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July 2001

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CLINICAL MICROBIOLOGY REVIEWS, July 2001, p. 489–512 Vol. 14, No. 3
0893-8512/01/$04.00+0 DOI: 10.1128/CMR.14.3.489–512.2001
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Mycobacterium avium subsp. *paratuberculosis* in Veterinary Medicine

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INTRODUCTION

Bacteria of the genus *Mycobacterium* are gram-positive, acid-fast organisms that include a number of significant human and animal pathogens. *Mycobacterium avium* subsp. *paratuberculosis* (basonym *M. paratuberculosis*) is the etiological agent of a severe gastroenteritis in ruminants, known as Johne's disease. H. A. Johne and L. Frothingham initially reported the disease in Germany in 1894. However, it was not until 1910 that F. W. Trowt successfully fulfilled Koch's postulates by growing *M. paratuberculosis* in the laboratory and reproducing the disease in experimentally infected cattle (46, 148).

Johne's disease is prevalent in domestic animals worldwide and has significant impact on the global economy (290). Its influence in the United States alone is staggering, causing an estimated loss of $1.5 billion to the agriculture industry every year (279). It is considered to be one of the most serious diseases affecting dairy cattle (200). However, accurately assessing losses in productivity and profit at the level of an individual herd is difficult, making it likely that the impact of this disease is underestimated nationwide (165, 223). Nonetheless, a Johne's disease regression model estimates this loss to be from $40 to $227 per cow inventoried per year, based on the percentage of culled cows with clinical signs (219).

Isolation of *M. paratuberculosis* from intestinal tissue of Crohn's disease patients has led to concern that it may be pathogenic for humans (204). This issue is still controversial, with several reports documenting either the presence or absence of this bacterium in Crohn's disease patients (36, 43, 58, 110, 167, 257). Given the difficulty that investigators have had in isolating a putative infectious agent for this disease from human tissues and the lack of suitable animal models, it is not surprising that Koch's postulates have not been fulfilled for Crohn's disease. Thus, a causal relationship between *M. paratuberculosis* and Crohn's disease has not been demonstrated. Similarly, chemotherapy with antimycobacterial agents has given mixed results in Crohn's disease patients (see references 61 and 151 for reviews). Finally, it has been hypothesized that

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only a subset of Crohn's disease cases have an infectious etiology (53). The reader is referred to a recent review by Hermon-Taylor et al. (151) for a thoughtful analysis of the involvement of *M. paratuberculosis* in Crohn's disease.

Another aspect of this controversy has surfaced recently with a report that in milk pasteurization trials, a clinical strain of *M. paratuberculosis* was more thermally tolerant than either *Mycobacterium bovis* or *Coxiella burnetii*, the current milk pasteurization standard microorganism (285). While some reports have indicated that high-temperature short-time pasteurization does not effectively kill *M. paratuberculosis* in milk (135, 136), others have demonstrated killing by turbulent-flow conditions (277). Thus, the pathogenic role of *M. paratuberculosis* in human disease and the identification of potential sources of infection are topics of intense debate.

Neonatal and juvenile animals are at the highest risk for acquiring an infection of *M. paratuberculosis* (56, 61). Young animals are most commonly infected through the fecal-oral route. This occurs either by ingesting the organism through contaminated milk or food products or by accidental ingestion of the microorganism from contaminated surfaces (61). *M. paratuberculosis*, similar to other pathogenic mycobacteria, targets the mucosa-associated lymphoid tissues of the host (189). It preferentially targets the mucosa-associated lymphoid tissues of the upper gastrointestinal tract, where it is endocytosed by the M cells of the ileal Peyer's patches and subsequently phagocytosed by subepithelial and intraepithelial macrophages (121, 189, 208). *M. paratuberculosis* bacilli probably remain in the phagosome, where they multiply intracellularly (168). Cytokine production and the initiation of a cellular immune response by the host causes the appearance of an intestinal granuloma, and a cellular response is initiated in the nearby lymph nodes in an attempt to clear the infection (56, 61, 189). This inflammatory process leads to the clinical manifestations of a corrugated intestinal epithelium and the corresponding characteristic malnutrition syndrome associated with Johne's disease.

CHARACTERISTICS OF *M. PARATUBERCULOSIS*

Taxonomic and Phylogenetic Analysis

The mycobacterial species *M. avium* is currently subdivided into three subspecies, *M. avium* subsp. *avium (M. avium), M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum (M. silvaticum)*. The subspecies designation of *M. paratuberculosis* is based on DNA-DNA hybridization studies (159, 259, 298, 328) and numerical taxonomy analysis (298). Although commonly grouped with *M. avium* in the *Mycobacterium avium-intracellulare* complex, *M. intracellulare* is a genetically distinct species (7, 256). At the subspecies level, *M. paratuberculosis* can be differentiated phenotypically from *M. avium* and *M. silvaticum* by its dependence on mycobactin (298) and genotypically by the presence of multiple copies of an insertion element, IS*900* (67, 137).

Analysis of the rRNA genes (rDNA) of mycobacteria has resulted in the division of this genus into two separate clusters. These correspond to the traditional fast-growing mycobacteria, represented by nonpathogenic environmental isolates, and the slow-growing mycobacteria, containing most of the overt pathogens (251, 280, 311). The rDNA gene copy number also reflects this division between fast- and slow-growing mycobacteria; fast-growing mycobacteria contain two sets of rRNA genes, whereas the slow growers, including *M. paratuberculosis* and *M. avium*, contain only one copy (19, 47, 48, 286). Like other mycobacteria, the rDNA genes are found in a single operon in *M. paratuberculosis* (120, 185, 186, 286). Interestingly, as in *Streptomyces* spp. (237, 287), this operon does not contain a tRNA gene, a feature found in the *Mycobacterium smegmatis rrnB* ribosomal operon (131).

An internal transcribed spacer region of approximately 280 bp separates the 16S and 23S rDNA genes, and a smaller internal transcribed spacer of 91 bp separates the 23S rDNA and 5S rDNA genes (186). Both *M. paratuberculosis* and *M. avium* have an unusual insertion of approximately 16 nucleotides in the 23S rRNA gene, which is also found in the 23S rRNA genes of other mycobacteria (186, 303). This region appears to be variable among mycobacteria. Sequence comparison shows a single mismatch between *M. paratuberculosis* and *M. avium*, whereas this sequence contains an additional 4 bp in *M. phlei* (303).

The *dnaJ* gene encodes a highly conserved heat shock protein and has been used in other bacterial genera for phylogenetic mapping (123, 329). This gene was sequenced from 19 mycobacterial species, and the phylogenetic relationship inferred from this data was in close agreement with the traditional classification based on 16S rRNA sequencing for slowly growing mycobacteria. As expected, the *M. avium* and *M. paratuberculosis dnaJ* genes were 99% homologous, substantiating the previously established close relationship between these two mycobacterial species (292).

The size of the *M. paratuberculosis* genome has been estimated to be 4.4 to 4.7 Mb (61). Compared to other mycobacteria, this is similar to the *M. tuberculosis* genome of 4.41 Mb (65) and the *M. bovis* genome of 4.4 Mb (Sanger Center; http://www.sanger.ac.uk) but slightly larger than the estimated size of the genome of *M. leprae* (3.3 Mb; sequencing recently completed) (Sanger Center). However, the *M. paratuberculosis* genome sequence is nearly complete and appears to be larger than expected at approximately 5 Mb (University of Minnesota; http://www.cbc.umn.edu/ResearchProjects/Ptb/) similar to that of the *M. avium* genome (The Institute for Genomic Research; sequencing recently completed http://www.tigr.org). *M. paratuberculosis* DNA has a base composition of 66 to 67% G+C, similar to *M. tuberculosis* and *M. bovis* (65 and 64%, respectively) (Sanger Center).

Extrachromosomal plasmid DNA was first reported in *Mycobacterium fortuitum* (178). This 5.0-kb plasmid was designated pAL5000 and has been used extensively for the construction of other recombinant plasmids in the molecular genetic studies of many other mycobacterial species. Naturally occurring plasmids have also been observed in clinical and environmental isolates of *M. avium, M. intracellulare*, and *M. scrofulaceum* (106). However, no endogenous plasmid DNA has yet been reported in any isolates of *M. paratuberculosis*.

