



"Paratuberculosis."

Cocito, Carlo ; Gilot, P. ; Coene, M. ; de Kesel, M. ; Poupart, P. ; Vannuffel, P.

ABSTRACT

Paratuberculosis (Johne's disease) is a chronic, wasting, widespread mycobacteriosis of ruminants. It involves extensive mycobacterial shedding, which accounts for the high contagiousness, and ends with a fatal enteritis. Decreases in weight, milk production, and fertility produce severe economic loss. The DNA of the etiological agent (*Mycobacterium paratuberculosis*) has a base composition (66 to 67% G+C) within the range of that of mycobacteria (62 to 70% G+C), a size (4.4×10^6 to 4.7×10^6 bp) larger than that of most pathogenic mycobacteria (2.0×10^6 to 4.2×10^6 bp), and a high relatedness (> 90%) to *Mycobacterium avium* DNA. However, the DNAs of the two organisms can be distinguished by restriction fragment length polymorphism analysis. *M. paratuberculosis* genes coding for a transposase, a cell wall-associated protein (P34), and two heat shock proteins have been cloned and sequenced. Nucleic acid probes (two of which are species specific) are used, after PCR amplification, for *M. paratuberculosis* identification in stools and milk. As in leprosy, with disease progression, cellular immune reactions decrease and humoral immune reactions increase. Cutaneous testing with sensitins, lymphocyte proliferation assays, and cytokine tests are used to monitor cellular immune reactions in paratuberculosis, but these tests lack specificity. Complement fixation, immunodiffusion, and enzymometric tests based on antibodies to *M. paratuberculosis* extracts, to mycobacterial antigen complex A36, to glycolipids, and to proteins help identify affected cattle but are not species s...

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Paratuberculosis

CARLO COCITO,* PHILIPPE GILOT, MARC COENE, MYRIAM DE KESEL,
PASCALE POUPART, AND PASCAL VANNUFFEL

*Microbiology and Genetics Unit, Institute of Cellular Pathology, University of Louvain,
Medical School, Brussels 1200, Belgium*

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HISTORICAL INTRODUCTION

The clinical symptoms and pathoanatomical picture of paratuberculosis were first described during the 19th century. The name Johne's disease comes from the work of H. A. Johne and L. Frothingham, who, in 1895, demonstrated a connection between cattle enteritis and the presence of acid-fast microorganisms in sections of the intestinal mucosa. In 1906, Bang distinguished between tuberculous and nontuberculous enteritis and proposed that the latter be called pseudotuberculous enteritis. The identification of the etiological agent is attributed to F. W. Twort, who, in 1912, succeeded in cultivating and characterizing a mycobacterium, which in 1914 was shown to produce experimental enteritis. After the full characterization of *Mycobacterium paratuberculosis* as a distinct species within the genus *Mycobacterium*, the disease was renamed paratuberculosis (40, 115).

It was only towards the middle of this century, however, that the prevalence and wide geographic distribution of paratuberculosis were realized, and its effect on the livestock farming economy became apparent. The yearly losses due to the disease were estimated to exceed \$1.5 billion in the United States (107). Moreover, since Johne's disease is one of the most widespread bacterial diseases of domestic animals, its impact on the world economy is enormous (10, 40, 146, 160).

The aim of the present review is to briefly summarize the most relevant early findings and to concentrate on the molecular biological aspects of more recent works. A book recently edited by R. J. Chiodini (39) contains the complete set of references pertinent to the history of Johne's disease.

CLINICAL STAGES OF JOHNE'S DISEASE AND HOST RANGE

Paratuberculosis affects domestic and wild animals (in particular, ruminants) on five continents. Although the veterinary world has focused on cattle, sheep, and goats, many wildlife ruminants (deer, antelopes, mountain goats, bison, camels, llamas, and others) are also affected by paratuberculosis. In addition, the etiological agent, *M. paratuberculosis*, multiplies in horses and mules, which become asymptomatic shedders. Laboratory animals (mice, rats, rabbits, hamsters, gerbils, and guinea pigs) and birds (pigeons and poultry) replicate experimentally injected *M. paratuberculosis* (40, 71, 86, 88, 89, 141, 151). A closely related species isolated from the wood pigeon (*M. avium* subsp. *silvaticum*) was reported to cause chronic enteritis in deer, goats, and pigeons (128, 205). The relationship between the agent of Johne's disease and similar organisms isolated from humans and birds is discussed in a following section. Here we focus on the disease in ruminants, which are the usual hosts.

Although it is commonly agreed that mycobacteria invade the intestinal tract when animals feed on contaminated pastures, there are grounds to believe that infection of newborn animals is the main cause of disease. Because of the incomplete development of their immune systems, neonates are particularly susceptible to the etiological agent. Intrauterine infection seems to be restricted to fetuses of females with advanced forms of the disease. In fact, this kind of transmission was claimed to occur in about 25% of the calves born to critically ill cows (222). Calves can also be infected by ingesting colostrum and milk from paratuberculous cows (40, 196). *M. paratuberculosis* crosses the intestinal mucosa, presumably by penetrating the M cells of the dome epithelium that covers ileal Peyer's patches. Mycobacteria are then phagocytosed by subepithelial and intraepithelial macrophages, wherein they multiply intracellularly. This results in the appearance of a

* Corresponding author. Mailing address: Microbiology and Genetics Unit, UCL 5225, Ave. Mounier 52, B-1200 Brussels, Belgium. Phone and fax: 32-2-764.5225.

TABLE 1. Clinical stages of paratuberculosis

Stage ^a	Mycobacterial shedding	Immune reactivity		Clinical symptoms
		Humoral	Cellular	
I	Undetectable	Low	High	Absent
II	Medium	Medium	Medium	Absent
III	High	High	Low	Present ^b

^a I, asymptomatic, nonexcretory; II, asymptomatic, excretory; III, symptomatic, excretory.

^b Impairment of lactation, of weight increase, and of reproduction; emaciation and diarrhea; anorexia, cachexia, and death.

granuloma at the site of entry and in the involvement of nearby lymph nodes (153, 233). The basic intestinal reaction in cattle with paratuberculosis is chronic catarrhal inflammation, along with hyperplasia and intense histiocyte infiltration of the lamina propria (143).

Infection may spread due to the repeated ingestion of organisms or to the gradual replication of bacteria ingested at an earlier time. Larger regions of the intestinal tract and other body systems become involved as the disease advances with time. The diffuse granulomatous reaction results in a protein-losing enteropathy, and hence, hypoproteinemia and edema due to decreased osmotic pressure, and in progressive thickening of the gut (161–163). At later stages, the congested epithelial cells of the mucosa are sloughed into the intestinal lumen, and large numbers of bacilli are discharged. Bacterial concentration in the stools of infected animals may exceed 10^8 cells per g of feces, and the daily number excreted can be higher than 10^{12} mycobacteria per animal (40). This massive number of organisms accounts for the rapid spread of the infection, from even a single infected animal, to the whole herd.

The disease progresses through three clinical stages (Table 1). In stage I (subclinical with undetectable excretion), the infectious process develops without appreciable shedding of bacteria. This is followed by a subclinical excretory phase (stage II), during which the concentration of mycobacteria in the intestinal mucosa and lumen progressively increases. The terminal phase (stage III, clinical and excretory) is characterized by an intractable chronic diarrhea and the symptoms of a generalized infectious process. Emaciation, decreased milk production, diffuse edema, anemia, and infertility are the dominant late signs. Abortion and alopecia are rare events, even in the advanced stage of the disease. Animals die in a cachectic state.

Information on disease development was gathered by experimental infection trials. After oral administration of *M. paratuberculosis* cells to calves, the majority of animals developed a subclinical form of disease, although they shed organisms (stage II). Clinical symptoms appeared after prolonged periods in only a minority of cattle (125). Intravenous injection of the etiological agent produced similar results, though the experimental route differed from the natural one (192).

There is evidence that animals with the asymptomatic form of the disease outnumber those with the symptomatic form (22). A Pennsylvania slaughterhouse survey of 14,440 cull dairy cattle reported a prevalence of 7.2% of the animals to be infected with *M. paratuberculosis* on the basis of the recovery of the organism from tissues and/or manure samples. It is estimated that for every animal with clinical disease, 9 other animals in the herd are culture positive and another 10 to 15 animals are infected but undetectable by current diagnostic methods (222). As much as 60% of certain herds were reported to be asymptotically infected (40, 198).

MICROBIOLOGICAL ASPECTS

The etiological agent of Johne's disease, *M. paratuberculosis*, is a gram-positive, acid-fast microorganism of 0.5 to 1.5 μm . On Watson-Reid agar plates, or the commonly used HEYM (Herrold's egg yolk medium) plates, it forms nonpigmented rough colonies. It is a very slowly growing, fastidious organism: to proliferate, many strains require the presence of mycobactin. Visible colony formation on solid media may require up to 4 months, irrespective of the presence of different supplements (40, 142).

While a dependence on metals for growth is common to all bacteria, the iron requirement of pathogenic mycobacteria is peculiar in that an organic source of this metal is needed for its uptake and utilization. The complexed iron is chelated by siderophores and undergoes transmembrane transport and cytoplasmic exchange. Two kinds of siderophores are known, mycobactins and exochelins: the former are the internal sequestering agents, and the latter are the external vectors. Exochelins are small protein molecules present in the extracellular fluid: high concentrations are released under conditions of iron deficiency. Liposoluble and hydrosoluble exochelins have been identified. In animals, the main storage form of ferric iron is ferritin, and the transport molecules for this metal are transferrin and lactoferrin. Mycobacterial exochelins remove Fe^{3+} from these animal chelators and transport it across the bacterial cell wall. An exchange of the chelated metal with intracellular mycobactin may then take place, thus yielding the iron storage form present in mycobacteria. Mycobactins are high-molecular-weight complex lipids containing a core to which Fe^{3+} is coordinately linked and alkyl side chains of different lengths. Being insoluble in water, these highly hydrophobic structures are confined to the cell membrane. Mycobactins are unique to mycobacteria and nocardiae: they have not been found in any other actinomycetes examined and are therefore of taxonomic importance. Moreover, only mycobacteria contain both mycobactins and exochelins. The absence of mycobactins from most strains of *M. paratuberculosis* and some *M. avium* isolates is intriguing. These strains multiply freely in parasitized hosts, while requiring an exogenous supplement of mycobactin for in vitro proliferation (5, 6, 145). Mycobactin dependence is no longer considered pathognomonic for identification of *M. paratuberculosis* since mycobactin-dependent wood pigeon mycobacteria and *M. avium* strains have been identified (128, 202).

No one test can identify a single mycobacterial species, and only after multiple tests are performed for specific markers (growth factors, growth inhibitors, specific enzymes, lipid and sugar components, protein profiles, restriction analysis of DNA, and host range pathogenicity) can a microorganism isolated from an animal source be classified as *M. paratuberculosis* (105, 106). Thus, different chromatographic methods have been used to identify specific lipid and sugar markers for diagnostic and taxonomic purposes (19, 58, 150, 199).

It is particularly difficult to distinguish among species closely related to *M. paratuberculosis*. Wood pigeon mycobacteria do not grow on egg medium and are inhibited by *para*-nitrobenzoate, whereas *M. paratuberculosis* and *M. avium* multiply in both instances. The test for production of alkaline phosphatase is negative for *M. paratuberculosis* but positive for both *M. avium* and wood pigeon mycobacteria. Wood pigeon mycobacteria are pathogenic for chickens but not for rabbits, *M. avium* is pathogenic for both, and *M. paratuberculosis* is pathogenic for neither (33, 58, 142, 204, 206–208). Restriction fragment length polymorphism (RFLP) analysis of mycobacterial DNA with nucleic acid probes is the most powerful diagnostic

procedure available to distinguish among genomes of these closely related species (119, 131, 156). Studies along these lines are described in the next section.

GENOMES OF *M. PARATUBERCULOSIS* AND RELATED MICROORGANISMS

Mycobacteria belong to the taxonomic group that includes the genera *Corynebacterium*, *Mycobacterium*, and *Nocardia* (CMN group) and the new genus *Rhodococcus* (7). The base composition of the genomes of different bacteria is an essential taxonomic parameter, which is currently measured by biophysical methods (thermal profile or buoyant density) and expressed as the percentage of guanine plus cytosine (G+C) in DNA. The base composition of CMN organisms varies from 48 to 73% G+C: it is 48 to 59% for corynebacteria, 63 to 73% for rhodococci, 62 to 70% for mycobacteria, and 64 to 69% for nocardiae (82). However, the etiologic agent of human leprosy, *M. leprae*, does not fit into this scheme: it has a 55 to 58% G+C composition (45, 98, 104). The base composition of *M. paratuberculosis* DNA is 66 to 67% G+C (103, 130), and that of other pathogenic mycobacteria is as follows: 66 to 68% for *M. avium*, 65 to 69% for *M. intracellulare*, 64% for *M. kansasii*, and 63 to 66% for *M. bovis* and *M. tuberculosis* (44, 60, 102).

The sizes of mycobacterial genomes have been determined by renaturation kinetics and, more recently, by pulsed-field gel electrophoresis. The *M. paratuberculosis* genome has a molecular size of 4.4×10^6 to 4.7×10^6 bp. Thus, it is larger than the genomes of closely related mycobacteria: 3.5×10^6 bp for *M. avium*, 3.0×10^6 to 4.2×10^6 bp for *M. tuberculosis*, and 2.0×10^6 to 3.3×10^6 bp for *M. leprae* (3, 4, 44, 45, 102–104, 130).

The genetic relatedness of *M. paratuberculosis* with other mycobacterial species has been established by DNA-DNA hybridization (Table 2). Nearly 100% homology between most strains of *M. paratuberculosis* and *M. avium* has been reported (100, 103, 174, 231). The DNA of *M. paratuberculosis* was also found to be highly homologous to the DNA from several wood pigeon mycobacteria, organisms genetically related to some *M. avium* and *M. intracellulare* strains (100, 231). On the other hand, *M. paratuberculosis* DNA is less related to DNA of other mycobacteria (100, 103, 130, 174, 231).

Hybridization with the complete genome does not distinguish among very closely related organisms (for instance, *M. paratuberculosis* isolates, wood pigeon mycobacteria, and some *M. avium* serovars). A deeper insight into the genetic relatedness of *M. paratuberculosis* with other mycobacterial species was obtained by RFLP analysis. Nucleic acid probes proved to be the appropriate tools for such sophisticated discrimination. With this technique, labelled probes from the DNA of one bacterial strain are hybridized with the restriction fragments obtained by electrophoretic fractionation of endonuclease-digested chromosomes from other mycobacterial species (Southern blot). The degree of similarity of patterns between the reference and the unknown organisms provides a measure of the genetic relatedness of the compared species. These data can be used to evaluate DNA base substitution levels in closely related species and strains. Accurate estimation is obtained with probes prepared from single-copy DNA segments, which are known to undergo little rearrangement. From base substitution data, phylogenetic trees, such as that shown in Fig. 1, can be constructed (134).

