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Detection and Isolation of *Mycobacterium avium* Subspecies *paratuberculosis* from Intestinal Mucosal Biopsies of Patients with and without Crohn's Disease in Sardinia

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OBJECTIVES: Sardinia is an island community of 1.6 million people. There are also about 3.5 million sheep and one hundred thousand cattle in which Johne's disease and *Mycobacterium avium* subspecies *paratuberculosis* infection are endemic. The present study was designed to determine what proportion of people in Sardinia attending for ileocolonoscopy with or without Crohn's disease were infected with this pathogen.

METHODS: *Mycobacterium avium* subspecies *paratuberculosis* was detected by IS*900* PCR on DNA extracts of fresh intestinal mucosal biopsies as well as by isolation in culture using supplemented MGIT media followed by PCR with amplicon sequencing.

RESULTS: Twenty five patients (83.3%) with Crohn's disease and 3 control patients (10.3%) were IS*900* PCR positive ($p = 0.000001$; Odds ratio 43.3). *Mycobacterium avium* subspecies *paratuberculosis* grew in cultures from 19 Crohn's patients (63.3%) and from 3 control patients (10.3%) ($p = 0.00001$; Odds ratio 14.9). All patients positive by culture had previously been positive by PCR. *Mycobacterium avium* subspecies *paratuberculosis* first appeared in the liquid cultures in a Ziehl Neelsen (ZN) staining negative form and partially reverted through a rhodamine-auramine positive staining form to the classical ZN positive form. This resulted in a stable mixed culture of all 3 forms illustrating the phenotypic versatility of these complex chronic enteric pathogens.

CONCLUSIONS: Mycobacterium avium subspecies paratuberculosis was detected in the majority of Sardinian Crohn's disease patients. The finding of the organism colonizing a proportion of people without Crohn's disease is consistent with what occurs in other conditions caused by a primary bacterial pathogen in susceptible hosts.

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INTRODUCTION

Mycobacterium avium (*M. avium*) subspecies *paratuberculosis* is a member of the *M. avium* complex MAC. The recently sequenced genome of the K10 bovine strain comprises 4.83 million base pairs (mbp) of GC-rich DNA encoding about 4,350 proteins (GenBank accession number AE016958). Of this, 4.54 mbp shares 96–100% homology in sequence and genetic organization with generally nonpathogenic *M. avium* sp. The remaining 289 kb, representing only 6% of the genome, is made up of genes that are either unique to *M. avium* subspecies *paratuberculosis* or homologous to genes many of which are related to pathogenicity in other microorganisms.

Unlike other environmental MAC, *M. avium* subspecies *paratuberculosis* has the specific ability to cause chronic inflammation of the intestine of a range of histopathological types in many animals including primates (1–4). The disease in animals ranges from pluribacillary to paucimicrobial with chronic granulomatous inflammation like leprosy in humans (5). First described in 1895 (6), *M. avium* subspecies *paratuberculosis* has amplified particularly in domestic livestock with the emergence of distinct bovine and ovine strains having differing phenotypic characteristics while retaining the ability to cross infect (7–11). Recent data are clarifying the nature of human Crohn's disease isolates of these pathogens which, while demonstrating some differences, have so far all been on a bovine background (12–14).

Despite its broad pathogenicity *M. avium* subspecies *paratuberculosis* can live in animals for years without causing clinical disease. Infection in farm animals is widespread particularly in Europe and North America (15). Wildlife reservoirs contribute to cycles of reinfection (16). Infected animals secrete *M. avium* subspecies *paratuberculosis* in their milk (17). These organisms are more thermotolerant than *M. bovis* (18) and have been so far been cultured from retail pasteurized milk supplies in the United Kingdom, Czech Republic, and the United States (19, 20) (http://www.johnes.org). *M. avium* subspecies *paratuberculosis* is therefore from time to time transmitted to people by this route. The possibility that these pathogens may also cause chronic inflammation of the intestine in humans is a public health issue of paramount importance (21).

The central question has been is *M. avium* subspecies *paratuberculosis* actually present in the inflamed gut particularly of people with Crohn's disease? This has not been an easy question to answer. The organisms are very slow growing and exceedingly difficult to isolate and passage in conventional culture. They are present in low abundance and can adopt a Ziehl Neelsen (ZN) staining negative form that cannot be seen in tissues by ordinary light microscopy (22–24). They appear to be able to minimize immune recognition and unlike conventional spheroplasts, their ZN negative form is highly resistant to the prerequisite chemical and enzymatic lysis procedures essential for reliable detection by PCR (25). As a consequence of these difficulties the results of much research carried out during the 1990s were conflicting (22).