Major Antigens

The search for *M. paratuberculosis*-specific antigens for diagnostic testing or preventive therapy has led to the discovery of several immunoreactive proteins (Table 1). Many of these

TABLE 1. Known immunogenic proteins of *M. paratuberculosis*

M. paratuberculosis protein	Characteristic	Size (kDa)	Reference(s)
GroES	Heat shock protein	10	60
AhpD	Alkyl hydroperoxide reductase D	19	222
32-kDa antigen	Fibronectin binding properties, secreted protein	32	100
34-kDa antigen	Cell wall antigen, B-cell epitope	34	62, 87, 88, 127, 264
34-kDa antigen	Serine protease	34	33
34.5-kDa antigen	Cytoplasmic protein, *M. paratuberculosis* species specific	34.5	214
35-kDa antigen	Immunodominant protein	35	102
42-kDa antigen	Cytoplasmic *M. paratuberculosis*-specific protein	42	320
44.3-kDa antigen	Soluble protein	44.3	214
AhpC	Alkyl hydroperoxide reductase C	45	222
65-kDa antigen	GroEL heat shock protein	65	100, 101

proteins have homology to other mycobacterial antigens, such as the GroES and GroEL proteins. The GroES antigens are highly antigenic, small (ca. 100-amino-acid), highly conserved heat shock proteins. Recently, the GroES antigens of *M. avium* and *M. paratuberculosis* have been cloned and sequenced (60). Not surprisingly, the *M. avium* and *M. paratuberculosis* GroES coding sequences and deduced amino acid sequences are 100% identical to each other and are over 90% identical to the *M. tuberculosis* and *M. leprae* sequences, differing by only 3 amino acids. The homologous GroES protein from *M. tuberculosis* has two T-cell epitopes, which are both conserved in *M. avium* and *M. paratuberculosis* (60).

Other immunoreactive proteins of *M. paratuberculosis* include a 32-kDa secreted protein with fibronectin binding properties implicated in protective immunity (4, 100) and a 34-kDa cell wall antigenic protein homologous to a similar protein in *M. leprae* (87, 88, 127, 264). This 34-kDa protein carries two species-specific B-cell epitopes that have been exploited in the histopathological diagnosis of Johne's disease (62). Cameron et al. (33) described a seroreactive 34-kDa serine protease expressed in vivo by *M. paratuberculosis*. This antigen is different from the 34-kDa antigen described above. Another strongly immunoreactive protein of 35 kDa has also been identified in *M. avium* complex isolates, including *M. paratuberculosis* (102). Sequencing data for this antigen are not available, and its relation to any of the above antigens has not been established. Nonetheless, it reacted with sera from cattle in both the subclinical and clinical stages of Johne's disease but was nonreactive with sera from cattle vaccinated with *M. bovis* BCG (102).

A more thoroughly characterized protein of 65 kDa from *M. paratuberculosis* is a member of the GroEL family of heat shock proteins (100, 101). Like the GroES proteins, the GroEL antigens from other mycobacteria are highly immunogenic (260, 296, 297). As expected, the *M. paratuberculosis* GroEL protein is homologous to similar proteins of *M. tuberculosis* (93%), *M. leprae* (89%), and *M. avium* (98%). Adsorbed antisera from infected animals failed to consistently recognize this antigen, however, precluding its use in the serodiagnosis of Johne's disease. Similarly, a DNA vaccine constructed from the *M. avium* 65-kDa antigen fused to the green fluorescent protein failed to confer protective immunity to vaccinated mice (308).

The alkyl hydroperoxide reductases C and D (AhpC and AhpD) are the most recently characterized immunogenic proteins of *M. paratuberculosis* (222). Unlike other mycobacteria, large amounts of these antigens are produced by *M. paratuberculosis* when the bacilli are grown without exposure to oxidative stress. AhpC is the larger of the two proteins and appears to exist as a homodimer in its native form since it migrates at both 45 and 24 kDa under denaturing conditions. In contrast, AhpD is a smaller monomer, with a molecular mass of about 19 kDa. Antiserum from rabbits immunized against AhpC and AhpD reacted only with *M. paratuberculosis* proteins and not with proteins from other mycobacterial species, indicating that antibodies against these proteins are not cross-reactive. Furthermore, peripheral blood monocytes from goats experimentally infected with *M. paratuberculosis* were capable of inducing gamma interferon (IFN-γ) responses after stimulation with AhpC and AhpD, confirming their immunogenicity (222). In conclusion, these proteins are potentially useful for developing future vaccines and diagnostic assays or monitoring disease progression.

Several putative *M. paratuberculosis*-specific antigenic proteins have been described in the literature (Table 1). These include a cellular antigen of 34.5 kDa, (214), a 42-kDa protein of unknown function (320), and a 44.3-kDa antigen (214). Unfortunately, the above-mentioned 34.5-kDa protein has not been compared to either the 34-kDa serine protease described by Cameron et al. (33) or the 35-kDa protein described by El-Zaatari et al. (102). Similarly, a comparative analysis of the 42- and 44.3-kDa antigens has not been reported.

Transcriptional Control

Mycobacteria, like other prokaryotes, have a plethora of DNA-dependent RNA polymerase sigma factors (130). The *M. tuberculosis* genome project annotates 14 genes encoding either confirmed or putative sigma factors (65). Sigma factors provide the core RNA polymerase with various recognition specificities, allowing the transcription of regular housekeeping, stress, and virulence-associated genes (193, 267). Interestingly, a point mutation in the principal sigma factor, *sigA*, was demonstrated to attenuate virulent *M. bovis* (73). In general, mycobacterial promoters function poorly in *Escherichia coli* and have a greater G+C content, reflecting their genome composition. A rather highly conserved −10 region and an extended TGN motif (12, 13) characterize an important set of mycobacterial promoters, while other promoters seem to possess a more highly conserved −35 region (212).

In the sole analysis of *M. paratuberculosis* promoters, Bannantine et al. (9) studied eight promoter regions of *M. paratuberculosis* and found significant differences from those of *E. coli*. In this study, gene fusions to the β-galactosidase reporter gene were analyzed in both *E. coli* and *M. smegmatis*. As expected, these fusions displayed lower or negligible levels of β-galactosidase activity in *E. coli* than in *M. smegmatis*. Primer extension and sequence analysis of upstream regions demonstrated −35 and −10 consensus sequences of TGMCGT (where M represents C or A) and GGGCCS (where S represents G or C), respectively. These *M. paratuberculosis* promoters are more closely related to each other than they are to other mycobacterial promoters. Interestingly, the −35 hexamer is more highly conserved than the −10 hexamer while the latter has a consensus that departs significantly from the proposed extended TGN motif mentioned above. The only other *M. paratuberculosis* promoter described is the P_{AN} promoter (213), which differs from the consensus described by Bannantine et al. (9) and also lacks a TGN motif. It is possible that *M. paratuberculosis*, one of the slowest-growing mycobacteria, may possess promoters that depart significantly from those of other mycobacteria. However, Bannantine et al. (9) may have identified a unique set of promoters that do not use the TGN motif. In this context, the various studies described above used different vectors, reporter genes, and selection or screening strategies that may have led to the identification of different types of promoters. More studies are necessary to ascertain whether the main *M. paratuberculosis* sigma factor uses promoters with TGN motifs or the special consensus identified by Bannantine et al. (9). In general, mycobacterial translation signals show a significant number of GTG start codons and a characteristic GC codon bias (212). Regarding *M. paratuberculosis* ribosome binding sites (RBS), all the corresponding mRNAs from the *lacZ* fusions analyzed by Bannantine et al. (9) possess a putative RBS complementary to the 3′ sequence of the 16S rRNA from *M. leprae*. In this study, a putative initiation codon and an open reading frame (ORF) followed the RBS sequences. The start codons were either ATG (5 of 8) or GTG.

Homologous Recombination

Allelic replacement is based on recombination between homologous sequences and is useful to generate mutations in a gene of interest. In *M. tuberculosis*, allelic exchange has presented major difficulties, which only recently have been overcome. Initially, the small number of homologous recombination events obtained in *M. tuberculosis* was attributed to a defect in the homologous recombination system, possibly related to the presence of an intein in the *recA* gene (86). Recent work demonstrated that homologous recombination seems to be functional in *M. tuberculosis*, but problems occur due to the high frequency of illegitimate recombination observed in that pathogen (118, 227). To circumvent these problems, a series of vectors with thermosensitive replicons (11, 233), sucrose selection (231–233), long concatomeric molecules (8), and pretreatment of DNA with restricition endonucleases (154, 224, 225) have been developed. These approaches have been successful in the more closely related mycobacterial species *M. intracellulare* (154, 195), suggesting that *M. paratuberculosis* may be amenable to them as well. Additionally, a study of the IS*900* insertion sites in *M. paratuberculosis* indicated that homologous recombination events may have occurred between IS*900* insertion elements present at different loci (29).