Probes containing repetitive DNA elements have been used to measure genetic relatedness among isolates of the same species (52, 134). Thus, the insertion sequence IS900 of *M. paratuberculosis* was found to produce similar patterns from

TABLE 2. Relatedness of *M. paratuberculosis* genome to other mycobacterial species

Mycobacterial species	% DNA-DNA hybridization ^a
<i>M. avium</i> D4	91
<i>M. avium</i> ATCC 25291	92–102 (95)
<i>M. avium</i> serotype 2	96–105
<i>M. avium</i> serotype 7	49
<i>M. avium</i> serotype 8	92
<i>M. avium</i> serotype 9	92
<i>M. avium</i> serotype 19	35
<i>M. paratuberculosis</i> 18 (<i>M. avium</i> st 2)	76–91
<i>M. paratuberculosis</i> 316 F	(100)
<i>M. paratuberculosis</i> Linda	92
<i>M. paratuberculosis</i> C286	90
<i>M. paratuberculosis</i> 21	90
Wood pigeon 64	(94)
Wood pigeon 36	(93)
Wood pigeon M21	99
Wood pigeon VI/72	98
<i>M. intracellulare</i> serotype 6	111
<i>M. intracellulare</i> serotype 9	65
<i>M. intracellulare</i> TMC 1406	53
<i>M. scrofulaceum</i> serotype 41	18–21
<i>M. scrofulaceum</i> serotype 42	11–18
<i>M. scrofulaceum</i> serotype 43	14–30
<i>M. fortuitum</i>	3–17
<i>M. tuberculosis</i>	52
<i>M. phlei</i>	2.7–19
<i>M. kansasii</i>	23
<i>M. goodii</i>	27

^a Data from references 100, 103, 174, and 231. Numbers without parentheses refer to hybridization with the ATCC 19698 strain and those within parentheses refer to hybridization with the 2E strain of *M. paratuberculosis*.

several *M. paratuberculosis* isolates (Fig. 2), suggesting homogeneity of this mycobacterial species (134). IS900, a 1.45-kb insertion sequence present in 15 to 20 copies in the *M. paratuberculosis* genome, has several unique features: (i) it lacks terminal inverted repeats, (ii) it does not induce duplication of the insertion sequences at the target site, (iii) it possesses homologous target site sequences within the ge-

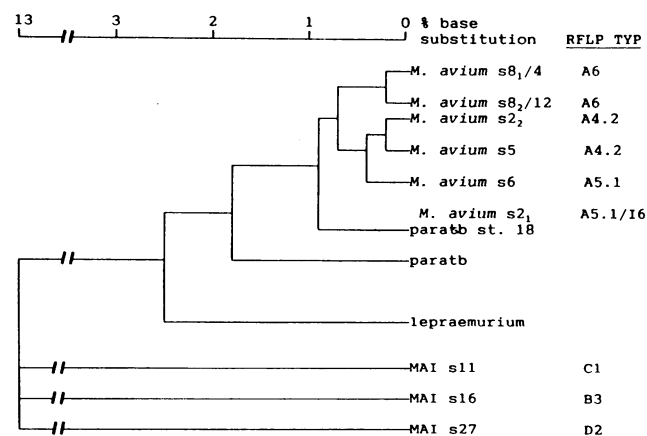


FIG. 1. Phylogenetic tree of a group of pathogenic mycobacteria. The dendrogram was constructed with DNA base substitution data as calculated from RFLP analysis of the genomes of *M. paratuberculosis* (paratb), *M. avium*, some strains of the *M. avium-M. intracellulare* (MAI) group (different serovars), and *M. lepraemurium*. Reprinted from reference 133 with permission of the publisher.

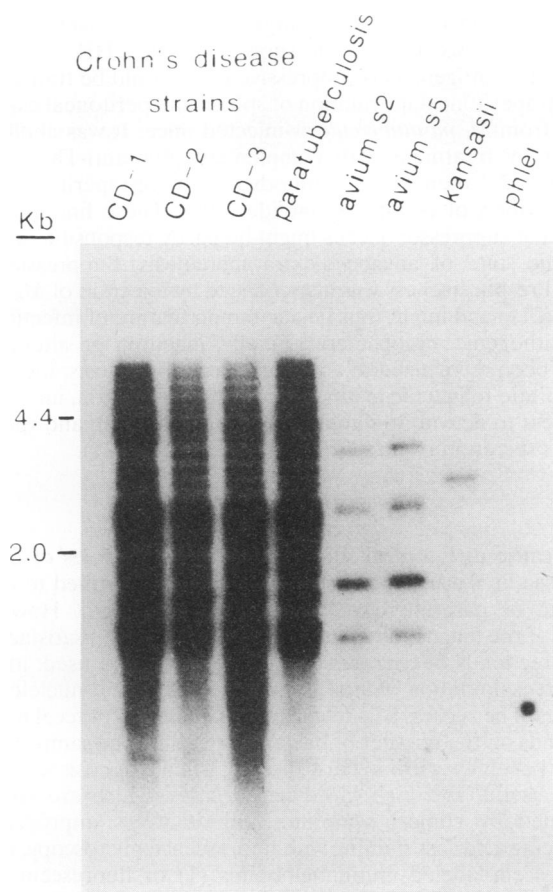


FIG. 2. RFLP analysis of mycobacterial genomes. *Pvu*II-digested DNA from *M. paratuberculosis* strains of human (Crohn's disease [CD]) and bovine (Johne's disease [paratuberculosis]) origin and other mycobacteria were probed with clone pMB22 containing the insertion sequence IS900 of *M. paratuberculosis*. Reprinted from reference 133 with permission of the publisher.

nome, and (iv) it has oriented insertion (85). The expression product of IS900, the p43 protein, is similar to the polypeptides encoded by transposable elements from streptomycetes (IS116 and IS110) (70). IS900 has recently been used as a transposing vector in *M. smegmatis* (70). Sequences related to IS900 were also found in wood pigeon mycobacteria (IS902) and *M. avium* (IS901) (119, 120, 156). IS900 and IS902 have been used in taxonomic studies of *M. paratuberculosis* and *M. avium* isolates, respectively (27, 53, 90, 119, 120, 134, 221).

rRNA sequences have also been used to evaluate the genetic relatedness of different bacterial genera and species. Indeed, these sequences are highly conserved in evolution. The number of rRNA genes is low in mycobacteria compared with that of the reference organism *Escherichia coli* (which has seven copies of rRNA genes). Two copies of rRNA genes per chromosome were identified in saprophytic mycobacteria, and a single copy was seen in pathogenic mycobacteria (15, 114). RFLP analysis of the 5S rRNA gene region clearly indicates that the strain closest to *M. paratuberculosis* is *M. avium* serovar 2 (36). The development of sequence analysis has provided taxonomists with very high resolution power. A comparison of 16S rRNA sequences from a large number of mycobacteria has shown that the 16S rRNA of *M. paratuberculosis* is 99.9% homologous to *M. avium*, 98.1% homologous

to *M. bovis*, more than 95% homologous to other mycobacteria, and 85 to 92% homologous to the related genera *Corynebacterium*, *Rhodococcus*, and *Nocardia* (188). This technique also revealed two clusters within the genus *Mycobacterium*: one constituted by the nonpathogenic rapid growers, and the other made up of the pathogenic slow growers (188). The latter group included *M. paratuberculosis*, *M. avium*, and *M. intracellulare*. *M. leprae* and *M. bovis* were the next closest species (57, 172, 184, 188, 213).

These studies indicate that the *M. avium* group is larger and more heterogeneous than the *M. paratuberculosis* group and that the two species are more closely related genetically than the other mycobacterial species.

MAJOR IMMUNOLOGICALLY ACTIVE COMPONENTS OF *M. PARATUBERCULOSIS* AND OTHER MYCOBACTERIA

Three groups of immunogenic mycobacterial glycolipids have been analyzed in detail. One group is composed of glycopeptidolipids that include polar mycoside C. These polymers contain monoglycosylated and acylated peptides linked to oligosaccharide residues endowed with serotype specificity. Glycopeptidolipid 1 of *M. paratuberculosis* was found to be strongly immunogenic and to cross-react with the glycopeptidolipid of *M. avium* serotype 2 (28–30).

Sophisticated structural studies have been recently carried out on lipoarabinomannan (LAM), a glycolipid anchored to the membrane and inserted in the mycobacterial cell wall. The latter is made up of two interconnected polymers: the arabinogalactanmycolate complex and peptidoglycan. In addition to arabinose (mostly in the furanose form) and mannose, LAM contains glycerol, polyolphosphates, and the diacyl-glycerol esters of hexadecanoate and methyloctadecanoate (7, 139). LAM is a dominant mycobacterial immunogen that strongly reacts with the sera from patients with leprosy and tuberculosis (18, 147) and with the sera from animals with paratuberculosis (137, 193–195). LAM-based immunoassays are very sensitive but lack species specificity. Mycobacteria excrete large quantities of hydrosoluble LAM complexes that exert various effects on the immune system: they stimulate B lymphocytes, induce suppressor T cells, and modulate lymphokine production (32, 67, 154, 182). The lateral arabinofuranose chains of this polymer seem to be mainly responsible for LAM's antigenicity (139).

The best-known members of the phenolic glycolipid group are the phenolic glycolipid 1 and 2 components of *M. leprae*, which accumulate in large quantities in the tissues of patients with lepromatous leprosy. They are highly immunogenic and species specific (99, 140). Some synthetic phenolic glycolipid 1 derivatives have been synthesized and used as reagents for serological tests in human leprosy (43, 126). These types of compounds have not been explored in *M. paratuberculosis*.

Mainly, two techniques are used to screen *M. paratuberculosis* antigens: two-dimensional immunoelectrophoresis on agarose gel, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. Protein antigens are fractionated under natural conditions by the former technique and under denaturing conditions by the latter technique.

Rabbit hyperimmune sera to *M. paratuberculosis* identified 44 *M. paratuberculosis* antigens in a two-dimensional immunoelectrophoretic reference system (87). Most of these antigens cross-reacted with components from other mycobacterial species. Thus, of the 44 antigens indexed, 27 reacted with anti-*M. avium* serum and 24 reacted with anti-*M. bovis* BCG serum,

but only antigen 17 was specific for *M. paratuberculosis*. The antigen complex A36, identified by this technique, is described in detail in a later section.

When similar investigations were carried out with paratuberculous animal sera, the number of antigens identified was smaller than that revealed by hyperimmunized rabbit antisera. Of the 15 main components recognized by paratuberculous bovine sera, only antigens 2 and 5 were absent from *M. avium* and *M. bovis* strains (118). In another work, seven antigens were identified by sera from paratuberculous sheep. Two of them, antigens A (31 kDa) and D (400 kDa), were used as reagents in enzyme-linked immunosorbent assays (ELISAs), but the tests lacked species specificity (20, 193). The NH₂-terminal portion of antigen A is similar to that of antigen 85 of *M. bovis* BCG, a 31-kDa fibronectin-binding protein (224, 225), and the amino acid sequence of antigen D closely resembles that of bacterioferritin (cytochrome *b_L*) from *E. coli* (21, 193). Another *M. paratuberculosis* component, antigen 16, which is involved in iron transport, is produced in large amounts when *M. paratuberculosis* is grown in the presence of a high concentration of iron, in mycobactin-free medium (54). Unfortunately, in the various publications cited, antigens are numbered according to different reference systems, which makes comparison difficult.

SDS-PAGE fractionation and Western blot (immunoblot) analysis afford higher resolution of mycobacterial proteins than agarose electrophoresis but yield denatured fractions. When *M. paratuberculosis* sonicates were fractionated by this procedure, 20 proteins within the 34- to 84-kDa region were recognized by paratuberculous bovine sera (9, 61), and fewer components of 30 to 43 kDa were recognized by sera of infected sheep (212).

IMMUNOLOGICAL ASPECTS

The immunological picture of Johne's disease mimics that of human leprosy: in both mycobacterioses, hyperreactive and anergic forms can be recognized. At the hyperreactive pole, which is characteristic of early stages of paratuberculosis, there are strong cell-mediated immune reactions that limit the proliferation of the etiological agent. This stage of Johne's disease resembles the tuberculoid form of human leprosy. At the opposite pole is anergy, which occurs in the terminal stage of paratuberculosis and resembles the lepromatous phase of human leprosy (11, 143).

Between the two poles there is an intermediate stage corresponding to the borderline form of human leprosy, which is characterized by a progressive weakening of cell-mediated immunity and increased blood levels of antimycobacterial immunoglobulin. The resemblance between the immunological spectra of leprosy and paratuberculosis is supported by numerous observations of low humoral response in subclinical cases of Johne's disease and weak cell-mediated reactions in advanced forms (11, 40, 59, 63, 125, 159). Indeed, in the early stages of paratuberculosis, there is a high proliferative response of lymphocytes to mycobacterial antigens and a positive response to cutaneous tests with johnin or with avian sensitin. These skin tests are based on delayed-type IV hypersensitivity reactions (DHR), which mimic those elicited by lepromin in leprosy and by tuberculin in tuberculosis (40, 83, 93).

On the other hand, lymphocytes from cattle with evident clinical symptoms of paratuberculosis yield weaker responses to mitogens than those from healthy animals (11, 40, 59). The occurrence in sera of paratuberculous cattle of a soluble factor suppressing mitogen-induced transformation of normal bovine lymphocytes has been reported (59). Moreover, intraperito-

neal injection of mice with large amounts of inactivated *M. paratuberculosis* induced the suppression of DHR against an unrelated antigen. This suppressive effect could be transferred by intraperitoneal inoculation of spleen and peritoneal exudate cells from *M. paratuberculosis*-injected mice. It was abolished either by treatment with complement plus anti-Thy 1-2 or anti-Lyt 2-2 monoclonal antibodies or by intraperitoneal administration of cyclophosphamide (109). These findings suggest that suppressor T cells might be partly responsible for the anergic state of advanced paratuberculosis. Suppression of T-cell responsiveness was also induced by injection of *M. bovis* cells (211) and might thus be a common feature of infection by all pathogenic mycobacteria. Finally, macrophage alterations have been recognized in advanced paratuberculosis, including the failure to inactivate phagocytosed mycobacteria, unresponsiveness to activating signals (gamma interferon), and spontaneous secretion of interleukin-1 (12, 59, 117, 234).

DIAGNOSTIC TOOLS

Because of the fecal shedding of large quantities of mycobacteria, it is crucial that infected cattle be identified to avoid spread of paratuberculosis throughout the herd. However, most of the diagnostic tools available are less than satisfactory.

Three kinds of coproanalytical procedures are used: microscopic examination of stool samples, culture, and nucleic acid probes. The success of microscopic examination of fecal matter depends on the number of bacteria present in the sample (113, 171). Positivity is low in early disease, when diagnosis would be most useful, and high in advanced cases, which are already revealed by clinical symptoms. Sensitivity is improved by replacing acid-fast staining with fluorescence microscopy, using fluorescein-labeled immunoglobulins (1) or fluorescent dyes (auramine, fluoresceine, and rhodamine). However, these procedures, which do not distinguish *M. paratuberculosis* from other mycobacteria, are of little practical use.

Culture, though more sensitive than microscopic examination of fecal samples, is a slow and exacting procedure because of the long generation time of *M. paratuberculosis*. Moreover, the decontamination step, which is required to remove the majority of organisms in the intestinal flora, may affect mycobacterial viability (64, 170). Biopsy materials and samples of milk have also been cultured for research purposes (197). Radiometric detection of *M. paratuberculosis* by culture of filter-concentrated fecal specimens was claimed to be more sensitive (>50% more positive cases) than conventional culture of sedimented bacteria on solid media (55). However, centrifugal concentration of mycobacteria from fecal samples seems to be a simpler and more sensitive coproanalytical procedure. The most advanced identification methods, based on nucleic acid probes, are described in a separate section.

Histopathological analysis of the terminal ileum and mesenteric lymph nodes has been carried out on biopsy material from paratuberculous cattle (13, 25, 116, 164). This approach, which allows rapid diagnosis, is mainly applied to examination of tissue obtained at necropsy. The presence of mycobacterial clumps in granulomatous tissue is confirmatory (223).

Immunological procedures such as immunodiffusion, complement fixation, and immunoenzymatic (ELISA) tests are described in the serology section.

Cutaneous testing with tuberculin-like preparations (sensitins) is used as a diagnostic procedure in several countries (40, 66, 112, 124) but not in the United States. *M. avium* extracts (avian tuberculin) often replace *M. paratuberculosis* extracts (johnin), being more easily available. Although the two bacteria are genetically related (35), there are some

differences in their antigens (78). Sensitins are heterogeneous mixtures of substances inducing nonspecific inflammatory reactions and of antigens displaying extensive cross-reactions among members of the genus *Mycobacterium* (40, 78, 79). Their use for diagnostic purposes entails obvious difficulties due to the lack of species specificity. Thus, in countries where cutaneous testing for tuberculosis is compulsory (European Community), comparative cutaneous testing of positive cattle with both avian and bovine tuberculin is the rule. Cattle displaying high reactivity to bovine tuberculin and low reactivity to avian tuberculin are considered tuberculous and thus are submitted to compulsory slaughtering.