The availability of improved culture media for *M. avium* subspecies *paratuberculosis* and advances in sample processing and molecular diagnostics are at last leading to a resolution of this uncertainty. The work of independent research groups in the United States (26, 27), Italy (23), the United Kingdom (25), and Germany (28) using appropriate laboratory methods has confirmed that the inflamed intestine of most people with Crohn's disease is infected with these chronic enteric pathogens. Further work from the United States has cultured *M. avium* subspecies *paratuberculosis* from the blood of 50% of patients with Crohn's disease (24) showing that, as in animals, the infection is often systemic. Despite this, negative reports still appear (29) and there is a need for more data from independent research centers particularly those with differing ethnicity, agriculture, dietary habits, and climatic conditions. The present study using validated laboratory methods was carried out to determine the *M. avium* subspecies *paratuberculosis* infection rate in the intestine of people with and without Crohn's disease in Sardinia, an island community of 1.6 million people, about 2.5 million mostly dairy sheep and 10,000 cattle.

METHODS

Samples

Endoscopic mucosal biopsies were obtained from people attending for ileocolonoscopy at the Clinic of Surgery, University of Sassari, Italy. Biopsies were taken both from visibly inflamed regions in patients with IBD and from several regions throughout the intestine in control subjects. Tissue samples were placed into liquid 7H9 medium, transported directly to the laboratory, and processed within 1 hr. One biopsy was used to make a smear and examine by ZN and rhodamine-auramine staining as previously described (5). Informed consent for sampling and publication of the results was obtained.

DNA Extraction

Samples assigned to PCR were processed as previously described (25). Tubes were centrifuged at 10,000 $\times$ *g* for 3 min and the supernatant was discarded. The tissue was resuspended in 600 μL of mycobacterial lysis buffer (2 mM sodium EDTA, 400 mM NaCl, 10 mM Tris HCl, 0.6% sodium dodecyl sulfate, and 33 μg of proteinase K [Sigma-Aldrich, St. Louis, MO] per mL) and transferred to a Lysing Matrix B Ribolyser tube (Qbiogene, Nottingham, UK) and incubated at 37°C for 2 hr. The Ribolyser tubes were then chilled on ice for 5 min and mechanically disrupted in the FastPrep Ribolyser (Qbiogene) at a setting of 6.5 ms^{-2} for 45 s and then immediately chilled again on ice for 15 min. A volume of 600 μL of phenol (pH 6.7) saturated in 1 $\times$ TE (10 mM Tris HCl, 1 mM sodium EDTA pH 8.0) was added, and the mixture was vortexed for 20 s and then centrifuged at 10,000 $\times$ *g* for 1 min. The aqueous layer was transferred to a new screw-cap reaction tube containing an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, Sigma), and the mixture was saturated in 1 $\times$ TE, vortexed for 20 s, and centrifuged (10,000 $\times$ *g* for 1 min). The aqueous layer was again transferred to a new tube containing 500 μL of chloroform-isoamyl alcohol (24:1), vortexed for 30 s, and centrifuged (10,000 $\times$ *g* for 1 min). The final aqueous layer (450 μL) was transferred to a new tube containing 90 μL of 10 M ammonium acetate and mixed thoroughly. One milliliter of ice-cold 100% ethanol was added to enable DNA precipitation at room temperature for 1 h. The samples were then centrifuged (10,000 $\times$ *g* for 20 min) at room temperature, and the pellets were washed in 750 μL of ice-cold 70% ethanol. The pellets were dried at room temperature for 30 min and resuspended in 50 μL of 1 $\times$ TE.

IS900 PCR

Primers p89 and p92 described in our previous work (23) were used to amplify a 284 bp fragment. The reaction mixture (final volume of 50 (μL) comprised primers at a concentration of 0.5 μM. Expand High fidelity reaction buffer 1$\times$ 200 μM (each) dNTPs and 3 U of Expand High Fidelity Taq polymerase (Expand High Fidelity PCR system; Roche, Lewes, UK). Cycling conditions were 1 cycle of 94°C for 3 min and 36 cycles of 94°C for 40 s, 62°C for 40 s, and 72°C for 40 s. Followed by a final step of 72°C for 5 min. Amplified fragments were visualized with ethidium bromide on 2.5% agarose-1000 gel (Life Technologies, Grand Island, NY)

and purified with a Quiaquick gel extraction kit (Quiagen, Crawley, UK). Each amplicon was sequenced in both directions by using p89 and p92 primers.