MOLECULAR EPIDEMIOLOGY

Restriction Fragment Length Polymorphism Analysis

Because *M. paratuberculosis* and *M. avium* are closely related genetically, they are difficult to differentiate. The application of molecular genetic methods in epidemiological studies allowed separation of clinical isolates of *M. avium* and *M. paratuberculosis* (64, 198, 309, 317). These analyses were instrumental in reassigning an isolate previously identified as *M. paratuberculosis* strain 18 as an *M. avium* strain (67, 105, 315, 316).

Restriction fragment length polymorphism (RFLP) analysis also has been used epidemiologically to ascertain a possible common clonal origin for *M. paratuberculosis* strains isolated from unrelated animal and human sources (54, 66, 89, 115, 228, 315). This technique can also separate sheep and cattle field isolates of *M. paratuberculosis* (15, 69), suggesting that *M. paratuberculosis* strains may be undergoing host adaptation. Interestingly, *M. paratuberculosis* isolates from other ruminants give similar RFLP profiles to cattle isolates (299). A single case study documented the isolation of two different strains of *M. paratuberculosis*, each with a unique RFLP profile, from the same animal, implying the possibility of a coinfection (228). An effort to standardize RFLP analysis for clinical isolates of *M. paratuberculosis* combined previously reported methods (69, 228) and resulted in 28 different RFLP types from 1,008 strains tested. These 28 RFLP types were derived from combining 13 RFLP (*Pst*I) types and 20 RFLP (*Bst*EII) types (229). Recently, a multiplex PCR based on specific IS*900* loci in the *M. paratuberculosis* genome was cross-referenced to these RFLP types (29), indicating that future typing of *M. paratuberculosis* isolates may not require bacterial culture, making large-scale epidemiological studies of Johne's disease possible.

IS*900*

Insertion sequence elements are small, mobile genetic elements containing genes related to transposition functions. IS*900* was the first example of an insertion sequence element to be found in mycobacteria (68, 137); it is a member of the IS*110* family of insertion sequences (191). An interesting aspect of the insertion sequences belonging to this family is their lack of both terminal inverted repeats and direct repeats commonly associated with other transposable DNA elements (191). Four insertion sequences in this family are from mycobacteria, IS*900* (*M. paratuberculosis*) (137), IS*901* (*M. avium*) (177), IS*902* (*M. silvaticum*) (210) , and IS*1110* (*M. avium)* (152). Two new insertion elements, IS*1547*, recently discovered in *M. tuberculosis* (107), and IS*1626*, from *M. avium* and *M. intracellulare* (244), are also related to the IS*110* family. All of these insertion elements, except for IS*1626*, which has been found only in 1 to 3 copies, are found in their respective genomes at 10 to 20 copies per chromosome (68, 107, 137, 177, 210).

IS*1626* is the most closely related insertion sequence to IS*900* (Table 2). These two insertion elements have 82% homology, with the greatest variability occurring at the 5′ end of

TABLE 2. Comparison of the putative mycobacterial insertion element transposase genes belonging to the IS*110* family

Insertion sequence	Mycobacterial species	Accession no.	Nucleotide sequence length (bp) of:		% Sequence similarity to IS*900* (no. of nucleotides)[c]	Sequence length of transposase (amino acids)	% Sequence similarity to IS*900* (no. of amino acids)[d]
			Insertion sequence[a]	Transposase[b]			
IS*900*	*M. paratuberculosis*	X16293	1,451	1,200		399	
IS*901*	*M. avium*	X59272	1,472	1,206	73% (232)	401	51% (212)
IS*902*	*M. silvaticum*	X58030	1,470	1,203	73% (233)	400	52% (213)
IS*1110*	*M. avium*	Z23003	1,462	1,395	74% (113)	464	51% (189)
IS*1547*	*M. tuberculosis*	Y13470	1,351	~1,182	NS[e]	~380[f]	31% (118)
IS*1626*	*M. avium*	AF071067	1,418	1,179[g]	82% (859)	393	66% (272)

[a] Nucleotide sequence length of the entire insertion element (107, 191).
[b] Nucleotide sequence length of the structural gene encoding the putative transposase for each insertion element.
[c] Nucleotide sequence similarities were determined using BLAST version BLASTN 2.0.9 (National Center for Biotechnology Information databases).
[d] Amino acid sequence similarities were determined using BLAST version BLASTP 2.0.9 (National Center for Biotechnology Information databases).
[e] NS, no significant similarity.
[f] Nucleotide and amino acid sequence lengths of putative transposase are variable, since the stop codon is not within the IS*147* sequence (107).
[g] The function of ORF1179 is unknown (244).

their nucleotide sequences. IS*1626* has a mechanism of transposition and consensus sequence remarkably similar to IS*900*. Furthermore, like IS*900*, IS*1626* inserts itself with a defined orientation with respect to its consensus sequence (244).

In contrast, the IS*900* and IS*901* transposase genes have a DNA homology of only 60% and an amino acid homology of 49% (177). Similarly, the homology between the IS*900* and IS*902* transposase genes is 60% at the nucleotide level and 50% at the amino acid level (210). The transposase from IS*900* appears to be quite distinct from the other mycobacterial insertion elements belonging to the IS*110* family, since only short regions of homology are found at the 3′ end of both the DNA and amino acid sequences.

The IS*900* transposase gene is functional, since an artificial transposon derived from IS*900* could stably integrate into the chromosomes of *M. smegmatis* (91, 103), *M. vaccae*, and *M. bovis* BCG (91). Furthermore, the method of transposition appears to involve both simple insertions and cointegrates, suggesting that IS*900* transposes by a replicative mechanism (91). IS*900* contains two ORFs encoded on opposite strands. The transposase protein, p43, is encoded by ORF1 and can be expressed in vitro (300). This protein is approximately 43 kDa but migrates at an apparent molecular mass of 44.5 to 45 kDa, perhaps due to its high basic character (predicted pI 10.4), which may retard its migration during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (300). Western blot analysis shows that antibodies against this protein react with both a 45-kDa protein and an apparent cleavage product of approximately 28 kDa. This antibody reacts with only the 28-kDa protein from clinical strains of *M. paratuberculosis*. Neither *M. avium* nor *M. silvaticum* extracts demonstrated positively staining bands at these molecular masses confirming that p43 is specific to IS*900*-bearing mycobacteria.

The P_{AN} promoter and an adjacent ORF called ORF2 were discovered by a search for *M. paratuberculosis* subspecies-specific genes and expression signals. The P_{AN} promoter is located adjacent to but outside of IS*900*. Its proximity to ORF2 suggested that P_{AN} is able to drive the expression of ORF2 (213). The P_{AN} promoter is present in a single copy in many pathogenic strains of mycobacteria but is absent from *M. marinum, M. terrae, M. gordonae*, and several fast-growing mycobacteria (133). The P_{AN} region of *M. paratuberculosis* has 70% similarity to *M. tuberculosis, M. bovis* and *M. bovis* BCG regions, and the −10 and −35 promoter regions are completely conserved (247). Although it is tempting to speculate that the corresponding genes driven by the P_{AN} promoter may have a similar function, no analysis of these regions has been performed to date.

ORF2 of IS*900* was designated as the *hed* (host expression-dependent) gene (95). The putative Hed protein encoded by IS*900* contains a hydrophobic sequence of 9 amino acids, which has significant homology to eight other prokaryotic and eukaryotic proteins involved in the transport of various essential growth requirements (94). Of the five prokaryotic transport proteins containing this conserved 9-amino-acid motif, three are involved in iron transport, leading to speculation that Hed may provide *M. paratuberculosis* with an alternate iron transport mechanism (94). Another possible role for Hed as a repressor of the native mycobactin pathway in *M. paratuberculosis* is implied by the observation that *M. smegmatis* transformants carrying a multicopy plasmid with IS*900* demonstrate a significantly decreased growth rate in broth cultures. This phenotype can be restored to wild-type levels by the supplementation with ferric mycobactin J (217). Clearly, these studies present intriguing hypotheses of iron utilization in *M. paratuberculosis* which merit further research.