The cellular immune status in mycobacterial diseases can also be explored by some in vitro immunoassays. Since gamma interferon is considered a basic mediator of DHR, its synthesis in response to *M. paratuberculosis* preparations and components can be considered a measure of cellular immune reactivity against the mycobacteria. An enzyme immunoassay and a bioassay for gamma interferon have been comparatively analyzed in paratuberculous cattle. The former test had a higher sensitivity, being able to detect 71.8 to 93.3% of subclinical cases, a small percentage of which (16.7 to 33.3%) was detected by the latter test (17). In the diagnostic kit from IDDEX (Westbrook, Portland, Maine), the level of gamma interferon synthesized by bovine lymphocytes in response to mycobacterial antigens is measured by an ELISA, which, however, lacks species specificity since it relies on cross-reacting antigens. Lymphocyte proliferation tests for Johne's disease are based on labeled thymidine incorporation into the DNA of lymphocytes from paratuberculous cattle which are incubated in vitro with mycobacterial components. Assays of this sort have been used in research laboratories (24, 63, 69, 79, 96) and, less frequently, in diagnostic veterinary laboratory work.

A36 ANTIGEN COMPLEX OF *M. PARATUBERCULOSIS* AND TMA COMPLEXES FROM OTHER MYCOBACTERIA

As mentioned, two-dimensional immunoelectrophoresis is a powerful tool for antigen fractionation. When a sample of mycobacterial cytoplasm fractionated in one dimension migrates in a second dimension in agarose gel containing anti-mycobacterial immunoglobulins, a series of precipitation arcs of antigen-antibody complexes are formed. After staining, antigens are numbered according to reference systems such as that of Closs et al. for *M. bovis* (46): antigen 60 (A60) is the major and least mobile component of *M. bovis* BCG. All mycobacteria (and some organisms of the CMN group) yield immunoelectrophoretic patterns similar to that of *M. bovis*. The term thermostable macromolecular antigen (TMA) complexes has been proposed for the A60-like components of various mycobacterial species (A7 in *M. leprae* and A36 in *M. paratuberculosis*) (Table 3). The term TMA highlights the unique properties of these complexes: thermostability and high molecular weight ($>10^6$) (46, 47, 49, 87, 92). The pattern of A36 from *M. paratuberculosis* is shown in Fig. 3.

Chemical analysis of TMA complexes revealed the presence of polysaccharides, proteins, and lipids. The branched glycan core and lipid components have negligible antigenicity (23). Upon dissociation with detergents and fractionation by gel electrophoresis (SDS-PAGE), TMA complexes yield 30 major protein components of 20 to 70 kDa, mainly responsible for the antigenicity of these complexes. Proteins within the 25- to 45-kDa region seem to be immunodominant in mycobacterioses (50, 61, 79).

TABLE 3. TMA complexes of pathogenic mycobacteria

Microorganism	Mycobacteriosis	TMA	
		Name	Antigenicity ^a
<i>M. bovis</i>	Tuberculosis	A60	High
<i>M. tuberculosis</i>	Tuberculosis	A60	High
<i>M. avium</i>	Nontuberculous mycobacteriosis		High
<i>M. leprae</i>	Leprosy	A7	High
<i>M. paratuberculosis</i>	Johne's disease (Crohn's disease)	A36	High

^a As determined by the level of immunoglobulins present in patients and infected animals.

TMA complexes elicit humoral and cellular immune reactions and are immunodominant in mycobacterioses, as shown by the observation that a large proportion of anti-mycobacterial immunoglobulin in the cerebrospinal fluid of tuberculous meningitis patients and in the blood of pulmonary tuberculosis cases is directed against A60 (14, 16, 31, 49, 50, 183). Sensitins, bacterial extracts used for cutaneous testing, contain TMA components that are partly responsible for the elicited DHR (79, 92). TMA-based immunoassays have thus been developed to explore the humoral and cellular immune reactions in infected hosts (48, 72). An A36-based serological test distinguished between healthy and paratuberculous cattle at all stages of the disease (61), and cutaneous testing with A36 in sensitized rabbits yielded DHR comparable to those elicited by johnin and avian tuberculin (79).

However, TMA-based immunoassays lack species specificity, owing to the pronounced cross-reactivity among complexes from different mycobacterial species (61, 79). The specificity lacking at the level of whole complexes was then sought at the level of their protein components. In practice, proteins used as reagents for immunoassay ought to possess B-lymphocyte-specific epitopes (serological assay) or T-cell-specific epitopes (cutaneous testing). To identify specific B-cell epitopes in A36 proteins, the reactivity of A36 antiserum, either preabsorbed or not absorbed with lysates of different mycobacteria, was compared by Western blot (Fig. 4). Five proteins of the *M.*

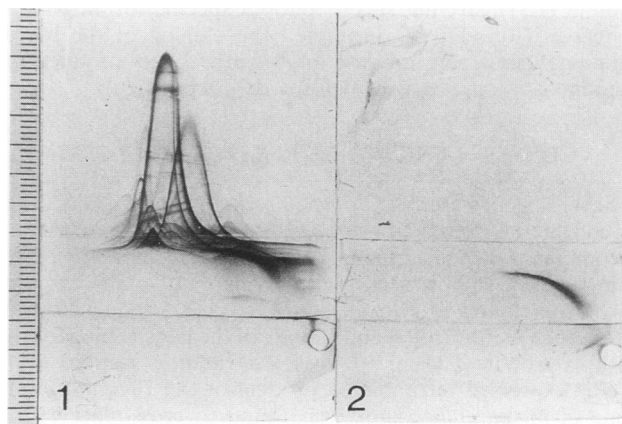


FIG. 3. Two-dimensional immunoelectrophoresis of *M. paratuberculosis* sonicate (1) and A36 complex (2). Migration: leftwards (first dimension) and upwards (second dimension). Rabbit antiserum directed against *M. paratuberculosis* sonicate was added to the second-dimension agarose gel. Reprinted from reference 61 with permission of the publisher.

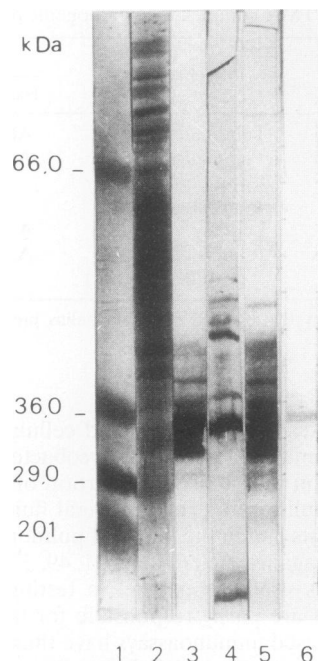


FIG. 4. Identification of immunodominant A36 proteins containing species-specific B-cell epitopes. Electrophoresed A36 proteins (SDS-PAGE), after blotting, were either stained with India ink (lane 2) or incubated with paratuberculous cattle sera (lanes 3 to 6) that were either preabsorbed (lane 6) or not (lanes 3 to 5) with a mixture of mycobacterial sonicates (*M. avium*, *M. bovis*, and *M. phlei*). Lanes 3 to 5 correspond, respectively, to stages I, II, and III. Bound primary immunoglobulins were revealed with peroxidase-labelled secondary immunoglobulin. Molecular mass markers are noted in lane 1.

paratuberculosis A36 complex (all within the 20- to 35-kDa region) contain epitopes specific for *M. phlei*, three contain epitopes specific for *M. bovis*, and only one protein (P34) contains epitopes specific for *M. avium* (Table 4) (61). The parallel between this immunological specificity and DNA relatedness is obvious: the more genetically related two organisms are, the fewer the proteins endowed with specific epitopes. The identification of a 34-kDa immunodominant protein (P34) carrying B-cell epitopes specific for *M. paratuberculosis* (61) (Fig. 4) prompted the cloning of the corresponding gene and the use of its expression product for immunoassays in paratuberculosis (described later).

CLONED GENES OF *M. PARATUBERCULOSIS*

Several genes from *M. tuberculosis*, *M. leprae*, and other mycobacterial genomes have been cloned, primarily to prepare recombinant proteins in amounts sufficient for immunological assays (232). In this section, we focus on the few cases of successful cloning of *M. paratuberculosis* DNA sequences (Table 5). The identification and cloning of the gene coding for the 34-kDa protein (P34) are discussed in the next section.

The expressed recombinant product of the 70-kDa protein gene of *M. paratuberculosis* was found to cross-react with a monoclonal antibody directed against the 71-kDa component of *M. tuberculosis* (190). The latter antigen is one of the heat shock proteins present in all bacteria and which have similar compositions, hence, the strong cross-reactions with even remote species. Indeed, the genes of heat shock proteins from both prokaryotes and eukaryotes are, to a large extent, homol-

TABLE 4. Antigenicity and specificity of some proteins of the A36 complex^a

Protein (kDa) ^b	Rabbit serum ^c	Antigenicity ^c			Specificity ^d		
		Infected bovine serum ^f			<i>M. avium</i>	<i>M. bovis</i>	<i>M. phlei</i>
		I	II	III			
74	++	—	—	+	—	—	—
52	+	—	—	—	—	—	—
41	+	+	+	+	—	—	—
40	+++	+	+	+	—	—	—
37	++	++	—	++	—	—	—
35	+	++	++	++	—	—	+
34	+++	+++	+++	+++	+	+	+
31	++	+++	—	+++	—	+	+
29	+++	—	—	+	—	—	—
23	+++	—	+	—	—	+	+
22	+	—	++	—	—	—	+

^a From reference 61 with permission of the publisher.

^b A36 components were fractionated by acrylamide gel electrophoresis (SDS-PAGE) and identified by immunoblotting.

^c The degree of antigenicity was evaluated from the immunoblot intensity as revealed by the corresponding sera: lack of (—) or low (+), medium (++), or high (+++) antigenicity.

^d The presence (+) or absence (—) in A36 proteins of epitopes specific towards three mycobacteria were tested with an anti-A36 rabbit serum preabsorbed on lysates of the indicated bacteria.

^e Recognition level of A36 proteins by an anti-A36 rabbit serum.

^f Sera from cattle at different stages of Johne's disease: I, asymptomatic, nonexcretory; II, asymptomatic, excretory; III, symptomatic, excretory forms.

ogous: they contain sequences that are highly conserved in the evolution of all living beings (127). For instance, the 70-kDa protein of *M. paratuberculosis* shows a 58% relatedness with *E. coli* DNA K, a 50% relatedness with the heat shock protein of *Drosophila melanogaster*, and a 49% relatedness with that of *Homo sapiens*. Some stretches of the 70-kDa protein of *M. paratuberculosis* are more than 90% related to the 70-kDa protein of *M. leprae* and the 71-kDa protein of *M. tuberculosis*. The carboxyl termini seem to be the most specific portions of these molecules (190).

The 65-kDa protein of *M. bovis*, another heat shock protein, is present in the cytoplasm in relatively large amounts and is highly antigenic. Proteins of comparable size and function have been found in all mycobacteria. The gene coding for the 65-kDa heat shock protein of *M. bovis* has been entirely sequenced (200). With two primers complementary to each strand of this gene, segments of the genomes of five other mycobacterial species, including *M. paratuberculosis*, have been amplified by PCR and shown to bear strikingly similar sequences (91).

The gene product (p43) of the insertion sequence IS900 has been produced in a recombinant strain of *E. coli*, using an exogenous promoter. An antiserum to p43 identified a 28-kDa processed product expressed at a level that is unusually high for classical transposases. If it were well recognized by bovine antisera, this recombinant protein could have a potential use in the diagnosis of Johne's disease (209).

Two promoters of unknown genes from *M. paratuberculosis* have been cloned. One induced the transcription of the promoterless *galK* gene in a promoter-probe plasmid (201). The cloned DNA segment contained the expected –35 and –10 promoter regions, and the Shine-Dalgarno ribosome binding sequence, preceding the ATG start codon of the *galK* gene. The other promoter sequence (P_{AN}), adjacent to the 3' end of the IS900 insertion element, was isolated from *M. paratuberculosis* and sequenced (158). The P_{AN} promoter was

TABLE 5. Proteins of *M. paratuberculosis* with known sequences

<i>M. paratuberculosis</i> protein (kDa)	Similar proteins (kDa) described in:			Characteristic	Reference(s)
	<i>M. leprae</i>	<i>M. bovis</i>	<i>M. tuberculosis</i>		
70	70	70	71	Heat shock	190, 232
65 ^a	65	64	65	Heat shock	61, 91, 232
43	No	No	No	Transposase	85
34	No	No	No	Cell wall associated	62, 80

^a Partly sequenced.

shown to control the expression of the reporter gene *lacZ* in *M. smegmatis* and *M. bovis*, hence, its possible use for the expression of heterologous genes in the BCG vaccine strain (158).

THE 34-kDa PROTEIN: IDENTIFICATION AND CLONING OF THE GENE

As mentioned, the 34-kDa protein (P34) of the A36 complex is immunodominant and contains B-cell species-specific epitopes (61) (Table 4). Three portions of the gene coding for P34 have been isolated from a λ gt11 genomic library of *M.*

paratuberculosis. The expression product of one of them (a362) was a 13.6-kDa polypeptide containing B-cell epitopes present in all tested *M. paratuberculosis* strains but not in other mycobacterial species (62). This recombinant polypeptide, which contained the carboxyl-terminal portion of P34, was used as a reagent for an ELISA for paratuberculosis. Infected cattle at all stages of the disease were correctly diagnosed (62, 80) (Fig. 5).

A portion of the cloned a362 DNA was then used as a probe to identify the 5' end of the P34 gene on a Southern blot containing restriction fragments of the *M. paratuberculosis*

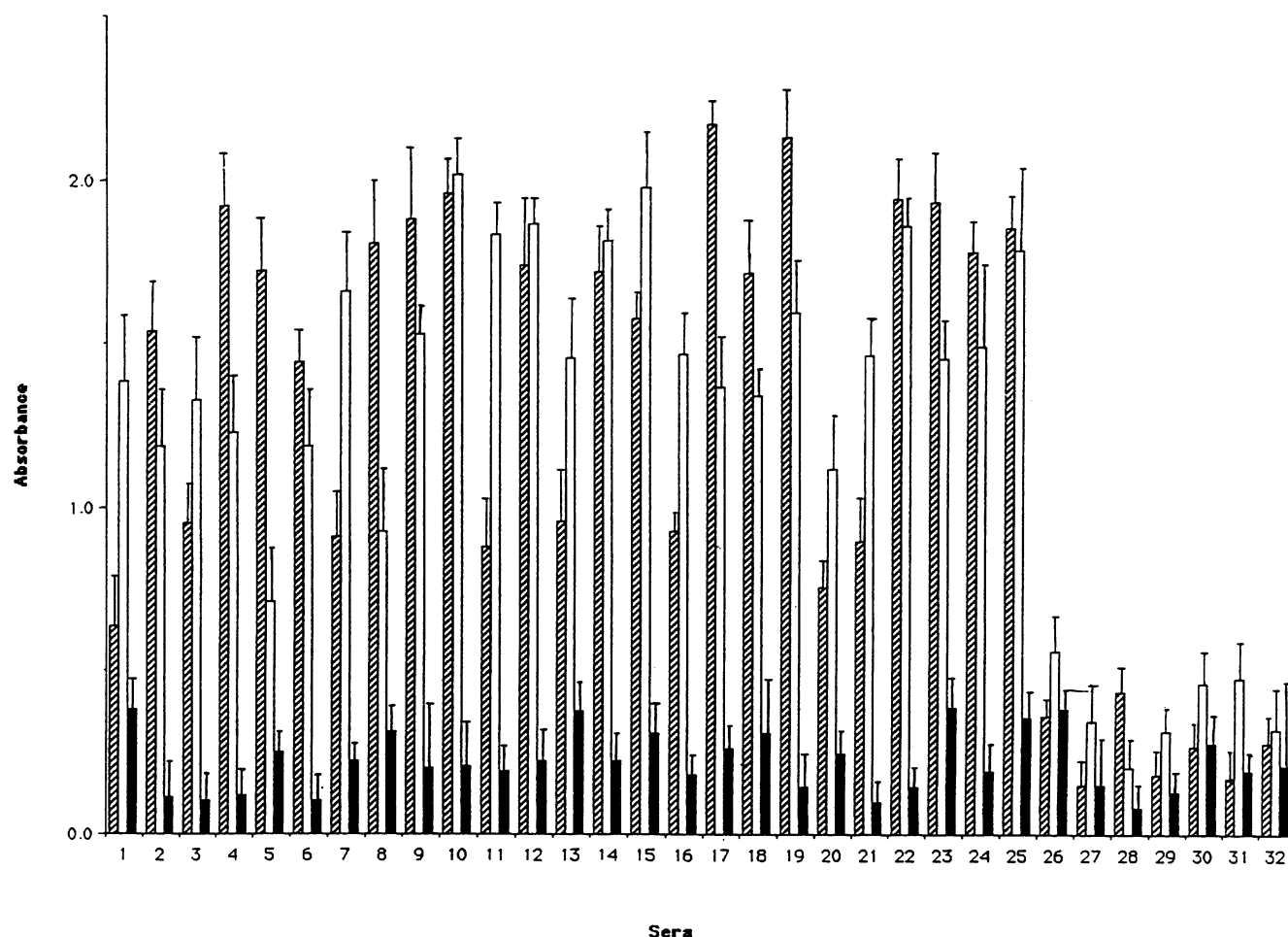


FIG. 5. Serological analysis of paratuberculous cattle. ELISAs were conducted on paratuberculous (samples 1 to 25) animals at stages I, II, and III and on healthy (samples 26 to 32) cattle. The antigens were the A36 complex (▨), a lysate of the recombinant *E. coli* MC1061 strain coding for the carboxy-terminal portion of the P34 protein (a362 polypeptide) (□), and a control of nonrecombinant *E. coli* MC1061 extract (■). Reprinted from reference 62 with permission of the publisher.