DNA extracts were also tested using Real Time PCR (5 μL of tissue DNA extract was added to the reaction performed by the iCycler iQ^{TM} instrument, Biorad, Hercules, CA). The PCR Primers used for the Real Time PCR (iCycler, Biorad were 5′-CCGACGCGATGATCGAGGAG for the sense primer and 5′-GAACTCAGCGCCCAGGATGA for the antisense primer and [6-FAM]CCGCCACCG CCACGCCGAAA[3′ TAMRA-5′ 6-FAM] for the internal Taqman probe sequence. As a standard for the Real Time, an internal fragment of IS*900* cloned into the vector PCR 2.1 (TA cloning Invitrogen) was used.

Mycobacterial Culture

Tissue samples were decontaminated by adding an equal volume of sodium hydroxide (2% final concentration) and incubating for 10 min. Tubes were then centrifuged at 3,000 $\times$ *g* for 30 min and the supernatant was discarded. The tissue pellet was washed with 10 mL of phosphate-buffered saline (0.067 M, pH 6.8) and resuspended in 0.2% fatty-acid-free albumin. The samples were inoculated into MGIT media supplemented with mycobactin J and egg yolk emulsion (20 μL using the MGIT 960 nonradioactive culture system (Becton Dickinson, Palo Alto, CA). The medium was then incubated in the MGIT instrument until growth was detected.

Electron Microscopy

Scanning electron microscopy was performed on the MGIT isolates. Bacteria grown in MGIT were fixed in 2.5% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer (pH 7.2), postfixed in 1% osmium tetroxide in 0.2 mol/L sodium cacodylate buffer (pH 7.2), and serially dehydrated in ethyl alcohol. After critical point drying, they were coated with gold using a sputter-coating system (sputter-coater 5150A, Edwards High Vacuum International, Crawley, UK) and then examined with a scanning electron microscope (DSM 962, Zeiss, Oberkochen, Germany) operating at kV20 20 kV. Images were digitized and stored as tagged image file format files in the microscope computer. In order to compare the appearances of these natural Crohn's disease isolates with the artificially induced ZN negative state a culture of *M. avium* subspecies *paratuberculosis* ATCC 43015 (human isolate) into 7H9 medium plus mycobactin J was treated with cycloserine (7 μg/mL) and lysozyme (1 μg/mL) for up to 3 months.

Statistics

The χ^2 Pearson test and odds ratios were used to compare the results of PCR and culture between CD and non-CD patients. A *p* value of less than 0.05 was judged to be significant.

RESULTS

The results are summarized in Table 1. The Crohn's disease group consisted of 30 patients (19 males and 11 females). Tissue samples from 25 (83.3%) of these patients were positive by IS*900* PCR. PCR positivity was independent of disease activity. Three CD patients were being treated with Prednisolone at the time of sampling and all were positive by PCR and culture. Cultures from 19 (63.3%) PCR positive patients became positive in the MGIT system and were confirmed as *M. avium* subsp. *paratuberculosis* by IS*900* PCR (Table 1). Sequencing of the amplicons from the PCRs on these cultures confirmed the presence of *M. avium* subsp. *paratuberculosis* with 100% sequence identity to IS*900* AF416985 locus available in the National Center for Biotechnology Information database. No sequence polymorphisms were detected.

The control group consisted of 29 patients (13 males and 16 females) classified as not having Crohn's disease. There were two additional patients (both male) diagnosed with ulcerative colitis (Table 1). Both patients with ulcerative colitis tested negative by IS*900* PCR and in the MGIT system. Of the 29 non-Crohn's disease patients, 3 (10.3%) were positive by IS*900* PCR and by MGIT culture and showed 100% identity to the isolates from Crohn's patients. These results (3/29 *vs* 25/30 CD) are significantly different from the Crohn's disease group (at the level of $p = 0.000001$; odds ratio 43.3%). One of the three positive non-Crohn's disease patients had diverticulitis, one had indeterminate colitis, and one was diagnosed with irritable bowel syndrome. Two additional MGIT cultures from the 29 control patients exhibited bacterial growth in the MGIT system but were negative by IS*900* PCR. These cultures were negative by ZN staining for acid fast bacilli after 16 wk of incubation.