The expression of *hed* requires that IS*900* be inserted into the chromosomal DNA in a directional manner (Fig. 1). This directionality is accomplished by IS*900* inserting itself between the putative RBS and start codon of a given gene in the chromosome, thus aligning the *hed* initiation codon next to a functional RBS from the target gene and downstream of an active promoter (29, 94) (Fig. 1). The target gene RBS (AGGAG) is in fact the complement of the IS*900* consensus sequence, $CATGN_{4–6}$ ∗CNCCTT (where ∗ denotes the site of insertion) (29, 94, 137). Since no termination codon is present in the *hed* sequence, the corresponding translational products of different IS*900* elements in the genome are of different lengths. Because the *hed* gene carries its own RBS at the 3′ terminus, the translation of the target gene may be restored by this replacement RBS (95) (Fig. 1). This hypothesis was confirmed recently by Bull et al., (29), who analyzed genomic regions immediately adjacent to 14 different IS*900* insertions and found that IS*900* was inserted in the *hed* orientation with respect to its consensus sequence in all loci. In five of these loci, these insertions resulted in the inactivation of the corresponding

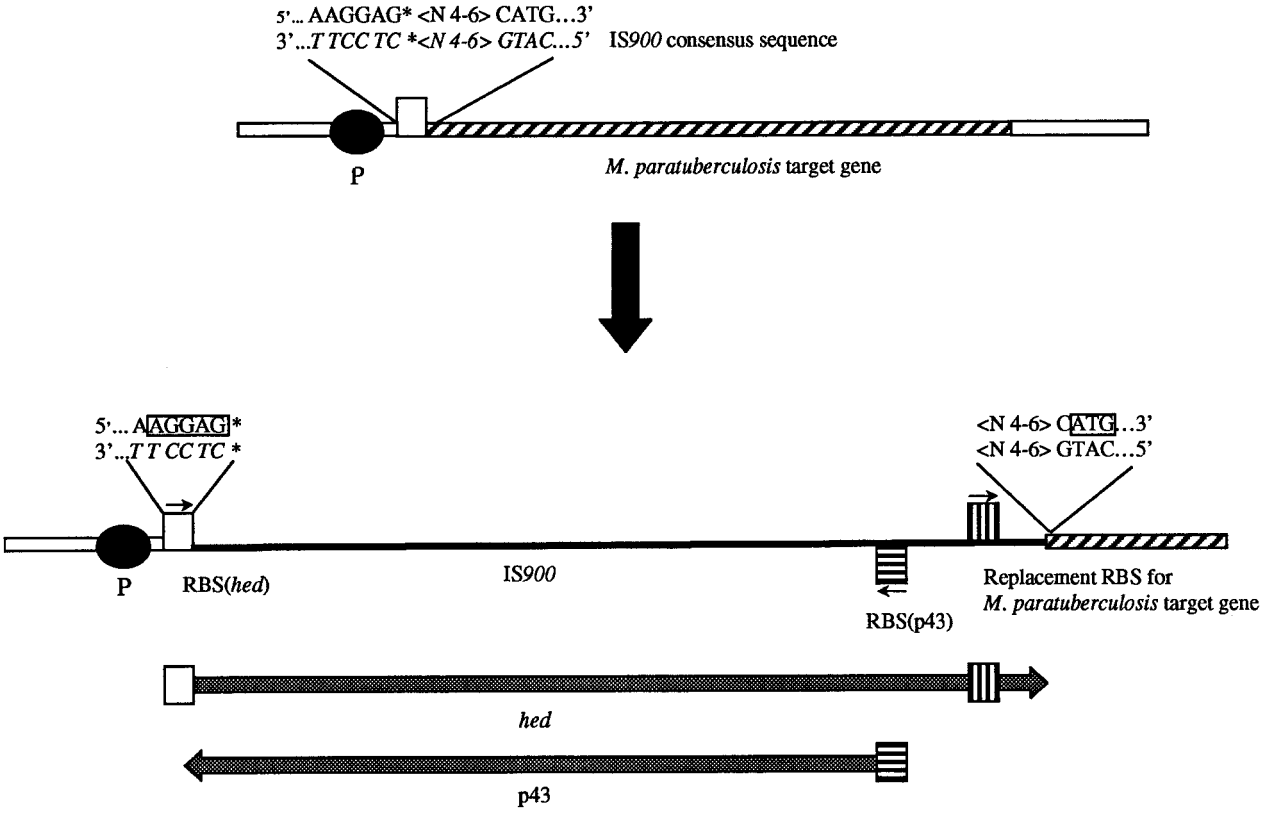

FIG. 1. IS*900* inserts in a target gene of *M. paratuberculosis* in an orientation-specific manner. A putative target gene of *M. paratuberculosis* is illustrated as a hatched line, with the corresponding upstream and downstream sequences of the genome shown as open lines. The IS*900* insertion consensus sequence from Green et al. (137) and Bull, et. al. (29) is italicized and is denoted in the 3′ to 5′ direction in the illustration. An asterisk indicates the site of IS*900* insertion (defines insertion in the *hed* orientation, according to the nomenclature of Bull et al. [29]). The RBS for the putative chromosomal target gene is represented by an open square, and its corresponding promoter (P) is shown as a black oval. A black line represents the IS*900* sequence. The DNA sequences for the putative target gene RBS and its start codon are boxed. The replacement RBS at the 3′ end of IS*900* is shown as a vertically striped square, and a horizontally striped square represents the RBS for the p43 gene. Small arrows above or below the RBS squares indicate the direction of translation. Black arrows represent the putative transposase (p43) and *hed* structural genes encoded for in IS*900*.

target genes, possibly altering gene expression. For 13 of these loci, 94 to 100% homology to the *M. avium* genome was found. However, one locus was absent from the *M. avium* genome and several other mycobacterial species, including *M. silvaticum*. This DNA region in the *M. paratuberculosis* genome appears to encode a transcriptional regulator and a polyketide synthase. Of the loci that had homology to accessions in GenBank, genes encoding a sigma factor, a nitrate reductase, and a methyl transferase were identified. ORFs of unknown functions were found in the other loci. Of the 14 loci, 7 were present in a panel of 81 *M. paratuberculosis* isolates tested, and 52 of these *M. paratuberculosis* strains contained all 14 IS*900* insertion loci.

IS*1311*

Only one other insertion element, IS*1311*, has been described in the *M. paratuberculosis* chromosome (74, 253). This insertion element is not unique to *M. paratuberculosis*, since it is also found in *M. avium* and *M. intracellulare* at approximately 7 to 10 copies throughout the genome (74, 321). IS*1311* is a member of the IS*256* family of insertion elements and differs from IS*900* in that it contains terminal inverted repeats (191). Although this has not been analyzed in IS*1311*, other members of the IS*256* family also generate an 8 to 9-bp direct target repeat. Of the 33 members in the IS*256* family of insertion sequences, 8 others are from mycobacteria (Table 3). These are IS*1081* (*M. bovis*) (70), IS*1245* (*M. avium*) (143), IS*1395* (*M. xenopi*) (238), IS*1407* (*M. celatum*) (239), IS*1408* (*M. branderi*) (191), IS*1511* (*M. gordonae*) (191), IS*1512* (*M. gordonae*) (191), and IS*6120* (*M. smegmatis*) (144). Significant similarity is seen throughout the entire nucleotide and amino acid sequences of IS*1311* and IS*1245* (Table 3). Conversely, the other mycobacterial members of the IS*256* family only have short (24 to 105-nucleotide) regions of similarity to the IS*1311* transposase gene. A slightly higher similarity (45 to 68%) is noted at the amino acid level (Table 3). IS*1311* is somewhat smaller than IS*900* (1,317 and 1,451 bp, respectively) and appears to contain a single ORF encoding a putative transposase. IS*1311* and IS*900* are not homologous at either the nucleotide or amino acid level. Analysis of *M. paratuberculosis* and *M. avium* IS*1311* sequences shows a slight difference of five point mutations (321). In addition, some copies of IS*1311* in the cattle strains of *M. paratuberculosis* contain an additional point mutation that can be used to distinguish them from the sheep strains (321).

TABLE 3. Comparison of the putative mycobacterial insertion element transposase genes belonging to the IS*256* family

Insertion element	Mycobacterial species	Accession no.	Nucleotide sequence length (bp) of:		% Sequence similarity to IS*1311* (no. of nucleotides)[c]	Protein sequence length (amino acids)	% Sequence similarity to IS*1311* (no. of amino acids)[d]
			Insertion sequence[a]	Transposase[b]			
IS*1311*	*M. paratuberculosis*	AJ223975	1,317	1,219		395	
IS*1081*	*M. bovis*	X84741	1,435	1,248	75% (102)	415	63% (227)
IS*1245*	*M. avium*	L33879	1,414	1,233	83% (976)	410	92% (366)
IS*1395*	*M. xenopi*	U35051	>1,323	1,248	NS[e]	415	63% (224)
IS*1407*	*M. celatum*	X97307	>1,399	1,248	92% (24)	415	66% (237)
IS*1408*	*M. branderi*	U62766	>1,325	1,254	NS	415	65% (237)
IS*1511*	*M. gordonae*	U95315	1,142	1,218	NS	405	61% (196)
IS*1512*	*M. gordonae*	U95314	1,428	1,236	76% (105)	411	68% (233)
IS*6120*	*M. smegmatis*	M69182	1,486	972	NS	323	45% (129)

[a] Nucleotide sequence length of entire insertion element (191).
[b] Nucleotide sequence length of ORF1 encoding the putative transposase gene.
[c] Nucleotide sequence similarities were determined using BLAST version BLASTN 2.0.9 (National Center for Biotechnology Information databases).
[d] Amino acid sequence similarities were determined using BLAST version BLASTP 2.0.9 (National Center for Biotechnology Information databases).
[e] NS, no significant similarity.