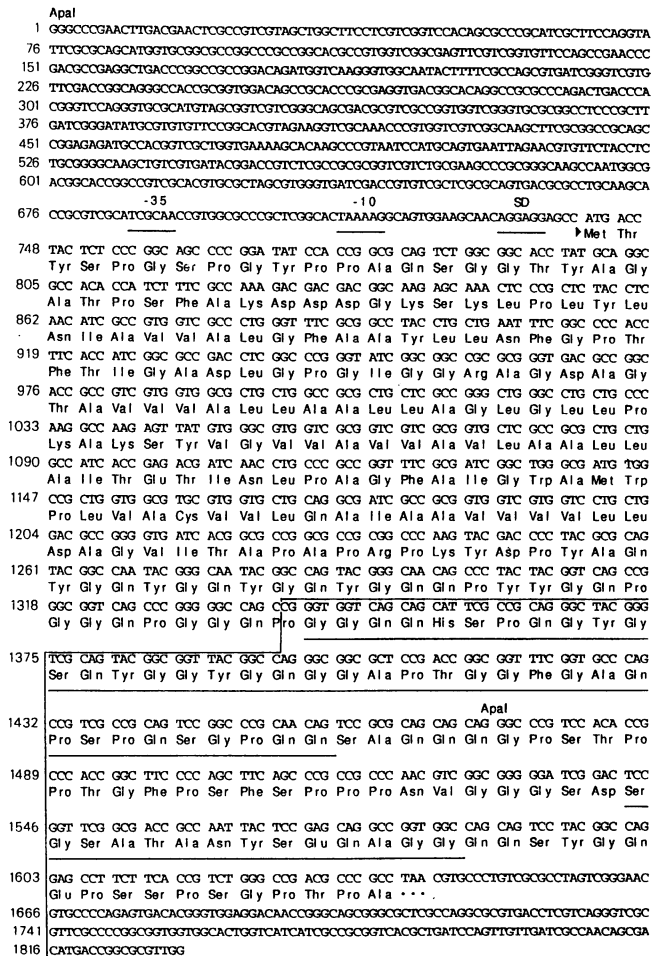


FIG. 6. Nucleotide sequence of the region containing the gene encoding the 34-kDa protein of *M. paratuberculosis*. The sequence of the gene, of its translation product, and of the putative promoter region (−10, −35) and the Shine-Dalgarno sequence (SD) are shown. The translation initiation codon is indicated by an arrow, and the stop codon is shown by dots. The amino acids confirmed by internal sequencing of the recombinant polypeptide are underlined. The sequence corresponding to the DNA fragment inserted in clone a362 is boxed. Reprinted from reference 80 with permission of the publisher.

genome. The DNA fragment of 1,500 bp thus detected was shown to contain the amino-terminal part of the P34 gene. The full sequence of the P34 gene is displayed in Fig. 6; a DNA bank search revealed its uniqueness. Putative regulatory sequences of the gene (start and stop codons, Shine-Dalgarno sequence, and promoter segments) have been identified. The high G+C content of the P34 gene (70%) agrees with that of the *M. paratuberculosis* genome (67%). P34 displayed a surprising hydrophilic profile: a strong hydrophobic portion corresponding to the NH₂ moiety of the molecule was followed by a highly hydrophilic part, the COOH moiety (80) (Fig. 7). The hydrophilic region is exposed at the outer cell surface, as shown by immune electron microscopy, while the hydrophobic portion is inserted in the envelope (62).

SEROLOGICAL ANALYSIS IN PARATUBERCULOSIS

In principle, proliferation of *M. paratuberculosis* in infected cattle induces the synthesis of specific immunoglobulins, serum

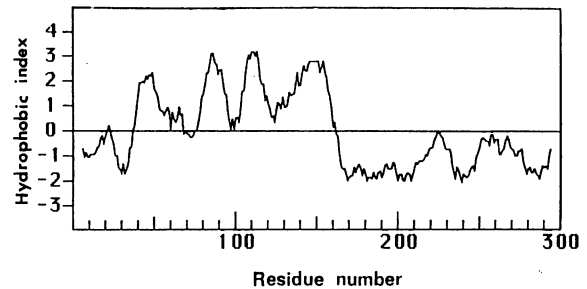


FIG. 7. Kyte-Doolittle hydrophobicity profile of the 34-kDa protein of *M. paratuberculosis*. This diagram, drawn with the DNA Strider computer program according to Kyte and Doolittle, identifies a strongly hydrophobic NH₂ moiety portion (amino acids 40 to 160) and a highly hydrophilic COOH terminal part (amino acids 161 to 298) exposed at the cell surface. Reprinted from reference 80 with permission of the publisher.

titer being proportional to the foreign antigen load. Thus, serological analysis would furnish both proof of infection (diagnosis) and information on disease evolution (prognosis). However, unless the antigen used for the test is specific for the chosen organism, diagnosis is impaired by cross-reactions with related bacteria of the CMN group. This is a serious obstacle because of the close genetic relatedness between *M. paratuberculosis* and other mycobacteria, particularly strains of the *M. avium* complex (30, 77, 135, 152, 165, 166). Moreover, the immunoglobulin titer is low in paratuberculous cattle at certain stages of the disease.

Complement fixation (CF) using crude mycobacterial preparations was widely used in the past. In spite of its lack of species specificity and poor sensitivity, CF is still the reference test for cattle export (51, 77, 136, 165, 227). The crude carbohydrate antigen used in the CF test in Canada differs from the antigen used in the United States (65, 223). A commercial CF assay (Parafix; Ifa-Mérieux, Lyon, France) is available in Europe. The agarose gel immunodiffusion (AGID) test has drawbacks similar to those of the CF test. The reagent of a commercial AGID immunoassay (Rapid John's Test; Immucell Corp., Portland, Maine) is whole-cell cytoplasm of *M. paratuberculosis* strain 18, recently identified as *M. avium* serotype 2. AGID is essentially used to confirm the diagnosis of paratuberculosis in an animal with suspected clinical symptoms, since this test becomes positive in cattle at advanced stages of the disease (178–181). An AGID test based on the D antigen is currently used in Canada (137). The performance of another immunoassay, the indirect immunofluorescence test, is comparable to that of CF (1, 74–76).

The sensitivity of the enzyme immunoassays is very high because of the amplification of the measured signal. In these tests, antimycobacterial immunoglobulins are captured by a selected antigen, and the antibody-antigen complex is revealed by a second antibody coupled to an enzyme that catalyzes a reaction which is measured spectrophotometrically. Some ELISAs incorporating crude mycobacterial extracts or partly purified antigens have been developed (2, 56, 108, 152, 229). To avoid the large number of false-positive results observed, a preabsorption step with *M. phlei* was introduced to remove some cross-reacting antibodies (8, 148, 149, 152, 230). Two such tests are commercially available: Allied ELISA (Allied Monitor Inc., Fayette, Mo.) and CSL-ELISA (John's absorbed EIA; Commonwealth Serum Laboratories, Melbourne, Australia [also distributed by Idexx Corp., Portland, Maine]). The former assay is based on a partly purified antigen prepa-

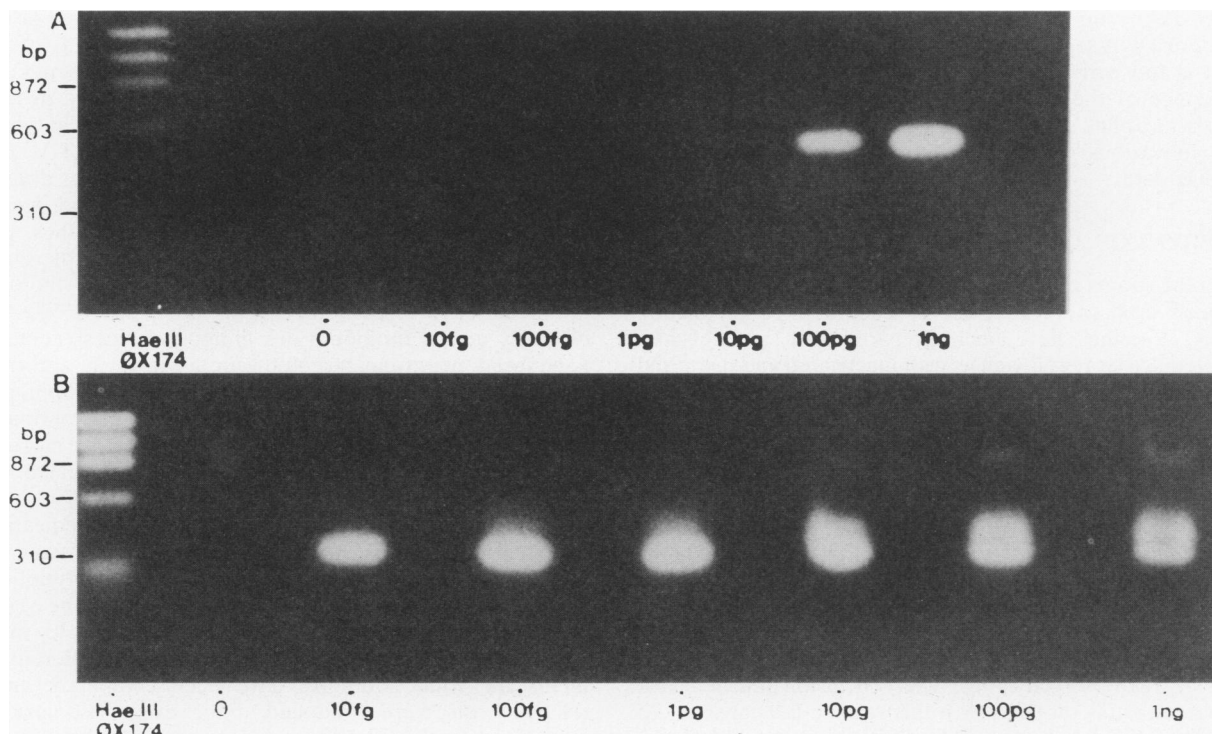


FIG. 8. Identification of amplified *M. paratuberculosis* DNA. Primary (A) and secondary nested (B) PCR-amplified *M. paratuberculosis* DNA samples, which were obtained with primers specific for the insertion sequence IS900, were electrophoresed and stained with ethidium bromide. The second amplification step leads to a 10^4 -fold increase in sensitivity. Reprinted from reference 133 with permission of the publisher.

ration of *M. paratuberculosis* strain 18, whereas the latter uses whole cytoplasm of strain VRI 316:102-2. Recently, a LAM-based assay (LAM-ELISA) has been submitted to field trials. This assay lacks species specificity due to cross-reactivity among LAM preparations from different mycobacterial and corynebacterial species (137, 138). The dot immunobinding assay uses whole cytoplasm or partly purified antigens of *M. paratuberculosis* ATCC 19698 as reagents and yields only qualitative results (infected or uninfected cattle) (210, 228).

The levels of sensitivity of the CF test (the standard CF test used in the United States), the AGID test (Rapid John's test), Allied ELISA, and CSL-ELISA were claimed to be 38.4, 26.6, 58.8, and 43.4%, respectively, for all subclinical cases of paratuberculosis (stages I and II) and: 14.5, 4.3, 47.8, and 24.6% for stage I and 54.6, 40.7, 65.7, and 56.5% for stage II of the disease. Specificity values were 99, 100, 95, and 99%, respectively (185, 186).

An ELISA based on the previously described A36 antigen was able to correctly detect infected cattle at all stages of the disease (61, 62) (Fig. 5). After identification of species-specific B-cell epitopes in the 34-kDa protein (P34) component of A36, the carboxy-terminal portion of this protein (a362), which contains only species-specific epitopes, was used as the reagent for a serological assay (62). The a362 ELISA was negative with tuberculous cattle and positive with paratuberculous cattle (Fig. 5) (62, 217).

NUCLEIC ACID PROBES

Preparation of a nucleic acid probe requires the identification of a species-specific DNA segment, which is then cloned and possibly sequenced. The probe, either labeled DNA or RNA complementary to the identified genomic segment, is

then hybridized with DNA released by lysis of unknown isolates. To circumvent the paucity of the infectious agent in biological samples, released DNA is amplified by PCR prior to probe hybridization. The PCR amplification of *M. paratuberculosis* DNA using IS900 sequences (134) is illustrated in Fig. 8. One hundred picograms of DNA could be detected after a first step of amplification, whereas a secondary nested amplification step led to a 10^4 -fold increase in sensitivity.

To date, only two species-specific DNA fragments have been identified in the *M. paratuberculosis* genome. Probes have been developed from them for the identification of *M. paratuberculosis* in stool samples (155, 157, 167, 218). One probe corresponds to a part of the IS900 insertion sequence, which is present in multiple copies in the *M. paratuberculosis* genome. Labeled IS900 probe was claimed to identify the DNA from 10^2 cells, after amplification by PCR. An IS900-based diagnostic kit, with nonisotopically labeled reagents, is now commercially available (Idexx Corp.). In studies performed in a paratuberculosis control program, this test had an 89% specificity but only a 13% sensitivity (214).

The other probe (F57) was prepared in our laboratory by cloning DNA segments from *M. paratuberculosis* in a transcription vector (167). Recombinant clone F57 proved to be specific for the John's disease agent. It failed to hybridize with the genomes of all mycobacteria tested, including *M. avium*, and it recognized all of the tested *M. paratuberculosis* strains isolated from paratuberculous animals and humans with Crohn's disease. Sequencing of the F57 insert yielded a 620-bp segment with a G+C content of 58.9%. Comparison with EMBL and UGEN banks revealed that the F57 sequence is unlike any other known gene, including IS900 (167). F57 is now being used as a diagnostic tool for both paratuberculous cattle and patients with Crohn's disease.

Other diagnostic probes with non-species-specific *M. paratuberculosis* DNA segments have also been described (101, 213, 214). It is too early to draw definitive conclusions as to the performance of these genetically engineered probes for the diagnosis of Johne's disease. Large clinical trials being conducted in various countries are expected to furnish more conclusive data.

PROPHYLAXIS AND THERAPY OF JOHNE'S DISEASE

Different strains of *M. paratuberculosis* and its components have been used to prevent the spread of infection among livestock. Vaccines have been prepared with virulent and attenuated live strains as well as with inactivated and disrupted organisms (40, 175).