The initial smears obtained from representative fresh biopsies from all patients were negative by ZN and rhodamine-auramine staining. After 8 wk of incubation, positive MGIT cultures showed positive rhodamine-auramine staining cells that were ZN staining negative (Fig. 1A). After 14 wk of incubation the MGIT cultures showed rhodamine-auramine and ZN staining cells characteristic of a mixture of prespheroplast, spheroplast, and acid fast bacilli (Fig. 1B). The average time for growth of *M. avium* subsp. *paratuberculosis* to be detected in the MGIT system was about 10 wk.

A scanning electron microscopy picture of one of the natural ZN negative *M. avium* subsp. *paratuberculosis* isolates from Crohn's disease is shown in Figure 2. The cells are seen to have lost their natural bacillary shape. This appearance is compared to that of *M. avium* subsp. *paratuberculosis* induced into the ZN negative state by treatment with cycloserine and lysozyme (Fig. 3).

As shown, *M. avium* subsp. *paratuberculosis* cells after treatment lose their bacillary shape and although they become ZN-negative they retain the ability to be stained by

Table 1. Patient Details

Group and Sex		Age (Years)	Disease Duration (Years)	Disease Location	Disease Activity	Drugs	PCR	Culture
Crohn's disease								
1	M	32	18	I/C	Active	No	+	+
2	M	29		TI	Active	No	+	+
3	F	33	4	I/C	Active	No	+	+
4	M	26		TI	Active	No	+	+
5	F	25		TI	Active	No	+	+
6	M	35	>5	TI	Inactive	No	+	+
7	M	45	3	TI	Active	P	+	+
8	M	44	0	TI	Active	No	+	+
9	M	26	0	TI	Active	No	+	+
10	M	44	4	I	Inactive	No	+	+
11	F	55	>6	I	Active	No	+	+
12	M	66		C	Active	P	+	+
13	M	28		TI	Active	No	+	+
14	M	20	5	C/R	Active	No	+	+
15	F	22	0	TI	Inactive	No	+	−
16	M	18		C	Active	No	−	−
17	F	50	>10	I/C/R	Active	No	+	+
18	F	39	1	C	Active	No	+	+
19	F	33	4	I	Active	No	+	+
20	M	14		I	Active	No	−	−
21	M	53		TI	Active	No	−	−
22	F	70		I	Active	No	+	−
23	M	25		TI	Inactive	No	+	−
24	F	73		C	Inactive	No	+	−
25	F	59		I	Active	No	+	−
26	F	34	0	TI	Active	No	−	−
27	M	74		I	Inactive	No	+	−
28	M	67		I	Active	No	+	+
29	M	14	0	I	Active	P	+	+
54	M	35		R	Active	No	−	−
Ulcerative colitis								
30	M	29		TI	Active	No	−	−
31	M	35		R	Active	No	−	−
No inflammatory bowel disease					Diagnosis			
32	M	30		TI	IBS	No	−	−
33	F	52		S	Gastritis	P	−	−
34	M	39		C	Diarrhea		−	+
35	F	26		TI	IBS	No	−	−
36	F	77		R	Adenocarcinoma		−	−
37	M	65		I	Diverticulitis		−	−
38	F	69		I	Screening		−	−
39	F	73		I	Adenocarcinoma		−	−
40	F	72		C	Diverticulitis		−	−
41	M	59		C	Adenocarcinoma		−	−
42	M	57		I	Adenocarcinoma		−	−
43	F	71		R	Adenocarcinoma		−	−
44	M	73		R	Adenocarcinoma		−	−
45	M	49		C	Adenocarcinoma		−	−
46	F	61		C	Diverticulitis		+	+
47	M	36		C/R	Adenocarcinoma		−	−
48	F	39		C/R	Colitis		+	+
49	M	78		I	Diverticulitis		−	+
50	M	59		I	Screening		−	−
51	M	66		C	Screening		−	−
52	F	73		C	Diverticulitis		−	−
53	F	39		I	IBS	No	+	+
55	F	25		I	IBS	No	−	−
56	F	40		I	Screening		−	−
57	F	55		I	Bleeding		−	−
58	M	75		TI	Screening		−	−
59	M	44		C/R	Screening		−	−
60	F	61		I	IBS	No	−	−
61	F	47		I	Colitis		−	−

Abbreviations: M = male; F = female; S = stomach; I = ileum; TI = terminal ileum; C = colon; R = rectum; P = prednisolone. Results of PCR for *M. avium* subsp. *paratuberculosis* DNA and culture shown as positive (+) or negative (–).