IS*1110*, IS*1626*, and Other Related Insertion Elements

The presence of other insertion elements in the *M. avium* genome that are closely related to IS*900* implies that *M. paratuberculosis* may also be harboring other undiscovered insertion elements. These insertion sequences, IS*1110* and IS*1626*, are not found in all strains of *M. avium*, suggesting that they are recent acquisitions by the *M. avium* genome (152, 244). Of interest is another insertion element from *M. avium* and *M. intracellulare*, IS*1613*, whose sequence has been submitted to GenBank (accession number AJ011838) and which displays 83% homology to IS*900* by BLAST analysis.

PATHOGENESIS AND ANIMAL MODELS

Interaction of *M. paratuberculosis* with Macrophages

Survival within macrophages is a hallmark of *M. paratuberculosis*. Recent evidence indicates that simultaneous intracellular multiplication and killing of *M. paratuberculosis* occurs, reflecting an initial T-helper 1 (Th1) cellular immune response of the host (331). The nutritional and hormonal status of an animal may also influence its susceptibility to *M. paratuberculosis* infections. Reduced dietary calcium (Ca^{2+}) protects beige mice from *M. paratuberculosis* infections (276), but a corresponding increase in endogenous 1,25-$(OH)_2D_3$ (vitamin D) levels reverses the beneficial effects of low Ca^{2+} levels (276, 278). Additionally, transient exposure of monocytes to growth hormone or prolactin enhances intracellular multiplication of *M. paratuberculosis* in primary bovine monocytes (109). Because the levels of these hormones also fluctuate during parturition and lactation, it is possible that similar alterations in the hormonal milieu might also influence the intracellular multiplication of *M. paratuberculosis* in bovine mononuclear phagocytes in vivo.

Other mycobacteria, including *M. avium* and *M. tuberculosis*, circumvent macrophage antigen processing by inhibiting phagolysosomal fusion (59, 221) and acidification (221, 283). In general, mycobacteria are relatively resistant to the bactericidal mechanisms of professional phagocytes. Resistance to reactive oxygen intermediates has been linked to catalase and peroxidase activities (38, 192), and alkyl hydroperoxidase reductase (AhpC) is implicated in resistance to both reactive oxygen and reactive nitrogen intermediates (41, 192). The gene products involved in these processes are candidate virulence determinants, which are likely to play an important role in disease. Treatment of *M. paratuberculosis*-infected bovine monocytes with IFN-γ induces the release of nitric oxide (NO) (330). However, the levels of NO produced by these monocytes are far below those needed to kill *M. paratuberculosis* in a cell-free system. This observation implies that the amount of NO produced by recombinant IFN-γ (rIFN-γ)-activated bovine monocytes is insufficient to kill intracellular *M. paratuberculosis* in vitro.

Regarding the use of macrophage-derived cell lines, both the activated murine J774.16 (272) and bovine BoMac (273) macrophages can restrict the growth of *M. paratuberculosis*. Surprisingly, no studies have been reported in the literature quantifying the ability of *M. paratuberculosis* to replicate and survive in cultures of primary human macrophages or human intestinal epithelial cell lines, important in the context of its potential role as the etiological agent of Crohn's disease.

Role of Cytokines in *M. paratuberculosis* Infections

M. paratuberculosis also influences cytokine production in infected animals. Both serum IFN-γ levels (274) and IFN-γ gene expression in ileal tissue and cecal lymph nodes (291) were higher in subclinically infected animals than in animals exhibiting clinical symptoms of Johne's disease. However, Adams et al. (3) did not find significant differences in mRNA IFN-γ expression from peripheral blood monocytes in subclinically infected cattle, suggesting that cytokine production is a local phenomenon restricted to infected tissues. Bovine macrophages can be induced to release interleukin 1 (IL-1) (1, 176), IL-6 (1, 2), and tumor necrosis factor alpha (TNF-α) (1, 2) upon stimulation with *M. paratuberculosis* antigens. These cytokines are also associated with granuloma formation and cachexia in other disease syndromes (42, 170, 184, 211). In particular, IL-1 seems to play an important role in protecting experimentally infected mice against *M. paratuberculosis* infection (169). These investigators show that in vivo administration of a monoclonal antibody against the IL-1 receptor hinders the elimination of *M. paratuberculosis* from these animals.

Cytokines appear to affect the ability of *M. paratuberculosis*

to survive within the macrophages. In the J774.16 murine macrophage cell line, preincubation with moderate levels (10 to 1,000 IU) of TNF-α prior to infection with *M. paratuberculosis* significantly increases the number of viable bacteria recovered whereas high doses (4,000 IU) of TNF-α reduce bacterial numbers (272). Pretreatment of bovine monocytes with IFN-γ slightly increases phagocytosis of *M. paratuberculosis* and inhibits its intracellular growth (331). Furthermore, continuous incubation of bovine monocytes with IFN-γ or human granulocyte-macrophage colony-stimulating factor significantly restricts the intracellular growth of *M. paratuberculosis* (333). Together, these studies demonstrate that local tissue cytokine concentrations are important in determining the outcome of a mycobacterial infection.

Role of Iron Uptake in *M. paratuberculosis* Infections

Mammalian hosts actively restrict iron supply to bacterial pathogens. Cytokines may function by causing macrophages to actively limit their intracellular iron concentration in an attempt to restrict bacterial growth (172, 312). Activated macrophages downregulate transferrin receptors, reducing iron levels (32). Nramp1, an integral membrane protein expressed exclusively in the lysosomal compartment of monocytes and macrophages, has been implicated in controlling mycobacterial replication by actively removing iron or divalent cations from the phagosomal space, further decreasing the amount of iron available to intracellular bacteria (34, 129). To scavenge iron in limiting environments, most mycobacterial species produce the lipid-soluble siderophore mycobactin and the water-soluble siderophore exochelin (10, 269). Since *M. paratuberculosis* is a mycobactin auxotroph, it can synthesize exochelin only (10, 269). These two siderophores appear to be relevant in the pathogenicity of mycobacteria, as gene mutations in the biosynthetic pathway of mycobactin T in *M. tuberculosis* impairs its ability to replicate in a human macrophage cell line (93). How *M. paratuberculosis* survives intracellularly when producing only exochelins is unclear.

Genetic Systems for *M. paratuberculosis*

The genetic manipulation of *M. paratuberculosis* is notoriously difficult. Its lengthy generation time, resistance to enzymatic or chemical lysis, and difficulty in performing genetic exchange commonly used for other mycobacteria all contribute to this problem. Therefore, the establishment of a gene transfer system in *M. paratuberculosis* was a significant breakthrough in the molecular genetic analysis of this organism (112). Prior to this, the ability to directly manipulate DNA elements from the *M. paratuberculosis* genome without the use of surrogate bacterial hosts had not been possible. This study demonstrated for the first time that *M. paratuberculosis* could be transformed with both foreign plasmid and bacteriophage DNA, providing a molecular genetics-based method for genetic manipulation. It also established that the aminoglycoside phosphotransferase gene (*aph*) from Tn*903*, which confers kanamycin resistance, could function as a selectable marker and that the firefly luciferase gene could be used as a reporter for gene expression. Based on this study, the firefly luciferase was subsequently used in *M. paratuberculosis* to determine antimicrobial susceptibilites (324).