The first vaccine for paratuberculosis was devised by Vallée and Rinjard in 1926. It consisted of a live virulent *M. paratuberculosis* strain suspended in liquid paraffin and olive oil to which pumice powder was added. This vaccine, which was inoculated subcutaneously, found wide application in France, apparently with success (140, 192). An attenuated strain of *M. paratuberculosis*, obtained after prolonged propagation in mycobactin-free medium, was used for vaccination in field trials conducted in France and England. When calves from infected herds were vaccinated within the first month of life, a protective effect was observed (192). The conclusion reached in a retrospective study on trials with this vaccine (all calves of 23 herds were vaccinated over prolonged periods) was that disease could be eradicated after an average period of 4 years (226). A commercial vaccine made of live attenuated *M. paratuberculosis* 316F suspended in an oil-based adjuvant (Neoprasec; Rhone-Mérieux) has been used extensively in France in field trials. After vaccination by subcutaneous injection, the animals produced long-lasting antimycobacterial antibodies and hence had positive reactions in the serological assays currently used for diagnostic purposes and export requirements (95).

The protective effects of vaccine preparations containing inactivated mycobacteria have been compared. A vaccine prepared with a virulent *M. paratuberculosis* strain afforded higher protection than that made with an attenuated strain (adapted to grow in mycobactin-free medium) (121, 123). The prophylactic practice in the United States is to restrict the injection of inactivated vaccines to calves less than 1 month old. Vaccinated cattle older than 1 year cannot be distinguished from infected cattle by means of the lymphocyte proliferation assay (96).

In vaccinated herds, there was a decrease in the number of both infected animals and those with advanced stages of the disease. A reduction in the percentage of shedders and in the quantity of excreted mycobacteria was also observed (40). However, the efficacy of vaccination should be viewed in light of environmental factors. Isolation of newborn animals immediately after birth and replacement of suckling with artificial nursing are probably as efficient prophylactic interventions as vaccination itself (122).

In some cases, vaccination produces cutaneous lesions (inflammation, swelling, and nodules) at the injection site (40). Moreover, inoculation of whole mycobacteria and crude extracts induces delayed hypersensitivity towards the sensitins from related species and positivity in serologic diagnostic tests (40, 95, 187). The use of purified proteins able to induce both cellular immune reactivity and protection from disease would be a more rational prophylactic approach. Ideally, different proteins should be used for vaccination and for diagnostic tests.

Treatment for paratuberculosis is rarely indicated or undertaken; however, it may be considered for animals of exceptional genetic value or companion animals (191). Drugs against *M. paratuberculosis* have been tested in vitro and in vivo. In short, most antimycobacterial agents, including antimetabolites and antibiotics, inhibit the in vitro growth of *M. paratuberculosis*. Antimetabolites include antituberculous drugs such as cycloserine, ethambutol, ethionamide, isoniazid, *para*-aminosalicylic acid, thiocarlide and thiosemicarbazones, phenazines, pyrazinamide, and antileprosy drugs such as dapsone and other sulfones. There are several reports on the use of antimetabolites in paratuberculosis (168, 169, 177). Among clinically active antibiotics are aminoglycosides (gentamicin, kanamycin, neomycin, streptomycin, tobramycin, and others), capreomycin, rifabutin, rifampin, and viomycin. Riminophenazine inhibited the development of the disease in experimentally infected mice and sheep (73). These antibiotics are more active on *M. tuberculosis* than on other pathogenic mycobacteria such as *M. leprae* and the *M. avium* complex (37, 38, 40).

Treatment of paratuberculosis requires daily medication for extended periods and results in palliation of the disease rather than a definitive cure (191). Clofazimine is the antibiotic most widely used for treatment of cattle (97, 144). In some cases, the addition of anti-inflammatory drugs appeared to improve symptomatology (141). In a recent work (97), different treatments for Johne's disease have been compared, and the relative costs were evaluated. Four drugs (isoniazid, clofazimine, rifampin, and gentamicin) were chosen as being both effective in vitro and in vivo and safe. It should be noted that none of the drugs used was approved for use in food animals in the United States and that these treatments did not eliminate infection, as shown by consistently positive fecal cultures and biopsies during therapeutic periods.

To conclude, in spite of the promising results of in vitro susceptibility tests, chemotherapy of paratuberculosis has been disappointing. The discrepancy between the in vitro and in vivo results is due to the inaccessibility of mycobacteria, which multiply within phagocytes and other cells of the intestinal mucosa and Peyer's patches. Antimycobacterial drugs penetrate such intracellular targets with difficulty.

CROHN'S DISEASE: RELATIONSHIP WITH PARATUBERCULOSIS

Although there had been earlier reports of chronic inflammatory bowel diseases, it was only in 1932 that B. Crohn provided the first description of the chronic granulomatous ileitis now known as Crohn's disease. Almost three decades later, a complete picture of the syndrome was established. It has been shown that every organ of the digestive tract may be involved and that, in some cases, there are alterations of the locomotor system and of the skin. It is still difficult to differentiate clinically Crohn's disease from such chronic gut infections as ulcerative colitis and intestinal tuberculosis (the inflammatory bowel diseases group). The basic clinical symptoms common to all of these disorders are enteralgia, diarrhea, and vomiting. Prolonged constipation may cause intestinal obstruction, and surgery is required to remove the obstructed ileum. Thus, the clinical symptoms of Crohn's disease mimic somewhat those of Johne's disease (34).

Histopathological analysis of biopsy material frequently shows diffuse granulomas and lymph node alterations resembling those of bovine paratuberculosis (176).

Acid-fast bacteria were rarely found in granulomas from Crohn's patients, and even the more sensitive immunohistochemical methods yielded negative results in most cases (110,

215). However, prolonged culture of biopsy specimens yielded three types of colonies: (i) acid-fast *M. paratuberculosis*; (ii) non-acid-fast, protoplast-like, wall-deficient organisms that eventually converted into acid-fast cells; and (iii) other mycobacterial species (26, 33, 34, 41, 81, 129, 203). More recently, PCR was used to amplify the DNA of organisms in biopsy material from patients with Crohn's disease: identification was made by probe hybridization. The probe was a portion of the *M. paratuberculosis* insertion sequence IS900 (85, 94, 132, 155, 157, 219). By this approach, *M. paratuberculosis* was identified in biopsy specimens from 65% of Crohn's disease patients compared with 4.3% of ulcerative colitis cases, and 12.5% of healthy persons (173).

The problem of etiology was also approached by exploring the humoral immune status of Crohn's disease patients. Serological assays based on antigenic preparations of lipids (111) and glycopeptidolipids (42) and on antigen complex A60 (133) yielded negative results. While in these studies immunoglobulin G (IgG) was essentially analyzed, other studies disclosed significantly higher titers of antimycobacterial IgA in Crohn's disease patients than in ulcerative colitis cases and healthy controls (68, 84, 189, 220). In a recent study with a species-specific ELISA (using the cited a362 recombinant polypeptide), significant differences in titers between sera from Crohn's disease patients and control sera were observed for both IgG and IgA. Higher titers of anti-a362 IgA than IgG were observed in 36% of Crohn's disease patients (216).

Chemotherapeutic approaches to Crohn's disease have been unsuccessful. Antimetabolites (dapsone, ethambutol, isoniazid, and sulfasalazine) and antibiotics (rifampin, streptomycin, and viomycin) proved to be almost ineffective. Some improvement was obtained only when chemotherapy was given in combination with certain anti-inflammatory steroidal drugs (cortisone, prednisolone, and methotrexate). The failure of the chemotherapeutic approach can be ascribed to the intracellular location of mycobacteria and to the peculiar features of the digestive apparatus (intestinal tuberculosis is far less susceptible to therapy than the pulmonary and pleural forms of the disease) (34).

CONCLUSIONS AND PERSPECTIVES

The etiological agent of Johne's disease is one of the lesser known pathogenic bacteria. The slow rate of growth and the complex requirements for axenic proliferation are undoubtedly at the origin of our limited knowledge of the topic.

Composition, properties, and the metabolism of different polymers (glycolipids, polysaccharides, and proteins) of *M. paratuberculosis* are to be further defined. Investigations of this kind are expected to identify components of medical value (specific antigens and immunomodulators) and to contribute to the development of numerical taxonomy.

Gene cloning and sequencing will presumably reveal similarities and differences of components from *M. paratuberculosis* and other mycobacteria. In turn, the purification of different antigens would promote the development of more specific and more sensitive assays for the diagnosis, prognosis, and prophylaxis of Johne's disease.

The development of new diagnostic tests (probes) and immunoassays (ELISA) will help to identify infected animals and thus prevent propagation of paratuberculosis. The IS900 probe provided by McFadden and coworkers (134) and the two specific tests developed in our laboratory, the F57 probe and the a362 ELISA, can be considered new effective diagnostic tools for experimental and clinical studies.

It is only through the convergence of different approaches

that the control of Johne's disease (which is perhaps the most diffused infectious disease of domestic animals) can be envisaged and its eradication successfully planned.

REFERENCES

1. Abbas, B., H. Riemann, and D. E. Behymer. 1983. Evaluation of the fluorescent antibody test for the diagnosis of paratuberculosis. *Am. J. Vet. Res.* **44**:720-721.
2. Abbas, B., H. P. Riemann, and B. Lonnerdal. 1983. Isolation of specific peptides from *Mycobacterium paratuberculosis* protoplasm and their use in an enzyme-linked immunosorbent assay for the detection of paratuberculosis (Johne's disease) in cattle. *Am. J. Vet. Res.* **44**:2229-2236.
3. Antoine, I., M. Coene, and C. Cocito. 1988. Size and homology of the genomes of leprosy-derived corynebacteria, *Mycobacterium leprae*, and other corynebacteria and mycobacteria. *J. Med. Microbiol.* **27**:45-50.
4. Athwal, R. S., S. S. Deo, and T. Imaeda. 1984. Deoxyribonucleic acid relatedness among *Mycobacterium leprae*, *M. lepraemurium*, and selected bacteria by dot blot and spectrophotometric deoxyribonucleic acid hybridization assays. *Int. J. Syst. Bacteriol.* **34**:371-375.
5. Barclay, R., D. F. Ewing, and C. Ratledge. 1985. Isolation, identification, and structural analysis of the mycobactins of *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, and *Mycobacterium paratuberculosis*. *J. Bacteriol.* **164**:896-903.
6. Barclay, R., and C. Ratledge. 1983. Iron-binding compounds of *Mycobacterium avium*, *M. intracellulare*, *M. scrofulaceum*, and mycobactin-dependent *M. paratuberculosis* and *M. avium*. *J. Bacteriol.* **153**:1138-1146.
7. Barksdale, L., and K. Kim. 1977. *Mycobacterium*. *Bacteriol. Rev.* **41**:217-332.
8. Bech-Nielsen, S., J. Berg Jorgensen, P. Ahrens, and N. C. Feld. 1992. Diagnostic accuracy of a *Mycobacterium phlei*-absorbed serum enzyme-linked immunosorbent assay for diagnosis of bovine paratuberculosis in dairy cows. *J. Clin. Microbiol.* **30**:613-618.
9. Bech-Nielsen, S., L. L. Burianek, E. Spangler, L. E. Heider, G. F. Hoffsis, and C. R. Dorn. 1985. Characterization of *Mycobacterium paratuberculosis* antigenic proteins. *Am. J. Vet. Res.* **46**:2418-2420.
10. Behymer, D., H. Riemann, W. Utterback, C. Elmi, and C. Franti. 1991. Mass screening of cattle sera against 14 infectious disease agents, using an ELISA system for monitoring health in livestock. *Am. J. Vet. Res.* **52**:1699-1704.
11. Bendixen, P. H. 1978. Immunological reactions caused by infection with *Mycobacterium paratuberculosis*. A review. *Nord. Vet. Med.* **30**:163-168.
12. Bendixen, P. H., B. Bloch, and J. Jorgensen. 1981. Lack of intracellular degradation of *Mycobacterium paratuberculosis* by bovine macrophages infected *in vitro* and *in vivo*: light microscopic and electron microscopic observations. *Am. J. Vet. Res.* **42**:109-113.
13. Benedictus, G., and J. Haagsma. 1986. The efficacy of mesenteric lymph node biopsy in the eradication of paratuberculosis from an infected dairy farm. *Vet. Q.* **8**:5-11.
14. Benoit, C., A. Beschin, M. Desmecht, P. Dekeyser, and C. Cocito. 1989. Delayed hypersensitivity reactions by mycobacterial antigen A60 and cutaneous testing in tuberculosis. *Med. Microbiol. Immunol.* **178**:105-112.
15. Bercovier, H., O. Kafri, and S. Sela. 1986. Mycobacteria possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. *Biochem. Biophys. Res. Commun.* **136**:1136-1141.
16. Beschin, A., L. Brijs, P. De Baetselier, and C. Cocito. 1991. Mycobacterial proliferation in macrophages is prevented by incubation with lymphocytes activated *in vitro* with a mycobacterial antigen complex. *Eur. J. Immunol.* **21**:793-797.
17. Billman, J., M. Carrigan, F. Cockram, L. Corner, I. Gill, J. Hill, T. Jessep, A. Milner, and P. Wood. 1992. A comparison of the interferon gamma assay with the absorbed ELISA for the diagnosis of Johne's disease in cattle. *Aust. Vet. J.* **69**:25-28.

