA from culture extract; Lanes 14 and 15: human diverticular disease.

Other evidence lends credence to this conclusion. Prior to this, there has been no clinical evidence that unequivocally shows an increased incidence of Crohn's disease in farmers or agricultural workers associated with dairy herds with a high incidence of Johne's disease. Nor has eating organs or tissues from infected animals been documented to cause infection of humans with *M. avium ss paratuberculosis*. Subclinically infected animals harbor the organisms in the lower small intestine and the regional lymph nodes, making them an unlikely source of infection for consumers. However, *M. avium ss paratuberculosis* can be widely disseminated in the latter stages of clinical disease, with viable organisms present in the liver, spleen, kidneys, lungs, pancreas, thymus gland, uterus and epicardium.⁷² Consumption of these organs by humans might transmit the organism, even though no infection occurs. No published information is available to date on whether *M. avium ss paratuberculosis* can be cultured from muscle tissue of infected cows.

The most worrisome avenue of potential spread of *M. avium ss paratuberculosis* to humans is thought to be through the consumption of inadequately pasteurized dairy products. Standard temperatures and times for heat pasteurization of milk, especially either via standard holder methods (63.5°C for 30 minutes) or high-temperature, short-time methods (71.7°C for 15 seconds), have been shown to be insufficient to kill all *M. avium ss paratuberculosis* in milk.^{73,74} Studies of raw milk inoculated with live *M. avium ss paratuberculosis* (104, 106 CFU/ml), have demonstrated that it is possible to effectively kill all the bacteria using laboratory-scale pasteurizers (72°C for 15 seconds).⁴³ A group in Australia has further corroborated this data in a study using a small-scale commercial pasteurizing unit.⁷⁵ These studies indicate the transmission of viable *M. avium ss paratuberculosis* from animals to humans via adequately pasteurized dairy products can be made even less likely, thus minimizing any remaining potential concern that it may act as a zoonotic agent in Crohn's disease.

Additionally, *M. avium ss paratuberculosis* has been reported to cause Johne's disease only in a variety of ungulate species. No non-ruminant animals have been found to be affected. Aside from the dramatic differences between ruminant and non-ruminant gastrointestinal physiologies, an explanation for this difference has not yet been forwarded.

Finally, human Crohn's disease may prove to be an immunity-mediated susceptibility to general bacterial infections in the gastrointestinal mucosa. A gene associated with susceptibility to Crohn's disease has now been identified. *CARD15/NOD2* situated within the IBD1 region of chromosome 16q12,⁷⁶⁻⁷⁹ is only expressed in monocytes and macrophages. *CARD15* encodes a protein composed of two caspase recruitment domains, a nucleotide-binding domain and leucine-rich repeats.^{80,81} The caspase recruitment domains enable a protein to induce apoptosis and the NF-κB signaling pathways. *CARD15* has been proposed to be an intracellular receptor for bacterial components in monocytes where it is mainly

expressed.⁸⁰ Leucine-rich repeats are involved in the interaction with the lipopolysaccharides of infecting bacteria.⁸² Three main mutations of *CARD15* have been found in patients with Crohn's disease, but not in patients with ulcerative colitis. All occur in the leucine-rich repeats domain or its vicinity.^{76,77,83} Thus these mutations may alter the recognition of the bacterial lipopolysaccharides. Other rare variants also occur.^{81,83}

However, this is not yet the complete mechanism. Crohn's may be stratified into ileal, colonic and perianal fistular. It has been independently demonstrated by two groups that the *CARD15/NOD2* mutations are seen only in the cases restricted to ileal Crohn's disease.⁸⁴⁻⁸⁶ It has been speculated that gut bacteria occur at much higher concentrations in the colon than in the ileum. Therefore additional mechanisms to combat higher levels of bacteria have evolved in the colon, whereas the ileum may be more dependent on the function of *NOD2*.⁸⁴ Other mutations are actively being sought, especially in the interleukin and major histocompatibility complex regions.⁸⁷⁻⁸⁹

M. avium ss paratuberculosis is resilient in the environment and is relatively ubiquitous. It is likely that *M. avium ss paratuberculosis* may commonly or transiently exist in the intestinal tract of the normal population.^{16,27} In light of the new genetic findings, it seems likely that patients with Crohn's disease have an altered immune reaction against *M. avium ss paratuberculosis* and a variety of other intestinal microorganisms. Some patient populations may be exposed to *M. avium ss paratuberculosis*, while others are not, and in the latter group some other offending organism triggers the aberrant immune response in the intestinal wall. Our patient population showed no evidence of *M. avium ss paratuberculosis* infection. However, this study accurately confirmed an already established association between *M. avium ss paratuberculosis* and Johne's disease lesions in ruminants, no etiology of Crohn's disease was determined.

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