The ability to mutate genes randomly is another requirement to study the underlying molecular genetic basis of pathogenesis in an organism. Based on the previous study, which established that *M. paratuberculosis* is susceptible to the bacteriophage TM4 (112), a transposon mutagenesis system utilizing phAE94 (a temperature-sensitive derivative of TM4) was developed (149). This system uses phAE94 as a vector to deliver a transposable element (Tn*5367*) randomly into the bacterial genome (11, 149, 233). The *aph* gene present in Tn*5367* provides a selectable marker to distinguish mutants carrying a transposon insertion. For this study, strain K-10, a virulent bovine *M. paratuberculosis* isolate with a small number of in vitro passages (112), was used to generate a transposon mutant bank with phAE94 and was shown to yield a collection of approximately 5,000 mutants. Theoretically, mutants from this bank that are attenuated in virulence can be isolated for further testing in animal or cell culture model systems (149). Recently, the usefulness of the phAE94 delivery vector system was confirmed using the *M. paratuberculosis* strains 989 and ATCC 19698 (TMC 1613), in which approximately 2,000 mutants were generated (35). The *M. paratuberculosis* strain K-10 mutant collection has been recently expanded by our laboratory to a representative bank of 13,500 independent mutants necessary for a 95% theoretical coverage of the genome with transposon insertions in all nonessential genes (N. B. Harris and R. G. Barletta, unpublished data).

Animal Models

The most relevant information on host-pathogen interactions is gathered by using the natural host in infection trials. Unfortunately for Johne's disease research, the natural ruminant hosts need to be maintained under containment for 2 to 3 years before they develop clinical paratuberculosis. Therefore, the use of ruminants for research is limited to the few institutions with facilities capable of meeting these needs. Consequently, several small-animal models, including chickens (181, 302), guinea pigs (114), hamsters (114, 148, 156), mice (39, 40, 114, 187), and rabbits (114, 148, 156, 205, 206, 245), have been developed for *M. paratuberculosis* infections. These small-animal models except mice (see below) are somewhat limited in their use for studying *M. paratuberculosis* infections because they do not consistently reproduce disease symptoms in experimentally infected animals. For example, oral inoculation of rabbits (205, 206) with approximately 10^8 CFU of *M. paratuberculosis* culminated in clinical and histopathological lesions in only 62 to 75% of the animals. In contrast, 100% of calves orally inoculated with 10^6 CFU of *M. paratuberculosis* developed disease (182). Possibly the inability of *M. paratuberculosis* to cause disease in other animal species may be attributed to the genetic background of the animals and/or the marginal virulence of *M. paratuberculosis* in these species.

More extensive work has been performed on the mouse model, with some promising developments. Early researchers observed that certain inbred and outbred strains of mice, such as the C57 black and Swiss white strains (39, 40, 187), were more susceptible to *M. paratuberculosis* infections than was the CBA strain (39). Unfortunately, some of the earlier work on *M. paratuberculosis* infections in mice is of limited value be-

cause the researchers did not report which mouse strain was used (114, 148).

Resistance to mycobacterial infections in mice, including *M. paratuberculosis*, is associated with the *Bcg* locus on mouse chromosome 1 (116, 265, 266). Two allelic forms of the *Bcg* gene, Bcg^s and Bcg^r, confer susceptibility or resistance to infection, respectively. As reviewed by Blackwell and Searle (24), the *Bcg* gene has been identified as *nramp1* (which encodes natural resistance-associated macrophage protein). A point mutation at position 169 in the amino acid sequence of the protein substitutes glycine for aspartic acid and leads to the susceptible phenotype. The Nramp1 protein is an integral membrane protein expressed in the lysosomal compartment of monocytes and macrophages and appears to function as an iron transporter. How this function influences bacterial survival is still unknown. Both C57/B6 mice (40, 306, 307) and BALB/c mice (50, 293) have the Bcg^s allele and are susceptible to *M. paratuberculosis* infections. Conversely, the C3H/HeJ strain is resistant to *M. paratuberculosis* and has the Bcg^r genotype (293).

Studies using C57/B6 and BALB/c mice confirm that these animals are capable of harboring 10^4 to 10^7 CFU of *M. paratuberculosis* per g of tissue in the liver and spleen for 6 months (50, 52, 306). Corresponding lesions typical of a mycobacterial infection are observed histologically in the mesenteric lymph node, liver, and spleen, but the intestine (ileum, cecum, and colon) does not contain lesions or acid-fast bacilli. The pattern of infection seen in BALB/c mice is somewhat atypical compared to natural infections in cattle, where the development of granulomas with acid-fast bacilli in the intestinal mucosa is common. However, liver lesions have been found in cattle naturally infected with *M. paratuberculosis* (27), and one report exists of a disseminated *M. paratuberculosis* infection in a cow (155). Nonetheless, the BALB/c mouse model may be useful for evaluating potential chemotherapeutic treatments, since Chiodini et al. (52) were able to significantly reduce bacterial counts in tissues of infected mice by prolonged rifabutin treatment.

Typical Johne's disease intestinal lesions have been induced in beige, SCID beige, and athymic nude mice. These mice have mutations that render them deficient in one or more components of the cellular immune system and thus are more susceptible to *M. paratuberculosis* infections, among others. BALB/c mice immunosuppressed with either cyclophosphamide or prednisolone also establish an intestinal infection following oral *M. paratuberculosis* inoculation (113). Beige mice are derived from C57/B6 mice and are deficient in lysosomal granules, type 2 pneumocytes and mast cells, and natural killer (NK) cells (122). Phenotypically, these deficiencies make them more susceptible to pyogenic bacterial infections. Beige mice have been utilized extensively to study *M. avium* infections and experimental chemotherapy in AIDS patients (21, 22, 122). When inoculated intravenously with ca. 10^8 CFU of *M. paratuberculosis*, these mice develop a disseminated infection by 2 months postinoculation, with concurrent granulomatous lesions in the liver and spleen, which are maintained for up to 12 months. Approximately 10^2 CFU of viable bacteria per g of tissue can be recovered from the small intestine by 1 month. This number increases to ca. 10^4 CFU/g of tissue by 6 months and is maintained through 12 months postinfection (276, 318). The beige mouse model has been used to demonstrate the influence of dietary calcium (Ca^{2+}) on *M. paratuberculosis* survival and cytokine secretion (275, 276, 278).

Severe combined immunodeficient beige (SCID beige) mice have a dual deficit which makes them attractive to study paratuberculosis (215, 216). Similar to SCID mice, SCID beige mice lack functional T and B cells, and, like the beige mice, they have decreased numbers and activity of NK cells (83, 190). Therefore, these mice provide the means of studing innate immunity in the absence of acquired immunity. As expected, SCID beige mice are also more susceptible than BALB/c or C57B/6 mice to *M. paratuberculosis*. The establishment of a progressive infection is possible in these mice, with gross pathological abnormalities, intestinal granuloma formation, and the clinical symptoms of weight loss and cachexia gradually increasing in severity over time (215). The route of inoculation (oral versus intraperitoneal) also significantly affects the onset and severity of the *M. paratuberculosis* infection in the SCID beige mice. Intraperitoneal injections at a dose of 10^5 CFU cause focal lesions and the detection of acid-fast bacilli in the liver by 4 weeks postinfection. By 12 weeks after infection, the average body weight of the intraperitoneally infected animals was significantly lower than that of controls, and all infected animals demonstrated generalized muscle wasting and depletion of abdominal fat, which progressed through the entire study (26 weeks). Conversely, oral inoculation with the same dose of *M. paratuberculosis* caused much milder symptoms. In this group, gross lesions were observed in only 1 of 18 infected animals by 26 weeks postinoculation. Similarly, histological lesions in the liver, spleen, and intestines were not observed until 26 weeks and then were found only in four of nine mice. Only one mouse had clinical symptoms by the termination of the study. Therefore, it can be concluded that the oral infection route causes a milder form of disease in these mice when low doses of *M. paratuberculosis* are used.

Athymic nude gnotobiotic mice have also been used in experiments examining *M. paratuberculosis* pathogenesis. Since these mice lack a mature thymus, they are congenitally immunodeficient (147). Intragastric inoculation of athymic nude mice with 10^{10} bacteria causes a progressive and severe enteritis, consistent with naturally occurring *M. paratuberculosis* infections seen in ruminants (113, 146, 147). Similar to the SCID beige mouse model, a drop in body weight and increase in mortality are observed over a period of months (113). Recovery of *M. paratuberculosis* from feces and tissues increases to approximately 10^4 to 10^5 CFU/g of feces and 10^6 to 10^8 CFU/g of tissue by 36 weeks postinfection. Similarly, a progressive intestinal granuloma formation and increased numbers of acid-fast bacilli are observed over the same period. These results are consistent with those obtained with SCID beige mice and confirm that a functional cellular immune response is necessary to contain *M. paratuberculosis* infections in the host.