18. Brennan, P. J. 1986. The carbohydrate-containing antigens of *Mycobacterium leprae*. *Lepr. Rev.* 57:39–51.
19. Brennan, P. J., M. Heifets, and B. P. Ullum. 1982. Thin-layer chromatography of lipid antigens as a means of identifying nontuberculous mycobacteria. *J. Clin. Microbiol.* 15:447–455.
20. Brooks, B., R. Robertson, A. Corner, B. Samagh, M. Garcia, C. Turcotte, and J. Duncan. 1988. Evaluation of the serological response of sheep in one flock to *Mycobacterium paratuberculosis* by crossed immunoelectrophoresis. *Can. J. Vet. Res.* 52:199–204.
21. Brooks, B., N. Young, D. Watson, R. Robertson, E. Sugden, K. Nielsen, and S. Becker. 1991. *Mycobacterium paratuberculosis* antigen D: characterization and evidence that it is a bacterioferitin. *J. Clin. Microbiol.* 29:1652–1658.
22. Brugère-Picoux, J. 1987. Le diagnostic de la paratuberculose chez les ruminants. *Rec. Med. Vet.* 163:539–546.
23. Bruneteau, M., J. Perret, F. Vanlinden, G. Michel, and C. Cocito. 1992. Composition and immunogenicity of the polysaccharide components of the thermostable antigen group of mycobacterial antigens. *Med. Microbiol. Immunol.* 181:13–23.
24. Buergelt, C. D., G. De Lisle, C. E. Hall, R. S. Merkal, and J. R. Duncan. 1978. *In vitro* lymphocyte transformation as a herd survey method for bovine paratuberculosis. *Am. J. Vet. Res.* 39:591–595.
25. Buergelt, C. D., C. Hall, K. McEntee, and J. R. Duncan. 1978. Pathological evaluation of paratuberculosis in naturally infected cattle. *Vet. Pathol.* 15:196–207.
26. Burnham, W. R., J. L. Stanford, and J. E. Lennard-Jones. 1977. Evidence for mycobacterial aetiology of Crohn's disease. *Gut* 18:965.
27. Butcher, P., J. J. McFadden, and J. Hermon-Taylor. 1988. Investigation of mycobacteria in Crohn's disease tissue by Southern blotting and DNA hybridisation with cloned mycobacterial genomic DNA probes from a Crohn's disease isolated mycobacteria. *Gut* 29:1122–1128.
28. Camphausen, R. T., R. L. Jones, and P. J. Brennan. 1985. A glycolipid antigen specific to *Mycobacterium paratuberculosis*: structure and antigenicity. *Proc. Natl. Acad. Sci. USA* 82:3068–3072.
29. Camphausen, R. T., R. L. Jones, and P. J. Brennan. 1986. Structure and relevance of the oligosaccharide hapten of *Mycobacterium avium* serotype 2. *J. Bacteriol.* 168:660–667.
30. Camphausen, R. T., R. L. Jones, and P. J. Brennan. 1988. Antigenic relationship between *Mycobacterium paratuberculosis* and *Mycobacterium avium*. *Am. J. Vet. Res.* 49:1307–1310.
31. Carlucci, S., A. Beschin, L. Tuosto, F. Ameglio, G. M. Gandolfo, C. Cocito, F. Fiorucci, C. Saltini, and E. Piccolella. 1993. Mycobacterial antigen complex A60-specific T-cell repertoire during the course of pulmonary tuberculosis. *Infect. Immun.* 61:439–447.
32. Chan, J., X. Fan, S. W. Hunter, P. J. Brennan, and B. R. Bloom. 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. *Infect. Immun.* 59:1755–1761.
33. Chiodini, R. J. 1986. Biochemical characteristics of various strains of *Mycobacterium paratuberculosis*. *Am. J. Vet. Res.* 47:1442–1445.
34. Chiodini, R. J. 1989. Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. *Clin. Microbiol. Rev.* 2:90–117.
35. Chiodini, R. J. 1989. The genetic relationship between *Mycobacterium paratuberculosis* and the *M. avium* complex. *Acta Leprol* 7(Suppl. 1):249–251.
36. Chiodini, R. J. 1990. Characterization of *Mycobacterium paratuberculosis* and organisms of the *Mycobacterium avium* complex by restriction polymorphism of the rRNA gene region. *J. Clin. Microbiol.* 28:489–494.
37. Chiodini, R. J. 1990. Bactericidal activities of various antimicrobial agents against human and animal isolates of *Mycobacterium paratuberculosis*. *Antimicrob. Agents Chemother.* 34:366–367.
38. Chiodini, R. J. 1991. Antimicrobial activity of rifabutin in combination with two and three other antimicrobial agents against strains of *Mycobacterium paratuberculosis*. *J. Antimicrob. Chemother.* 27:171–176.
39. Chiodini, R. J. 1993. The history of paratuberculosis (Johne's disease). A review of the literature 1895 to 1992, p. 1–658. The International Association for Paratuberculosis, Inc., Providence, R.I.
40. Chiodini, R. J., H. J. Van Kruiningen, and R. S. Merkal. 1984. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Vet.* 74:218–262.
41. Chiodini, R. J., H. J. Van Kruiningen, W. R. Thayer, and J. A. Couto. 1986. The spheroplastic phase of mycobacteria isolated from patients with Crohn's disease. *J. Clin. Microbiol.* 24:357–363.
42. Cho, S. N., P. J. Brennan, H. H. Yoshimura, B. I. Korelitz, and D. Y. Graham. 1986. Mycobacterial aetiology of Crohn's disease: serologic study using common mycobacterial antigens and a species-specific glycolipid antigen from *Mycobacterium paratuberculosis*. *Gut* 27:1353–1356.
43. Cho, S. N., D. L. Yanagihara, S. W. Hunter, R. H. Gether, and P. J. Brennan. 1983. Serological specificity of phenolic glycolipid I from *Mycobacterium leprae* and use in serodiagnosis of leprosy. *Infect. Immun.* 41:1077–1083.
44. Clark-Curtiss, J. E. 1990. Genome structure of mycobacteria, p. 77–96. In J. McFadden (ed.), *Molecular biology of the mycobacteria*. Surrey University Press, London.
45. Clark-Curtiss, J. E., W. R. Jacobs, M. A. Docherty, L. N. Ritchie, and R. Curtiss III. 1985. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae*. *J. Bacteriol.* 161:1093–1102.
46. Closs, O., M. Harboe, N. Axelsen, K. Bunch-Christensen, and M. Magnusson. 1980. The antigens of *Mycobacterium bovis* strain BCG, studied by crossed immunoelectrophoresis: a reference system. *Scand. J. Immunol.* 12:249–263.
47. Closs, O., R. N. Mshana, and M. Harboe. 1979. Antigenic analysis of *Mycobacterium leprae*. *Scand. J. Immunol.* 9:297–302.
48. Cocito, C. 1991. Properties of the mycobacterial antigen complex A60 and its application to the diagnosis and prognosis of tuberculosis. *Chest* 100:1687–1693.
49. Cocito, C., M.-C. Baelden, and C. Benoit. 1987. Immunological properties of antigen 60 of BCG. Induction of humoral and cellular immune reactions. *Scand. J. Immunol.* 25:579–585.
50. Coetsier, C., M.-C. Baelden, M. Coene, and C. Cocito. 1994. Immunological analysis of the components of the antigen complex A60 of *Mycobacterium bovis* BCG. *Clin. Diagn. Lab. Immunol.* 1:139–144.
51. Colgrove, G. S., C. O. Thoen, B. O. Blackburn, and C. D. Murphy. 1989. Paratuberculosis in cattle: a comparison of three serologic tests with results of fecal culture. *Vet. Microbiol.* 19:183–187.
52. Collins, D. M., D. M. Gabric, and G. W. De Lisle. 1989. Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. *FEMS Microbiol. Lett.* 60:175–178.
53. Collins, D. M., D. M. Gabric, and G. W. De Lisle. 1990. Identification of two groups of *Mycobacterium paratuberculosis* strains by restriction endonuclease analysis and DNA hybridization. *J. Clin. Microbiol.* 28:1591–1596.
54. Collins, M. T., N. Hoiby, J. B. Jorgensen, H. Bercovier, R. S. Lambrecht, and E. Jorgensen. 1991. Crossed immunoelectrophoretic analysis of *Mycobacterium paratuberculosis*. *Acta Pathol. Microbiol. Immunol. Scand.* 99:83–92.
55. Collins, M. T., K. B. Kenefick, D. C. Sockett, R. S. Lambrecht, J. McDonald, and J. B. Jorgensen. 1990. Enhanced radiometric detection of *Mycobacterium paratuberculosis* by using filter-concentrated bovine fecal specimens. *J. Clin. Microbiol.* 28:2514–2519.
56. Collins, M. T., D. C. Sockett, S. Ridge, and J. Cox. 1991. Evaluation of a commercial enzyme-linked immunosorbent assay for Johne's disease. *J. Clin. Microbiol.* 29:272–276.
57. Cox, R., K. Kempell, L. Fairclough, and M. J. Colston. 1991. The 16S ribosomal RNA of *Mycobacterium leprae* contains a unique sequence which can be used for identification by the polymerase chain reaction. *J. Med. Microbiol.* 35:284–290.
58. Damato, J. J., C. Knisley, and M. T. Collins. 1987. Characterization of *Mycobacterium paratuberculosis* by gas-liquid and thin-layer chromatography and rapid demonstration of mycobactin

- dependence using radiometric methods. *J. Clin. Microbiol.* **25**: 2380–2383.
59. Davies, D., L. Corbeil, D. Ward, and J. Duncan. 1974. A humoral suppressor of *in vitro* lymphocyte transformation responses in cattle with Johne's disease. *Proc. Soc. Exp. Biol. Med.* **145**:1372–1377.
 60. De Kesel, M., M. Coene, F. Portaels, and C. Cocito. 1987. Analysis of DNA from armadillo-derived mycobacteria. *Int. J. Syst. Bacteriol.* **37**:317–322.
 61. De Kesel, M., P. Gilot, M. Coene, and C. Cocito. 1992. Composition and immunological properties of the protein fraction of A36, a major antigen complex of *Mycobacterium paratuberculosis*. *Scand. J. Immunol.* **36**:201–212.
 62. De Kesel, M., P. Gilot, M.-C. Misonne, M. Coene, and C. Cocito. 1993. Cloning and expression of portions of the 34-kilodalton-protein gene of *Mycobacterium paratuberculosis*: its application to serological analysis of Johne's disease. *J. Clin. Microbiol.* **31**:947–954.
 63. De Lisle, G. W., and J. R. Duncan. 1981. Bovine paratuberculosis. III. An evaluation of a whole blood lymphocyte transformation test. *Can. J. Comp. Med.* **45**:304–309.
 64. De Lisle, G. W., B. S. Samagh, and J. R. Duncan. 1980. Bovine paratuberculosis. II. A comparison of fecal culture and the antibody response. *Can. J. Comp. Med.* **44**:183–191.
 65. De Lisle, G. W., P. Seguin, B. S. Samagh, A. H. Corner, and J. R. Duncan. 1980. Bovine paratuberculosis. I. A herd study using complement fixation and intradermal tests. *Can. J. Comp. Med.* **44**:177–182.
 66. Desmecht, M. 1980. Comparative cervical tuberculin testing in cattle. *Vlaams Diergeneesk. Tijdschr.* **49**:94–99.
 67. Ellner, J. J., and T. M. Daniel. 1979. Immunosuppression by mycobacterial arabinomannan. *Clin. Exp. Immunol.* **35**:250.
 68. Elsaghier, A., C. Prantera, C. Moreno, and J. Ivanyi. 1992. Antibodies to *Mycobacterium paratuberculosis*-specific protein antigens in Crohn's disease. *Clin. Exp. Immunol.* **90**:503–508.
 69. Elsken, L. A., and B. J. Nonnecke. 1986. *In vitro* transformation of lymphocytes from blood and milk of cows with subclinical paratuberculosis. *Am. J. Vet. Res.* **47**:1513–1516.
 70. England, P. M., and J. J. McFadden. 1991. IS 900-promoted stable integration of a foreign gene into mycobacteria. *Mol. Microbiol.* **5**:2047–2052.
 71. Frelier, P. F., J. W. Templeton, M. Estes, H. W. Whitford, and R. D. Kienle. 1990. Genetic regulation of *Mycobacterium paratuberculosis* infection in recombinant inbred mice. *Vet. Pathol.* **27**:362–364.
 72. Gevaudan, M. J., C. Bollet, D. Charpin, M. N. Mallet, and P. De Micco. 1992. Serological response of tuberculosis patients to antigen 60 of BCG. *Eur. J. Epidemiol.* **8**:666–676.
 73. Gilmour, N. 1966. Studies on the effect of the rimino phenazine B663 (G30320) on *Mycobacterium johnei*. *Br. Vet. J.* **122**:517–521.
 74. Gilmour, N. J. L., and K. W. Angus. 1976. The specificity and sensitivity of the fluorescent antibody test in cattle experimentally infected with *Mycobacterium avium* and *Mycobacterium johnei*. *Res. Vet. Sci.* **20**:6–9.
 75. Gilmour, N. J. L., and K. W. Angus. 1976. The specificity and sensitivity of the fluorescent antibody test for *Mycobacterium johnei* in abattoir and culled cattle. *Res. Vet. Sci.* **20**:10–12.
 76. Gilmour, N. J. L., and A. C. Gardiner. 1969. Fluorescent-antibody test in preclinical experimental *Mycobacterium johnei* infections in cattle. *J. Comp. Pathol.* **79**:71–77.
 77. Gilmour, N. J. L., and J. Goudswaard. 1972. *Corynebacterium renale* as a cause of reactions to the complement fixation test for Johne's disease. *J. Comp. Pathol.* **82**:333–336.
 78. Gilot, P., and C. Cocito. 1993. Comparative analysis of three sensitins used in cutaneous testing for tuberculosis and paratuberculosis in cattle. *FEMS Microbiol. Lett.* **110**:307–312.
 79. Gilot, P., M. De Kesel, M. Coene, and C. Cocito. 1992. Induction of cellular immune reactions by A36, an antigen complex of *Mycobacterium paratuberculosis*. Comparison of A36 and johnin components. *Scand. J. Immunol.* **36**:811–821.
 80. Gilot, P., M. De Kesel, L. Machtelinckx, M. Coene, and C. Cocito. 1993. Isolation and sequencing of the gene coding for an antigenic 34-kilodalton protein of *Mycobacterium paratuberculosis*. *J. Bacteriol.* **175**:4930–4935.
 81. Gitnick, G., J. Collins, B. Beaman, D. Brooks, M. Arthur, T. Imaeda, and M. Palieschesky. 1989. Preliminary report on isolation of mycobacteria from patients with Crohn's disease. *Dig. Dis. Sci.* **34**:925–932.
 82. Goodfellow, M., and L. G. Wayne. 1982. Taxonomy and nomenclature, p. 471–521. In C. Ratledge and J. Stanford (ed.), *The biology of the mycobacteria*. Academic Press, New York.
 83. Grange, J. M. 1988. Mycobacteria and human disease, p. 63–89. Edwards Arnold, London.
 84. Grange, J. M., J. Gibson, and E. Nassau. 1980. Enzyme-linked immunosorbent assay (ELISA). A study of antibodies to *Mycobacterium tuberculosis* in the IgG, IgA, and IgM classes in tuberculosis, sarcoidosis, and Crohn's disease. *Tubercle* **61**:145–152.
 85. Green, E. P., M. L. V. Tizard, M. T. Moss, J. Thompson, D. J. Winterbourne, J. J. McFadden, and J. Hermon-Taylor. 1989. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Res.* **17**:9063–9073.
 86. Griffin, J. F. T. 1988. The aetiology of tuberculosis and mycobacterial diseases in farmed deer. *Ir. Vet. J.* **42**:23–26.
 87. Gunnarsson, E., and F. Fodstad. 1979. Analysis of antigens in *Mycobacterium paratuberculosis*. *Acta Vet. Scand.* **20**:200–215.
 88. Hamilton, H. L., A. J. Cooley, J. L. Adams, and C. J. Czuprynski. 1991. *Mycobacterium paratuberculosis* monoassociated nude mice as a paratuberculosis model. *Vet. Pathol.* **28**:146–155.
 89. Hamilton, H. L., D. M. Follett, L. M. Siegfried, and C. J. Czuprynski. 1989. Intestinal multiplication of *Mycobacterium paratuberculosis* in athymic nude gnotobiotic mice. *Infect. Immun.* **57**:225–230.
 90. Hampson, S. J., J. Thompson, M. T. Moss, F. Portaels, E. P. Green, J. Hermon-Taylor, and J. J. McFadden. 1989. DNA probes demonstrate a single highly conserved strain of *Mycobacterium avium* infecting AIDS patients. *Lancet* **i**:65–68.
 91. Hance, A. J., B. Grandchamp, V. Lévy-Frébault, D. Lecossier, J. Rauzier, D. Bocart, and B. Gicquel. 1989. Detection and identification of mycobacteria by amplification of mycobacterial DNA. *Mol. Microbiol.* **3**:843–849.
 92. Harboe, M. 1981. Antigens of PPD, old tuberculin, and auto-claved *Mycobacterium bovis* BCG studied by crossed immunoelectrophoresis. *Am. Rev. Respir. Dis.* **124**:80–87.
 93. Hastings, R. C., T. P. Gillis, J. L. Krahenbueh, and S. G. Franzblau. 1988. Leprosy. *Clin. Microbiol. Rev.* **1**:330–348.
 94. Hermon-Taylor, J., M. Moss, M. Tizard, Z. Malik, and J. Sanderson. 1990. Molecular biology of Crohn's disease mycobacteria. *Clin. Gastroenterol.* **4**:23–42.
 95. Hilbink, F., and D. West. 1990. The antibody response of sheep to vaccination against Johne's disease. *N. Z. Vet. J.* **38**:168–169.
 96. Hintz, A. M. 1981. Comparative lymphocyte stimulation studies on whole blood from vaccinated and nonvaccinated cattle with paratuberculosis. *Am. J. Vet. Res.* **42**:507–510.
 97. Hoffsis, G. F., R. N. Streeter, D. M. Rings, and G. St. Jean. 1990. Therapy for Johne's disease. *Bovine Pract.* **25**:55–58.
 98. Hottat, F., M. Coene, and C. Cocito. 1988. DNA methylation in leprosy-associated bacteria: *Mycobacterium leprae* and *Corynebacterium tuberculostrictum*. *Med. Microbiol. Immunol.* **177**:33–45.
 99. Hunter, S. W., T. Fujiwara, and P. J. Brennan. 1982. Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. *J. Biol. Chem.* **257**:15072–15078.
 100. Hurley, S. S., G. A. Splitter, and R. A. Welch. 1988. Deoxyribonucleic acid relatedness of *Mycobacterium paratuberculosis* to other members of the family *Mycobacteriaceae*. *Int. J. Syst. Bacteriol.* **38**:143–146.
 101. Hurley, S. S., G. A. Splitter, and R. A. Welch. 1989. Development of a diagnostic test for Johne's disease using a DNA hybridization probe. *J. Clin. Microbiol.* **27**:1582–1587.
 102. Imaeda, T. 1985. Deoxyribonucleic acid relatedness among selected strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*. *Int. J. Syst. Microbiol.* **35**:147–150.
 103. Imaeda, T., G. Broslawski, and S. Imaeda. 1988. Genomic relatedness among mycobacterial species by nonisotopic blot