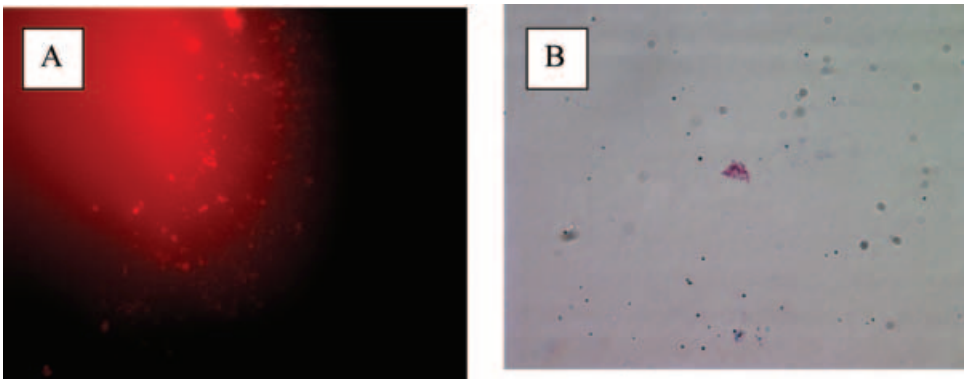

Figure 1. Mycobacteria grown in MGIT cultures from fresh biopsies, if stained at 8 wk from the inoculum were positive rhodamine auramine staining (*A*) whereas Ziehl Neelsen positive cells could be visualized only after 14 wk of growth (*B*).

auramine rodhamine. Cell shape can be appreciated by looking at the electron microscope appearance (Fig. 4). Treated cells are in aggregates and round in shape very similar to that of wild type *M. avium* subsp. *paratuberculosis* isolated from Crohn's disease patients (Fig. 2 and Fig. 4B). When observed by the electron microscopy untreated cells appear as bacilli (Fig. 4A), whereas after lysozyme treatment the same cells have a more rounded shape similar to those isolated from Crohn's disease patients (Fig. 4B) and the cell wall is thicker (Fig. 4C).

DISCUSSION

In the present study, we have found by PCR and by culture that a highly significant majority of patients with Crohn's disease living on the island of Sardinia are infected with *M. avium* subspecies *paratuberculosis.* This adds to the increasing body of reliable evidence contributed independently from several laboratories elsewhere in Europe and North America which implicates these chronic enteric pathogens in disease causation (23–28). As in our previous study (23) the two patients with ulcerative colitis tested negative, although in some cases it is probable that a *M. avium* subspecies *paratuberculosis* superinfection on an underlying ulcerative colitis may occur giving rise to a mixed clinico-pathological picture (30).

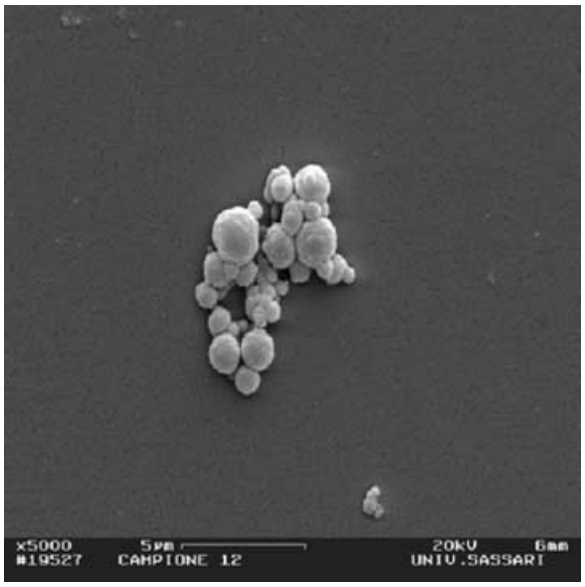

Figure 2. Electron microscopy of a strain IS*900* positive isolated from a fresh biopsy after 75 days of growth in the MGIT medium.

Testing the tissue samples fresh and the inclusion of optimized mechanical disruption of the sample lysate are again demonstrated to be important for PCR detection (25, 31). Our finding that these very slow growing pathogens will usually replicate in liquid MGIT medium sufficiently over 10–14 wk of incubation to become detectable, is in agreement with the previous work (24, 26). The organisms in culture are initially in their ZN staining negative *in vivo* form and appear slowly to revert through a rhodamine-auramine staining form, to a partial or complete ZN positive form classically seen in mycobacteria. The resulting culture appears to be a stable mixture of all three forms demonstrating the phenotypic versatility and plasticity of these complex pathogens. The acid fast negative induced forms of *M. avium* subspecies *paratuberculosis* in culture were seen to have a shape very similar to the *M. avium* subspecies *paratuberculosis* cells isolated from fresh Crohn's disease tissues. *M. avium* subspecies *paratuberculosis* infection is widespread in the island's dairy sheep flocks and it will be important to compare the genotypes of human and ovine isolates in Sardinia taking advantage of the improved PCR-based typing procedures which have become available (11, 14, 32, 33).