Immunologically chimeric mice are a recently developed animal model in which bovine fetal tissue or peripheral blood cells are engrafted into SCID mice or SCID beige mice. SCID mice are preferentially used as recipients for these xenografts since their lack of functionally mature B and T lymphocytes prevents tissue rejection. However, once reconstituted with either bovine fetal hematopoietic tissues or peripheral blood leukocytes, these mice can both produce antibodies, indicative

of a functional B-cell response, and reject subsequent bovine tissue allografts, demonstrating a functional T-cell response (25, 92, 138, 139). Therefore, these mice can mimic normal bovine immune function. In the sole study analyzing the bovine immune response to mycobacterial infections with this model, Smith et al. (268) concluded that both $CD8^+$ and γ/δT cells help protect cattle from infection with *M. bovis*.

In summary, more experimentation is necessary to improve the animal models to study the various aspects of Johne's disease pathogenesis. However, the mouse models currently available offer interesting alternatives for the study of different aspects of this disease. The more accurate animal model for use in the study of Johne's disease would be the one that parallels the features of ruminant paratuberculosis pathogenesis, including the protracted manifestation of clinical signs. For these studies, it is important to identify not only the appropriate genetically defined strain of mice but also the dose and route of inoculation, optimal age for infection, and frequency of repeated challenges with virulent or attenuated microorganisms. For example, an experimental model resulting in higher multiplication of *M. paratuberculosis* in the liver and spleen than is the ileum would indicate that the infection has become systematic, which is not a relevant model of ruminant paratuberculosis. Conditions that lead to preferential bacterial multiplication in the intestinal tract should be thoroughly explored experimentally. Nonetheless, a systemic infection model may be suitable for a preliminary examination of candidate attenuated strains, if they show a considerable decrease in virulence compared with wild-type strains. Evidently an immunologically chimeric model would be more useful for studying the response of bovine immune cells to subunit or live vaccines.

DIAGNOSTICS

Identification of *M. paratuberculosis* Subspecies-Specific Genes

Identification of either subspecies-specific genes or unique modifications in conserved genes (such as insertions, deletions, or hypervariable regions) of *M. paratuberculosis* is necessary for the development of DNA-based diagnostic tests. Two candidates reported in the literature to date besides IS*900* are the *hspX* gene (98) and a 620-bp DNA fragment designated F57 (61). The *hspX* gene encodes a putative heat shock-like protein possessing an arginyl-glycyl-aspartic acid (RGD) peptide motif. Proteins with this type of motif have been implicated in mediating cell attachment (240) and stimulating phagocytosis and are likely to be virulence determinants (26, 142). BLAST analysis of *hspX* against *M. avium* and other mycobacterial genomes show that *hspX* has approximately 60% homology to similar sequences present in other mycobacteria, indicating that it may be unique to *M. paratuberculosis*. A PCR panel assay using this gene, the 16S rRNA sequence, and insertion elements IS*901* (*M. avium*), IS*1245* (*M. avium* complex), and IS*900* (*M. paratuberculosis*) has been developed recently and could differentiate between *M. avium* and *M. paratuberculosis* with an accuracy of 99.2% (99). However, two of the 12 *M. paratuberculosis* strains tested in this study were negative for *hspX* by PCR. Further analysis of these two strains was not reported, so it is unknown if they were devoid of the *hspX* gene or if the PCR failed to amplify the appropriate gene product. Although the functional analysis of *hspX* regarding macrophage adhesion and entry has yet to be performed, it does appear to have immediate merit as a diagnostic tool.

Poupart et al. (241), first published the isolation and use of F57 as a diagnostic probe for detecting *M. paratuberculosis*. This fragment does not hybridize with DNA from other mycobacterial species and is not related to any known *M. paratuberculosis* sequence including IS*900* (241) and *hspX* (98). BLAST analysis of this DNA sequence further support these data, since only 56% homology is found to a DNA region from *M. avium*. This F57 fragment and the upstream sequence of the gene encoding a 34-kDa antigenic protein have been used recently to develop a diagnostic duplex PCR capable of differentiating *M. bovis, M. avium*, and *M. paratuberculosis* (63). Further characterization and functional analysis of this gene fragment is necessary to ascertain its role in *M. paratuberculosis* pathogenesis.

Although not specific for *M. paratuberculosis*, a set of genes located in a 6.5-kb region of the *M. paratuberculosis* genome have been cloned and sequenced (301). These genes are also found in *M. sylvaticum*, and they have a G+C content of 58%, which is significantly lower than the 67% average for the *M. paratuberculosis* genome. The five ORFs within this region are *gsa, gsbA, gsbB, gsc*, and *gsd* and are homologous to genes that encode functions related to extracellular polysaccharide or lipopolysaccharide biosynthesis from *M. tuberculosis* and other gram-negative bacteria (301). Finally, as the subspecies-specific antigens described earlier in this review are sequenced and characterized, more unique genes of the *M. paratuberculosis* genome will be discovered. The sequencing and analysis of the entire *M. paratuberculosis* genome, currently under way at the University of Minnesota (http://www.cbc.umn.edu/ResearchProjects/Ptb/), will also uncover more regions specific to this pathogen.

Molecular Genetic-Based Diagnostic Tests

The advent of diagnostic probes based on specific bacterial DNA sequences has allowed fastidious microorganisms, such as *M. paratuberculosis*, to be rapidly identified. This strategy requires the identification of a DNA sequence present only in *M. paratuberculosis* but not in *M. avium* or other closely related mycobacteria. Initially, this approach was based on Southern hybridization analysis, but soon thereafter PCR tests based on these principles were developed (305). The unique regions of the *M. paratuberculosis* genome most widely used for PCR are the 16S rRNA gene and the insertion element IS*900* (see the following section for a detailed discussion of IS*900*-based PCR tests). Additionally, an experimental PCR test for *hspX* has been recently described (99).

In general, PCR analysis has been unable to match the sensitivity of fecal culture for identifying small numbers of bacteria (319, 332), but attempts to increase the sensitivity of this diagnostic test have yielded promising results (72, 202). In part, the lack of sensitivity of the PCR technique is due to the extreme difficulty of removing PCR inhibitors when preparing DNA from fecal extracts. Englund et al. (104) combined fecal culture and PCR in an attempt to circumvent this problem. In this study, plasmid DNA containing human β-actin was incor-

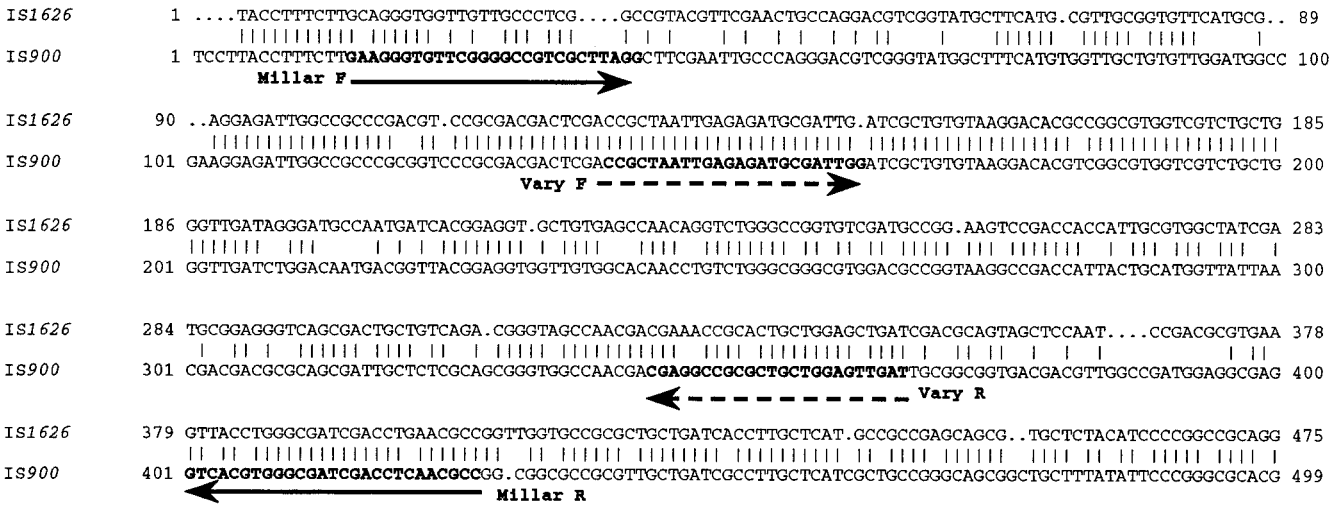

FIG. 2. Sequence comparison of the 5′ ends of IS*1626* and IS*900*. The sequences of the primer sets for *M. paratuberculosis* IS*900* published by Vary et al. (305) (Vary primers) and Millar et. al. (202) (Millar primers) are shown in boldface type. The Millar primers (Millar F and Millar R) are represented by solid arrows, and the Vary primers (Vary F and Vary R) are represented by dashed arrows.

porated into DNA extracted from primary cultures of *M. paratuberculosis*-suspect colonies, after which a nested PCR was performed using primers against both β-actin and the IS*900* element of *M. paratuberculosis*. Using this method, false-negative PCR results could be identified by the lack of amplification of both β-actin and the IS*900* product. However, it was still judged to be less sensitive than the culture method alone, highlighting the difficulty in removing inhibitors from primary samples. PCR has proven more useful for detecting the presence of *M. paratuberculosis* in infected sheep, a significant benefit since recovery of *M. paratuberculosis* from ovine fecal or intestinal samples is notoriously difficult (54, 71, 89). This tool is also valuable for differentiating species of *M. avium*. The IS*900* sequence is the most commonly chosen target gene and is used to distinguish *M. paratuberculosis* from *M. avium* (209) and *M. silvaticum* (202), with the limitations described below.