- hybridization. *Int. J. Syst. Bacteriol.* **38**:151-156.
104. Imaeda, T., W. F. Kirchheimer, and L. Barksdale. 1982. DNA isolated from *Mycobacterium leprae*: genome size, base ratio, and homology with other related bacteria as determined by optical DNA-DNA reassociation. *J. Bacteriol.* **150**:414-417.
 105. Jenkins, P. A. 1981. Lipid analysis in the identification of mycobacteria—an appraisal. *Rev. Infect. Dis.* **3**:862-866.
 106. Jenkins, P. A., S. R. Pattyn, and F. Portaels. 1982. Diagnostic bacteriology, p. 441-470. In C. Ratledge and J. Stanford (ed.), *The biology of the mycobacteria*, vol. 1. Academic Press, Inc., New York.
 107. Jones, R. L. 1989. Review of the economic impact of Johne's disease in the United States, p. 46-50. In A. R. Milner and P. R. Wood (ed.), *Johne's disease. Current trends in research, diagnosis and management*. Commonwealth Scientific and Industrial Research Organisation, Melbourne, Australia.
 108. Jorgensen, J. B., and P. T. Jensen. 1978. Enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to *Mycobacterium paratuberculosis* in cattle. *Acta Vet. Scand.* **19**:310-312.
 109. Kishima, M., Y. Yokomizo, I. Nonomura, and N. Goto. 1991. Suppressive effect of nonviable *Mycobacterium paratuberculosis* on the delayed-type hypersensitivity reaction to sheep erythrocytes in mice. *Lab. Anim.* **25**:310-318.
 110. Kobayashi, K., M. J. Blaser, and W. R. Brown. 1989. Immunohistochemical examination for mycobacteria in intestinal tissues from patients with Crohn's disease. *Gastroenterology* **96**:1009-1015.
 111. Kobayashi, K., W. R. Brown, P. J. Brennan, and M. J. Blaser. 1988. Serum antibodies to mycobacterial antigens in active Crohn's disease. *Gastroenterology* **94**:1404-1411.
 112. Körmendy, B. 1988. Diagnostic value of mammalian, avian and johnin PPD tuberculin in cattle herds infected by *Mycobacterium paratuberculosis*. *Acta Vet. Hung.* **36**:177-183.
 113. Körmendy, B. 1990. Paratuberculosis in a cattle herd: comparison of allergic, serologic and faecal microscopic tests. *Acta Microbiol. Hung.* **37**:219-222.
 114. Krawiec, S., and M. Riley. 1990. Organization of the bacterial chromosome. *Microbiol. Rev.* **54**:502-539.
 115. Kreeger, J. 1991. Ruminant paratuberculosis—a century of progress and frustration. *J. Vet. Diagn. Invest.* **3**:373-382.
 116. Kreeger, J., T. Snider, and B. Olcott. 1987. Rectal biopsy as an adjunct to the diagnosis of clinical paratuberculosis, p. 61. *Proc. 38th Annu. Meet. Am. Coll. Vet. Pathol.*
 117. Kreeger, J., T. Snider, and B. Olcott. 1991. Spontaneous murine thymocyte mitogenic activity consistent with interleukin-1 in cattle naturally infected with *Mycobacterium paratuberculosis*. *Vet. Immunol. Immunopathol.* **28**:317-326.
 118. Krishnappa, G., C. Jagannath, and B. Rao. 1989. The specificity of antibody response in experimental and natural bovine paratuberculosis studied by crossed immunoelectrophoresis with intermediate gel. *Vet. Microbiol.* **21**:67-78.
 119. Kunze, Z. M., F. Portaels, and J. J. McFadden. 1992. Biologically distinct subtypes of *Mycobacterium avium* differ in possession of insertion sequence IS901. *J. Clin. Microbiol.* **30**:2366-2372.
 120. Kunze, Z. M., S. Wall, R. Appelberg, M. T. Silva, F. Portaels, and J. J. McFadden. 1991. IS 901, a new member of a wide-spread class of atypical insertion sequences, is associated with pathogenicity in *Mycobacterium avium*. *Mol. Microbiol.* **5**:2265-2272.
 121. Larsen, A. B., and R. S. Merkal. 1979. Effectiveness of killed virulent cell vaccine against paratuberculosis, p. 480-484. *Proc. U.S. Anim. Health Assoc.* 1979.
 122. Larsen, A. B., R. S. Merkal, K. E. Kopecky, and H. W. Moon. 1969. Hypersensitivity and serologic responses in cattle vaccinated with disrupted *Mycobacterium paratuberculosis* cells and subsequently infected with *Mycobacterium bovis*. *Am. J. Vet. Res.* **30**:2167-2172.
 123. Larsen, A. B., A. I. Moyle, and E. M. Himes. 1978. Experimental vaccination of cattle against paratuberculosis (Johne's disease) with killed bacterial vaccines: a control field study. *Am. J. Vet. Res.* **39**:65-69.
 124. Larsen, A. B., T. H. Vardaman, and R. S. Merkal. 1963. An extended study of a herd of cattle naturally infected with Johne's disease. I. The significance of the intradermic johnin test. *Am. J. Vet. Res.* **24**:91-93.
 125. Lepper, A. W. D., C. R. Wilks, M. Kotiw, J. T. Whitehead, and K. S. Swart. 1989. Sequential bacteriological observations in relation to cell-mediated and humoral antibody responses of cattle infected with *Mycobacterium paratuberculosis* and maintained on normal or high iron intake. *Aust. Vet. J.* **66**:50-55.
 126. Levis, W. R., H. C. Mecker, G. B. Schuller-Levis, T. P. Gillis, L. J. Mario, Jr., and J. Zabriskie. 1986. Serodiagnosis of leprosy: relationships between antibodies to *Mycobacterium leprae* phenolic glycolipid I and protein antigens. *J. Clin. Microbiol.* **24**:917-921.
 127. Lindquist, S., and E. A. Craig. 1988. The heat-shock proteins. *Annu. Rev. Genet.* **22**:631-677.
 128. Matthews, P., and A. McDiarmid. 1979. The production in bovine calves of a disease resembling paratuberculosis with a *Mycobacterium sp.* isolated from a woodpigeon (*Columba palumbus* L.). *Vet. Rec.* **104**:286.
 129. McFadden, J. J., P. D. Butcher, R. J. Chiodini, and J. Hermon-Taylor. 1987. Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. *J. Clin. Microbiol.* **25**:796-801.
 130. McFadden, J. J., P. D. Butcher, R. J. Chiodini, and J. Hermon-Taylor. 1987. Determination of genome size and DNA homology between an unclassified *Mycobacterium* species isolated from patients with Crohn's disease and other mycobacteria. *J. Gen. Microbiol.* **133**:211-214.
 131. McFadden, J. J., P. D. Butcher, J. Thompson, R. J. Chiodini, and J. Hermon-Taylor. 1987. The use of DNA probes identifying restriction-fragment-length polymorphisms to examine the *Mycobacterium avium* complex. *Mol. Microbiol.* **1**:283-291.
 132. McFadden, J. J., J. Collins, B. Beaman, M. Arthur, and G. Gitnick. 1992. Mycobacteria in Crohn's disease: DNA probes identify the wood pigeon strain of *Mycobacterium avium* and *Mycobacterium paratuberculosis* from human tissue. *J. Clin. Microbiol.* **30**:3070-3073.
 133. McFadden, J. J., and C. Houdayer. 1988. No evidence for antibodies to mycobacterial A60 antigen in Crohn's disease sera by enzyme-linked immunoabsorbent assay (ELISA). *J. Med. Microbiol.* **25**:295-298.
 134. McFadden, J. J., Z. Kunze, and P. Seechurn. 1990. DNA probes for detection and identification, p. 139-172. In J. McFadden (ed.), *Molecular biology of the mycobacteria*. Surrey University Press, London.
 135. McIntyre, G., and J. L. Stanford. 1986. Immunodiffusion analysis shows that *Mycobacterium paratuberculosis* and other mycobactin-dependent mycobacteria are variants of *Mycobacterium avium*. *J. Appl. Bacteriol.* **61**:295-298.
 136. McKenzie, R. A., and W. H. Ward. 1981. *Rhodococcus (Corynebacterium) equi*: a possible cause of reactions to the complement fixation test for Johne's disease of cattle. *Aust. Vet. J.* **57**:200-201.
 137. McNab, W. B., A. H. Meek, J. R. Duncan, B. W. Brooks, A. A. Van Dreumel, S. W. Martin, K. H. Nielsen, E. A. Sugden, and C. Turcotte. 1991. An evaluation of selected screening tests for bovine paratuberculosis. *Can. J. Vet. Res.* **55**:252-259.
 138. McNab, W. B., A. H. Meek, J. R. Duncan, S. W. Martin, and A. Van Dreumel. 1991. An epidemiological study of paratuberculosis in dairy cattle in Ontario: study design and prevalence estimates. *Can. J. Vet. Res.* **55**:246-251.
 139. McNeil, M. R., and P. J. Brennan. 1991. Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibility arising from recent structural information. *Res. Microbiol.* **142**:451-463.
 140. Mehra, V., P. Brennan, E. Rada, J. Convit, and B. R. Bloom. 1984. Lymphocyte suppression in leprosy induced by unique *M. leprae* glycolipid. *Nature (London)* **308**:194-196.
 141. Merkal, R. S. 1973. Paratuberculosis, p. 1237-1249. In G. P. Kubica and L. G. Wayne (ed.), *The mycobacteria*. M. Dekker, New York.
 142. Merkal, R. S., and B. Curran. 1974. Growth and metabolic characteristics of *Mycobacterium paratuberculosis*. *Appl. Microbiol.* **28**:276-279.

143. Merkal, R. S., K. Kopecky, A. Larsen, and R. Ness. 1970. Immunologic mechanisms in bovine paratuberculosis. *Am. J. Vet. Res.* **31**:475-485.
144. Merkal, R. S., and A. B. Larsen. 1973. Clofazimine treatment of cows naturally infected with *Mycobacterium paratuberculosis*. *Am. J. Vet. Res.* **34**:27-28.
145. Merkal, R. S., W. G. McCullough, and K. Takayama. 1981. Mycobactins, the state of the art. *Bull. Inst. Pasteur* **79**:251-259.
146. Merkal, R. S., D. L. Whipple, J. M. Sacks, and G. R. Snyder. 1987. Prevalence of *Mycobacterium paratuberculosis* in ileocecal lymph nodes of cattle culled in the United States. *J. Am. Vet. Med. Assoc.* **190**:676-680.
147. Miller, R. A., S. Dissanayake, and T. M. Buchanan. 1983. Development of an enzyme-linked immunosorbent assay using arabinomannan from *Mycobacterium smegmatis*: a potential useful screening test for the diagnosis of incubating leprosy. *Am. J. Trop. Med. Hyg.* **32**:554-564.
148. Milner, A. R., A. W. Lepper, W. N. Symonds, and E. Gruner. 1987. Analysis by ELISA and Western blotting of antibody reactivities in cattle infected with *Mycobacterium paratuberculosis* after absorption of serum with *M. phlei*. *Res. Vet. Sci.* **42**:140-144.
149. Milner, A. R., W. N. Mack, K. J. Coates, J. Hill, I. Gill, and P. Sheldrick. 1990. The sensitivity and specificity of a modified ELISA for the diagnosis of Johne's disease from a field trial in cattle. *Vet. Microbiol.* **25**:193-198.
150. Minnikin, D. E., and M. Goodfellow. 1980. Lipid composition in the classification of acid-fast bacteria, p. 189-256. In M. Goodfellow and R. G. Board (ed.), *Microbiological classification and identification*. Academic Press, London.
151. Mokresh, A. H., C. J. Czuprynski, and D. G. Butler. 1989. A rabbit model for study of *Mycobacterium paratuberculosis* infection. *Infect. Immun.* **57**:3798-3807.
152. Molina, A., L. Morera, and D. Llanes. 1991. Enzyme-linked immunosorbent assay for detection of antibodies against *Mycobacterium paratuberculosis* in goats. *Am. J. Vet. Res.* **52**:863-868.
153. Momotani, E., D. Whipple, A. Thiermann, and N. Cheville. 1988. Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. *Vet. Pathol.* **25**:131-137.
154. Moreno, C., A. Melhert, and J. Lamb. 1988. The inhibitory effects of lipoarabinomannan and polysaccharides upon polyclonal and monoclonal human T cell proliferation. *Clin. Exp. Immunol.* **74**:206-210.
155. Moss, M. T., E. P. Green, M. L. Tizard, Z. P. Malik, and J. Hermon-Taylor. 1991. Specific detection of *Mycobacterium paratuberculosis* by DNA hybridisation with a fragment of the insertion element IS900. *Gut* **32**:395-398.
156. Moss, M. T., Z. P. Malik, M. L. V. Tizard, E. P. Green, J. D. Sanderson, and J. Hermon-Taylor. 1992. IS 902, an insertion element of the chronic-enteritis-causing *Mycobacterium avium* subsp. *silvaticum*. *J. Gen. Microbiol.* **138**:139-145.
157. Moss, M. T., J. D. Sanderson, M. L. V. Tizard, J. Hermon-Taylor, F. A. K. El-Zaatari, D. C. Markesich, and D. Y. Graham. 1992. Polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum* in long term cultures from Crohn's disease and control tissues. *Gut* **33**:1209-1213.
158. Murray, A., N. Winter, M. Lagranderie, D. F. Hill, J. Rauzier, J. Timm, C. Leclerc, K. M. Moriarty, M. Gheorghiu, and B. Gicquel. 1992. Expression of *Escherichia coli* β -galactosidase in *Mycobacterium bovis* BCG using an expression system isolated from *Mycobacterium paratuberculosis* which induced humoral and cellular immune responses. *Mol. Microbiol.* **6**:3331-3342.
159. Paliwal, O. P., R. Kumar, and R. Somvanshi. 1985. The immune spectrum of *Mycobacterium johnei* infections in goats. *Indian Vet. J.* **69**:743-747.
160. Pandey, G. S., T. L. Musonda, H. G. B. Chizyuka, and M. Schneebeli. 1987. Paratuberculosis (Johne's disease) in a herd of Friesian cattle in Zambia. *Vet. Rec.* **120**:369.
161. Patterson, D. S., W. M. Allen, and M. K. Lloyd. 1967. Clinical Johne's disease as a protein losing enteropathy. *Vet. Rec.* **81**:717-718.
162. Patterson, D. S., and S. Berrett. 1968. Malabsorption in Johne's disease of cattle: depressed *in vitro* amino-acid uptake by isolated intestinal tissue preparations. *Vet. Rec.* **83**:55-56.
163. Patterson, D. S., and S. Berrett. 1969. Malabsorption in Johne's disease in cattle: an *in vitro* study of L-histidine uptake by isolated intestinal tissue preparations. *J. Med. Microbiol.* **2**:327-334.
164. Pemberton, D. H. 1979. Diagnosis of Johne's disease in cattle using mesenteric lymph node biopsy: accuracy in clinical suspects. *Aust. Vet. J.* **55**:217-219.
165. Pepin, M., J. Marly, and P. Pardon. 1987. *Corynebacterium pseudotuberculosis* infection in sheep and the complement fixation test for paratuberculosis. *Vet. Rec.* **120**:236.
166. Pier, A., J. Thurston, and A. Larsen. 1968. A diagnostic antigen for nocardiosis: comparative tests in cattle with nocardiosis and mycobacteriosis. *Am. J. Vet. Res.* **29**:397-403.
167. Poupart, P., M. Coene, H. Van Heuverswyn, and C. Cocito. 1993. Preparation of a specific RNA probe for detection of *Mycobacterium paratuberculosis* and diagnosis of Johne's disease. *J. Clin. Microbiol.* **31**:1601-1605.
168. Rankin, J. D. 1953. Isoniazid: its effects on *Mycobacterium johnei* *in vitro* and its failure to cure clinical Johne's disease in cattle. *Vet. Rec.* **65**:649-651.
169. Rankin, J. D. 1955. An attempt to prevent the establishment of *Mycobacterium johnei* in calves by means of isoniazid alone and in combination with streptomycin. *Vet. Rec.* **67**:1105-1107.
170. Ridge, S. E. 1993. Cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples using elements of the Roche MB Check system. *J. Clin. Microbiol.* **31**:400-405.
171. Ris, D. R., K. L. Hamel, and J. M. Ayling. 1988. The detection of *Mycobacterium paratuberculosis* in bovine faeces by isolation and the comparison of isolation with the examination of stained smears by light microscopy. *N. Z. Vet. J.* **36**:112-114.
172. Rogall, T., J. Wolters, T. Flohr, and E. C. Böttger. 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. *Int. J. Syst. Bacteriol.* **40**:323-330.
173. Sanderson, J. D., M. T. Moss, M. L. Tizard, and J. Hermon-Taylor. 1992. *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. *Gut* **33**:890-896.
174. Saxegaard, F., and I. Baess. 1988. Relationship between *Mycobacterium avium*, *Mycobacterium paratuberculosis* and wood-pigeon mycobacteria: determinations by DNA-DNA hybridization. *Acta Pathol. Microbiol. Immunol. Scand.* **96**:37-42.
175. Saxegaard, F., and F. H. Fodstad. 1985. Control of paratuberculosis (Johne's disease) in goats by vaccination. *Vet. Rec.* **116**:439-441.
176. Schmitz-Moormann, P., and P. M. Pittner. 1984. The granuloma in Crohn's disease. A bioptical study. *Pathol. Res. Pract.* **178**:467-476.
177. Shaffer, J. L., S. Hughes, B. D. Linaker, R. D. Baker, and L. A. Turnberg. 1984. Controlled trial of rifampicin and ethambutol in Crohn's disease. *Gut* **25**:203-205.
178. Sherman, D. M., B. Bray, J. M. Gay, and F. Bates. 1989. Evaluation of the agar gel immunodiffusion test for diagnosis of subclinical paratuberculosis in cattle. *Am. J. Vet. Res.* **50**:525-530.
179. Sherman, D. M., J. M. Gay, D. S. Bouley, and G. H. Nelson. 1990. Comparison of the complement-fixation and agar gel immunodiffusion tests for diagnosis of subclinical bovine paratuberculosis. *Am. J. Vet. Res.* **51**:461-465.
180. Sherman, D. M., and H. M. Gezon. 1980. Comparison of agar gel immunodiffusion and fecal culture for identification of goats with paratuberculosis. *J. Am. Vet. Med. Assoc.* **177**:1208-1211.
181. Sherman, D. M., R. J. F. Markham, and F. Bates. 1984. Agar gel immunodiffusion test for diagnosis of clinical paratuberculosis in cattle. *J. Am. Vet. Med. Assoc.* **185**:179-182.
182. Sibley, L. D., S. W. Hunter, P. J. Brennan, and J. L. Krahenbuhl. 1988. Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. *Infect. Immun.* **55**:680-685.
183. Sindic, C. J. M., D. Boucquoy, M. P. Van Antwerpen, M. C. Baelden, C. Laterre, and C. Cocito. 1990. Intrathecal synthesis of antimycobacterial antibodies in patients with tuberculous meningitis. An immunoblotting study. *J. Neurol. Neurosurg. Psychiatry* **53**:662-666.
184. Smida, J., J. Kazda, and E. Stackebrandt. 1988. Molecular-