The isolation of *M. avium* subspecies *paratuberculosis* from 10% of people in Sardinia having ileocolonoscopies for reasons other than Crohn's disease is consistent with a fairly widespread human exposure in milk supplies or from the environment, and is in line with what is commonly found with *Helicobacter pylori*, *M. tuberculosis*, *Strep pneumoniae*, and many other bacterial pathogens. The finding in people without Crohn's disease that *M. avium* subspecies

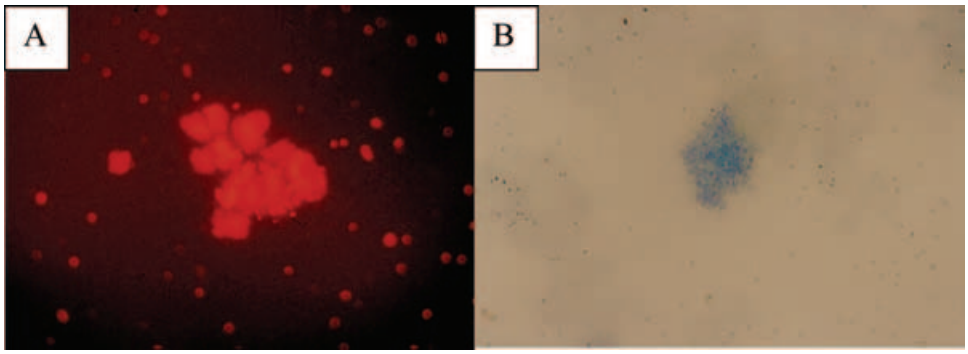

Figure 3. Auramine rhodamine staining (*A*) and Ziehl Neelsen staining (*B*) of *Mycobacterium avium* subsp. *paratuberculosis* cells after 2 months of growth in MGIT medium plus Lysozyme.

paratuberculosis colonization occurred in the presence of an established colonic abnormality is also in agreement with previous studies (25) particularly in relation to diverticulitis (34, 35).

In Sardinia, the 2.5 million sheep and 200,000 cattle make a big contribution to the island's economy. As happens elsewhere, clinically diseased animals in flocks and herds indicate the presence of a much wider subclinical burden of *M. avium* subspecies *paratuberculosis* infection (36). These organisms can survive for long periods in the environment and in surface waters (37, 38), as well as in pasteurized milk and soft cheeses (39, 40). Our present study suggests that they may also causing disease in the human population and much further work needs to be done including the development and introduction of new vaccines for animals and humans.