- genetic evidence for the relationship of *Mycobacterium leprae* to slow-growing pathogenic mycobacteria. *Int. J. Lepr.* **56**:449–454.
185. Sockett, D. C., D. J. Carr, W. D. Richards, and M. T. Collins. 1992. A repository of specimens for comparison of diagnostic testing procedures for bovine paratuberculosis. *J. Vet. Diagn. Invest.* **4**:188–191.
 186. Sockett, D. C., T. Conrad, C. Thomas, and M. T. Collins. 1992. Evaluation of four serological tests for bovine paratuberculosis. *J. Clin. Microbiol.* **30**:1134–1139.
 187. Spangler, E., L. E. Heider, S. Bech-Nielsen, and C. R. Dorn. 1991. Serologic enzyme-linked immunosorbent assay responses of calves vaccinated with a killed *Mycobacterium paratuberculosis* vaccine. *Am. J. Vet. Res.* **52**:1197–1200.
 188. Stahl, D. A., and J. W. Urbance. 1990. The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria. *J. Bacteriol.* **172**:116–124.
 189. Stevens, T. R. J., V. R. Winrow, D. R. Blake, and D. S. Rampton. 1992. Circulating antibodies to heat-shock protein 60 in Crohn's disease and ulcerative colitis. *Clin. Exp. Immunol.* **90**:271–274.
 190. Stevenson, K., N. F. Inglis, B. Rae, W. Donachie, and J. M. Sharp. 1991. Complete nucleotide sequence of a gene encoding the 70 kd heat shock protein of *Mycobacterium paratuberculosis*. *Nucleic Acids Res.* **19**:4552.
 191. St. Jean, G., and A. D. Jernigan. 1991. Treatment of *Mycobacterium paratuberculosis* infection in ruminants. *Vet. Clin. North Am. Small Anim. Pract.* **7**:793–804.
 192. Stuart, P. Vaccination against Johne's disease in cattle exposed to experimental infection. 1965. *Br. Vet. J.* **121**:289–318.
 193. Sugden, E., B. Brooks, N. Young, D. Watson, K. Nielsen, A. Corner, C. Turcotte, A. Michaelides, and R. Stewart. 1991. Chromatographic purification and characterization of antigens A and D from *Mycobacterium paratuberculosis* and their use in enzyme-linked immunosorbent assays for diagnosis of paratuberculosis in sheep. *J. Clin. Microbiol.* **29**:1659–1664.
 194. Sugden, E., A. Corner, B. Samagh, B. Brooks, C. Turcotte, K. Nielsen, R. Stewart, and J. Duncan. 1989. Serodiagnosis of ovine paratuberculosis, using lipoarabinomannan in an enzyme-linked immunosorbent assays. *Am. J. Vet. Res.* **50**:850–854.
 195. Sugden, E., B. Samagh, D. Bundle, and J. Duncan. 1987. Lipoarabinomannan and lipid-free arabinomannan antigens of *Mycobacterium paratuberculosis*. *Infect. Immun.* **55**:762–770.
 196. Sweeney, R. W., R. H. Whitlock, and A. E. Rosenberg. 1992. *Mycobacterium paratuberculosis* isolated from fetuses of infected cows not manifesting signs of the disease. *Am. J. Vet. Res.* **53**:477–480.
 197. Taylor, T. K., C. R. Wilks, and D. S. McQueen. 1981. Isolation of *Mycobacterium paratuberculosis* from milk of a cow with Johne's disease. *Vet. Rec.* **109**:532–533.
 198. Thoen, C. O., and K. H. Baum. 1988. Current knowledge on paratuberculosis. *J. Am. Vet. Med. Assoc.* **192**:1609–1611.
 199. Thoen, C. O., A. G. Karlson, and R. D. Ellefson. 1971. Comparison by gas-liquid chromatography of the fatty acids of *Mycobacterium avium* and some other nonphotochromogenic mycobacteria. *Appl. Microbiol.* **22**:560–563.
 200. Thole, J. E. R., W. J. Keulen, A. H. J. Kolk, D. G. Groothuis, L. G. Berwald, R. H. Tiesjema, and J. D. A. Van Embden. 1987. Characterization, sequence determination, and immunogenicity of a 64-kilodalton protein of *Mycobacterium bovis* BCG expressed in *Escherichia coli* K-12. *Infect. Immun.* **55**:1466–1475.
 201. Thomas, T. J., R. E. Andrews, Jr., and C. O. Thoen. 1992. Molecular cloning and characterization of *Mycobacterium paratuberculosis* promoters in *Escherichia coli*. *Vet. Microbiol.* **32**:351–362.
 202. Thorel, M. F. 1984. Review of the occurrence of mycobactin dependence among mycobacteria species. *Ann. Rech. Vet.* **15**:405–409.
 203. Thorel, M. F. 1989. Relationship between *Mycobacterium avium*, *M. paratuberculosis* and mycobacteria associated with Crohn's disease. *Ann. Rech. Vet.* **20**:417–429.
 204. Thorel, M. F., and P. Desmettre. 1982. Comparative study of mycobactin-dependent strains of mycobacteria isolated from the wood pigeon with *Mycobacterium avium* and *M. paratuberculosis*: study of biological and antigenic characteristics. *Ann. Microbiol.* **133B**:291–302.
 205. Thorel, M. F., M. Krichevsky, and V. Lévy-Frébault. 1990. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *Int. J. Syst. Bacteriol.* **40**:254–260.
 206. Thorel, M. F., and V. Lévy-Frébault. 1988. Classification et identification de *Mycobacterium paratuberculosis* et des mycobactéries apparentées mycobactin-dépendantes, p. 283–292. In M. F. Thorel and R. S. Merkal (ed.), *Proceedings of the Second International Colloquium on Paratuberculosis*. Maisons-Alfort, Paris, France.
 207. Thorel, M. F., J. Marly, D. Trap, P. Pardon, and P. Lechopier. 1985. Paratuberculose expérimentale: diagnostic biologique chez des veaux inoculés avec des souches de mycobactéries mycobactine-dépendantes. *Ann. Rech. Vet.* **16**:7–16.
 208. Thorel, M. F., and L. Valette. 1976. Etude de quelques souches de *Mycobacterium paratuberculosis*: caractères biochimiques et activité allergique. *Annu. Rev. Vet.* **7**:207–213.
 209. Tizard, M. L. V., M. T. Moss, J. D. Sanderson, B. M. Austen, and J. Hermon-Taylor. 1992. p43, the protein product of the atypical insertion sequence IS 900, is expressed in *Mycobacterium paratuberculosis*. *J. Gen. Microbiol.* **138**:1729–1736.
 210. Tsai, S. J., L. J. Hutchinson, and A. Zarkower. 1989. Comparison of dot immunobinding assay, enzyme-linked immunosorbent assay and immunodiffusion for serodiagnosis of paratuberculosis. *Can. J. Vet. Res.* **53**:405–410.
 211. Turcotte, R., and S. Lemieux. 1982. Mechanism of action of *Mycobacterium bovis* BCG-induced suppressor cells in mitogen-induced blastogenes. *Infect. Immun.* **36**:263–270.
 212. Valentin-Weigand, P., C. Murray, and K. Moriarty. 1991. Antibody reactivities of *Mycobacterium paratuberculosis* infected sheep as analyzed by enzyme-linked immunosorbent assay and Western blotting. *FEMS Microbiol. Lett.* **78**:145–148.
 213. Van der Giessen, J. W. B., A. Eger, J. Haagsma, R. M. Haring, W. Gastra, and B. A. M. van der Zeijst. 1992. Amplification of 16S rRNA sequences to detect *Mycobacterium paratuberculosis*. *J. Med. Microbiol.* **36**:255–263.
 214. Van der Giessen, J. W. B., R. M. Haring, E. Vaulclare, A. Eger, J. Haagsma, and B. A. M. van der Zeijst. 1992. Evaluation of the abilities of three diagnostic tests based on the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in cattle: application in a control program. *J. Clin. Microbiol.* **30**:1216–1219.
 215. Van Kruiningen, H. J., W. R. Tahayer, R. J. Chiodini, D. J. Meuton, and J. A. Coutu. 1988. An immunoperoxidase search for mycobacteria in Crohn's disease, p. 547–552. In R. P. MacDermott (ed.), *Inflammatory bowel disease. Current status and future approach*. Elsevier Science Publishing, Inc., Amsterdam.
 216. Vannuffel, P., C. Dieterich, B. Naerhuyzen, P. Gilot, M. Coene, R. Fiasse, and C. Cocito. 1994. Occurrence, in Crohn's disease, of antibodies directed against a species-specific recombinant polypeptide of *Mycobacterium paratuberculosis*. *Clin. Diagn. Lab. Immunol.* **1**:241–243.
 217. Vannuffel, P., P. Gilot, B. Limbourg, B. Naerhuyzen, C. Dieterich, M. Coene, and C. Cocito. 1994. Development of species-specific enzyme-linked immunosorbent assay for diagnosis of Johne's disease in cattle. *J. Clin. Microbiol.* **32**:1211–1216.
 218. Vary, P. H., P. R. Andersen, E. Green, J. Hermon-Taylor, and J. J. McFadden. 1990. Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *J. Clin. Microbiol.* **28**:933–937.
 219. Wall, S., Z. M. Kunze, S. Saboor, I. Soufferi, P. Seechurn, R. Chiodini, and J. J. McFadden. 1993. Identification of spheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction. *J. Clin. Microbiol.* **31**:1241–1245.
 220. Wayne, L. G., D. Hollander, B. Anderson, H. A. Sramek, C. M. Vadheim, and J. I. Rotter. 1992. Immunoglobulin A (IgA) and IgG serum antibodies to mycobacterial antigens in Crohn's disease patients and their relatives. *J. Clin. Microbiol.* **30**:2013–2018.

221. Whipple, D., P. Kapke, and C. Vary. 1990. Identification of restriction fragment length polymorphisms in DNA from *Mycobacterium paratuberculosis*. *J. Clin. Microbiol.* **28**:2561-2564.
222. Whitlock, R. Personal communication.
223. Whitlock, R. 1991. Laboratory diagnosis of Johne's disease, p. 1-11. In R. Chiodini and J. K. Kreeger (ed.), *Proceedings of the Third International Colloquium on Paratuberculosis*, Orlando, Florida.
224. Wiker, G., and M. Harboe. 1992. The antigen 85-complex: a major secretion product of *Mycobacterium tuberculosis*. *Microbiol. Rev.* **56**:648-661.
225. Wiker, H., K. Sletten, S. Nagai, and M. Harboe. 1990. Evidence for three separate genes encoding the proteins of the mycobacterial antigen 85 complex. *Infect. Immun.* **58**:272-274.
226. Wilesmith, J. W. 1982. Johne's disease: a retrospective study of vaccinated herds in Great Britain. *Br. Vet. J.* **138**:321-331.
227. Wilks, C. R., T. K. Taylor, E. G. Russell, and T. R. Thomas. 1981. Isolation of mycobacteria inducing cross-reactions in the complement fixation test for Johne's disease. *Res. Vet. Sci.* **30**:323-327.
228. Woodruff, T. S., W. P. Shulaw, S. Bech-Nielsen, G. F. Hoffsis, E. Spangler, and L. E. Heider. 1991. Serodiagnosis of bovine paratuberculosis by use of a dot enzyme-linked immunosorbent assay. *Am. J. Vet. Res.* **52**:217-221.
229. Yokomizo, Y., R. S. Merkal, and P. A. S. Lyle. 1983. Enzyme-linked immunosorbent assay for detection of bovine immunoglobulin G1 antibody to a protoplasmic antigen of *Mycobacterium paratuberculosis*. *Am. J. Vet. Res.* **44**:2205-2207.
230. Yokomizo, Y., H. Yugi, and R. S. Merkal. 1985. A method for avoiding false-positive reactions in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis. *Jpn. J. Vet. Sci.* **47**:111-119.
231. Yoshimura, H. H., and D. Y. Graham. 1988. Nucleic acid hybridization studies of mycobactin-dependent mycobacteria. *J. Clin. Microbiol.* **26**:1309-1312.
232. Young, D. B., S. H. E. Kaufmann, P. W. M. Hermans, and J. E. R. Thole. 1992. Mycobacterial protein antigens: a compilation. *Mol. Microbiol.* **6**:133-145.
233. Zurbrick, B., and C. Czuprynski. 1987. Ingestion and intracellular growth of *Mycobacterium paratuberculosis* within bovine blood monocytes and monocyte-derived macrophages. *Infect. Immun.* **55**:1588-1593.
234. Zurbrick, B., D. Follett, and C. Czuprynski. 1988. Cytokine regulation of the intracellular growth of *Mycobacterium paratuberculosis* in bovine monocytes. *Infect. Immun.* **56**:1692-1697.