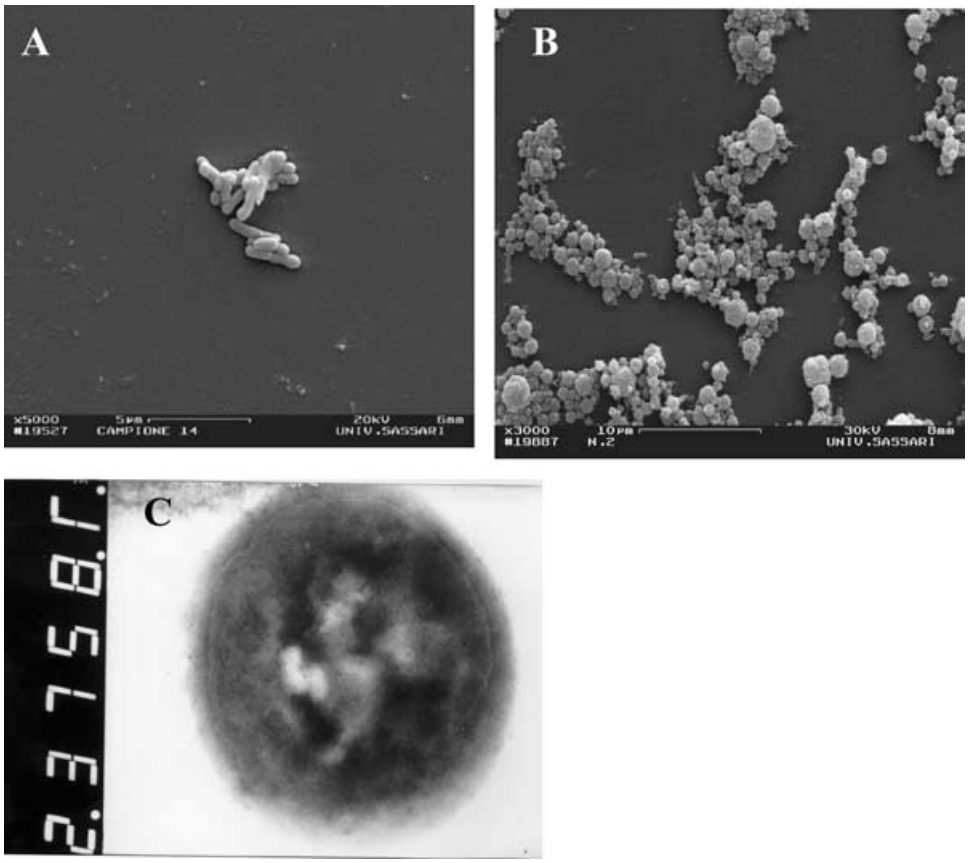

Figure 4. Scanning electron microscopy of *Mycobacterium avium* subsp. *paratuberculosis* cells grow in absence of Lysozyme (*A*) and after 2 months of growth in MGIT medium plus Lysozyme (*B*). Transmission electron microscopy of *Mycobacterium avium* subsp. *paratuberculosis* cells after 2 months of growth in MGIT medium plus Lysozyme (*C*).

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REFERENCES

1. Chiodini RJ, Van Kruiningen HJ, Merkal RS. Ruminant paratuberculosis (Johne's disease): The current status and future prospects. Cornell Vet 1984;74:218–62.
2. Riemann HP, Abbas B. Diagnosis and control of bovine paratuberculosis (Johne's disease). Adv Vet Sci Comp Med 1983;27:481–506.
3. Cocito C, Gilot P, Coene M, et al. Paratuberculosis. Clin Microbiol Rev 1994;7:328–45.
4. Gerlach GF. Paratuberculosis: The pathogen and routes of infection. Dtsch Tierarztl Wochenschr 2002;109:504–6.
5. Clarke CJ. The pathology and pathogenesis of paratuberculosis in ruminants and other species. J Comp Pathol 1997;116:217–61.
6. Johne HA, Frothingham L. Ein eigenthümlicher Fall von Tuberculose beim Rind. Deutsche Zeitschr Tierm Path 1895;21:438–54.
7. Collins DM, Gabric DM, de Lisle GW. Identification of two groups of Mycobacterium paratuberculosis strains by restriction endonuclease analysis and DNA hybridization. J Clin Microbiol 1990;28:1591–96.
8. Pavlik I, Horvathova A, Dvorska L, et al. Standardisation of restriction fragment length polymorphism analysis for Mycobacterium avium subspecies paratuberculosis. J Microbiol Methods 1999;38:155–67.
9. Bull TJ, Hermon-Taylor J, Pavlik I, et al. Characterization of IS900 loci in Mycobacterium avium subsp. paratuberculosis and development of multiplex PCR typing. Microbiology 2000;146:2185–97.
10. Dohmann K, Strommenger B, Stevenson K, et al. Characterization of genetic differences between Mycobacterium avium subsp. paratuberculosis type I and type II isolates. J Clin Microbiol 2003;41:5215–23.
11. Amonsin A, Li LL, Zhang Q, et al. Multilocus short sequence repeat sequencing approach for differentiating among Mycobacterium avium subsp. paratuberculosis strains. J Clin Microbiol 2004;42:1694–702.
12. Francois B, Krishnamoorthy R, Elion J. Comparative study of Mycobacterium paratuberculosis strains isolated from Crohn's disease and Johne's disease using restriction fragment length polymorphism and arbitrarily primed polymerase chain reaction. Epidemiol Infect 1997;118:227–33.
13. Whittington RJ, Hope AF, Marshall DJ, et al. 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.
14. Ghadiali AH, Strother M, Naser SA, et al. Mycobacterium avium subsp. paratuberculosis strains isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns. J Clin Microbiol 2004;42:5345–8.
15. Manning EJ, Collins MT. Mycobacterium avium subspecies paratuberculosis: Pathogen, pathogenicity and diagnosis. Rev Sci Technol 2001;20:133–50.
16. Daniels MJ, Henderson D, Greig A, et al. The potential role of wild rabbits Oryctolagus cuniculus in the epidemiology of paratuberculosis in domestic ruminants. Epidemiol Infect 2003;130:553–9.
17. Sweeney RW, Whitlock RH, Rosenberger AE. Mycobacterium paratuberculosis cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J Clin Microbiol 1992;30:166–71.
18. Sung N, Collins MT. Thermal tolerance of Mycobacterium paratuberculosis. Appl Environ Microbiol 1998;64:999–1005.
19. Grant IR, Ball HJ, Rowe MT. Incidence of Mycobacterium paratuberculosis in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. Appl Environ Microbiol 2002;68:2428–35.
20. Wuhib YA, Svastova P, Roubal P, et al. Mycobacterium avium subspecies paratuberculosis cultured from locally and commercially pasteurized cow's milk in the Czech Republic. Appl Environ Microbiol 2005;71:1210–4.
21. Hermon-Taylor J, El-Zaatari FAK. The Mycobacterium avium subspecies paratuberculosis problem and its relation to the causation of Crohn disease. In: Bartram J, Cotruvo J, Dufour A, Rees G, Pedley S, eds. Pathogenic Mycobacteria in water: A guide to public health consequences, monitoring and management. London, UK: IWA Publishing, 2000: 74–94.
22. Hermon-Taylor J, Bull TJ, Sheridan JM, et al. Causation of Crohn's disease by Mycobacterium avium subspecies paratuberculosis. Can J Gastroenterol 2000;14:521–39.
23. Sechi LA, Mura M Tanda F, et al. Identification of Mycobacterium avium subp. paratuberculosis in biopsies from patients with Crohn's disease identified by in situ hybridization. J Clin Microbiol 39:4514–7.
24. Naser SA, Ghobrial G, Romero C, et al. Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. Lancet 2004;364:1039–44.
25. 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.
26. Schwartz D, Shafran I, Romero C, et al. Use of short-term culture for identification of Mycobacterium avium subsp. paratuberculosis in tissue from Crohn's disease patients. Clin Microbiol Infect 2000;6:303–7.
27. Romero C, Hamdi A, Valentine JF, et al. 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 (In press).
28. 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;11:116–25.
29. Bernstein CN, Nayar G, Hamel A, et al. Study of animal-borne infections in the mucosas of patients with inflammatory bowel disease and population-based controls. J Clin Microbiol 2003;41:4986–90.

30. Hermon-Taylor J. Treatment with drugs active against Mycobacterium avium subspecies paratuberculosis can heal Crohn's disease: More evidence for a neglected public health tragedy. Dig Liver Dis 2002;34:9–12.
31. Odumeru J, Gao A, Chen S, et al. Use of the bead beater for preparation of Mycobacterium paratuberculosis template DNA in milk. Can J Vet Res 2001;65:201–5.
32. Bull TJ, Sidi-Boumedine K, McMinn EJ, et al. Mycobacterial interspersed repetitive units (MIRU) differentiate Mycobacterium avium subspecies paratuberculosis from other species of the Mycobacterium avium complex. Mol Cell Probes 2003;17:157–64.
33. O'Shea B, Khare S, Bliss K, et al. Amplified fragment length polymorphism reveals genomic variability among Mycobacterium avium subsp. paratuberculosis isolates. J Clin Microbiol 2004;42:3600–6.
34. Gledhill A, Dixon MF. Crohn's-like reaction in diverticular disease. Gut 1998;42:392–5.
35. Peppercorn MA. The overlap of inflammatory bowel disease and diverticular disease. J Clin Gastroenterol 2004;38(5 Suppl):S8–10.
36. Whitlock RH, Buergelt C. Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet Clin North Am Food Anim Pract 1996;12:345–56.
37. Whittington RJ, Marshall DJ, Nicholls PJ, et al. Survival and dormancy of Mycobacterium avium subsp. paratuberculosis in the environment. Appl Environ Microbiol 2004;70:2989–3004.
38. Pickup RW, Rhodes G, Arnott S, et al. Mycobacterium avium subspecies paratuberculosis in the Catchment Area and Water of the River Taff in South Wales, United Kingdom, and its potential relationship to clustering of Crohn's disease in the City of Cardiff. Appl Environ Microbiol 2005;71:2130–9.
39. Donaghy JA, Totton NL, Rowe MT. Persistence of Mycobacterium paratuberculosis during manufacture and ripening of cheddar cheese. Appl Environ Microbiol 2004;70:4899–905.
40. Grant IR, Ball HJ, Rowe MT. Incidence of Mycobacterium paratuberculosis in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. Appl Environ Microbiol 2002;68:2428–35.