IS*900* PCR

The first commercial diagnostic PCR test for Johne's disease based on the IS*900* sequence was developed by Idexx Laboratories, Inc. (305). The primers reported in that paper by Vary et al. (305) (Vary primers) are widely used in variations of the IS*900* PCR test (80, 99, 196, 203). Other primers that amplify a region spanning the Vary primers have also been reported (203). The location of these primer sets in relation to the IS*900* sequence is illustrated in Fig. 2. Using these primers, Cousins et al. (80) could amplify IS*900*-like PCR products from environmental mycobacteria, thus demonstrating that false-positive results are obtainable from strains other than *M. paratuberculosis*.

Using a variation of the primers described by Millar et al. (202), Naser et al. (218) amplified PCR products from *M. avium* complex (MAC) strains originating from AIDS patients. Although these PCR products hybridized with an IS*900*-specific probe, it was not established whether these MAC isolates contained a sequence identical to IS*900* or a novel sequence with high homology to IS*900*. This study supports recent findings that nearly one-third of all isolates of *M. avium* subsp. *avium* recovered from human sources have readily amplifiable fragments corresponding to the IS*900* insertion element when a variety of previously published IS*900* primer sets were used (V. Kapur, personal communication). In addition, a BLAST analysis of the 229-bp region amplified by the Vary primers revealed that both primers overlap regions of high homology between IS*900* and IS*1626*, present in *M. avium*. In particular, 23 of 24 nucleotides of the forward primer and 20 of 24 nucleotides of the reverse primer are conserved in IS*1626* (Fig. 2). All these data indicate that PCR results based on the IS*900* sequence alone should be interpreted with caution and that the IS*900* primers used may not be specific for *M. avium* subsp. *paratuberculosis*, since IS*900* and/or IS*900*-like elements may be present in other closely related mycobacteria.

A variation of the IS*900* PCR has recently been introduced by Bull et al. (29), in which they developed a multiplex PCR of IS*900* loci (MPIL), based on flanking genomic DNA sequences at 14 different IS*900* insertion sites throughout the *M. paratuberculosis* genome. Since this MPIL assay is dependent on DNA flanking IS*900* and since these researchers also determined that seven different IS*900* loci are conserved among all *M. paratuberculosis* strains they tested, it is feasible that a rapid diagnostic test could be developed based on multiple IS*900* loci, which would eliminate the potential for false-positive results.

Combined PCR and Restriction Enzyme Digestion Tests

Restriction enzyme analysis has been coupled with PCR to differentiate *M. avium* and *M. paratuberculosis* and to identify different strains of *M. paratuberculosis*. The basis for this test involves point mutations within the ORF of a highly conserved gene, which results in the loss of a restriction enzyme recognition site. When a portion of the gene is amplified using PCR and subsequently digested with the appropriate restriction enzymes, fragments of different lengths are obtained as a result of the specific polymorphisms. Eriks et al. (105) first used this

technique to successfully differentiate *M. paratuberculosis* from *M. avium* by using a region of the 65-kDa heat shock protein gene. This strategy was also used with point mutations in the IS*1311* mobile transposable element to differentiate *M. avium* from *M. paratuberculosis* and to differentiate cattle strains of *M. paratuberculosis* from sheep strains (196, 321). This principle has also been applied to IS*900* PCR (80). However, given the recent findings of IS*900*-like sequences described above in MAC isolates, it remains to be determined whether the restriction enzyme polymorphisms of IS*900* can differentiate *M. paratuberculosis* from all closely related mycobacteria.

In general, the latest information regarding the specificity of IS*900* suggests that the search for *M. paratuberculosis*-specific regions useful for diagnostic purposes is unfinished. Undoubtedly, the information that will be provided by analyzing the completed *M. avium* and *M. paratuberculosis* genomes is likely to yield more conclusive answers.

Diagnostic Serology Tests

During an *M. paratuberculosis* infection, the animal develops both humoral and a cell-mediated immune (CMI) responses, which can be correlated with the stage of disease and the type of observed lesion. Overall, *M. paratuberculosis* lesions are described histopathologically as granulomatous inflammatory reactions. This classification has been further subdivided into a spectrum of histological manifestations ranging from tuberculoid (discrete) granulomas to lepromatous (diffuse) lesions (46) and is based on a similar classification first described in leprosy (250). The tuberculoid form of the disease is associated with a strong CMI response early in the course of infection (56, 78, 236). This CMI response involves the recruitment and proliferation of $CD4^+$ Th1 cells, $CD8^+$ cells, and peripheral blood mononuclear cells that secrete high levels of cytokines, including IFN-γ (56), which can be detected serologically (14, 234, 274, 291). Paralleling this CMI response is a strong B-cell proliferative response to *M. paratuberculosis* antigens (310). The lepromatous form of paratuberculosis appears in the later stages of the disease and is characterized by a decreasing CMI response, an increasing humoral response, and high antibody titers (56, 234, 263). Moreover, a transition from subclinical infection to clinical disease is accompanied by a marked antigen-specific B-cell unresponsiveness (310).

Early diagnostic testing for *M. paratuberculosis* was limited to the intradermal (skin) test. This test evaluates the delayed-type hypersensitivity (DTH) reaction of an animal to injected *M. paratuberculosis* extracts and is an indication of the CMI response of the animal (46, 78). However, problems with antigenic cross-reactivity of environmental mycobacteria have recently precluded its use as a diagnostic tool for paratuberculosis (78). Because of the importance in determining the infection status of subclinically infected animals, research in this area has led to the development of a bioassay (326) and enzyme-linked immunosorbent assay (ELISA) (255) that detects elevated IFN-γ levels in response to *M. bovis* infections. This test was further modified for identifying *M. paratuberculosis*-infected animals (23). Evaluation of the IFN-γ test by using experimentally and naturally infected cattle and sheep demonstrated its utility for distinguishing animals in the initial stages of infection (23, 234, 236, 274). However, in a recent study by McDonald et al. (197), all noninfected control animals tested positive at least once during the course of their study, suggesting that the IFN-γ ELISA may require further optimization.

Three different tests are currently available for measuring antibodies against *M. paratuberculosis* in the serum of infected animals. These are the complement fixation (CF) test, the agar gel immunodiffusion (AGID) test, and ELISA. Since a strong humoral response does not occur until the later stage of Johne's disease, the sensitivity of these tests is the highest for animals with lepromatous lesions (57, 235, 262), those with clinical symptoms (96, 153, 289), or those that shed large numbers of bacteria (17, 18, 270, 289) (Table 4). Therefore, the main limitation of these antibody tests is their inability to accurately identify animals early in the course of an infection. Conversely, all of these tests are highly specific, with false-positive results occurring at low frequency.

Among the antibody tests, ELISA is more sensitive than either the AGID or CF test (61, 78). It performs similarly for cattle, sheep, and goats (30, 97) and can be used with comparable sensitivity for either milk or serum samples (288). Comparative studies of the CF and AGID tests and ELISA repeatedly show discrepancies in the ability of these tests to identify all infected animals (84, 85, 153, 199, 270). As suggested by Sugden et al. (284), this may be due to genetic variation of the individual animal or the lack of representation of the entire range of immunodominant antigens for *M. paratuberculosis* within a given test.

Many variations of the ELISA method have been used to detect antibodies against *M. paratuberculosis* (81, 160, 288, 327). One of the crucial components of this test is the antigen preparation used to capture antibodies from the test sera. Therefore, the method of antigen preparation directly affects both the sensitivity and specificity of the ELISA (81). This was shown by an inhibition study performed with three commercially available ELISA kits, in which the *M. avium* strain 18 protoplasmic antigen (PPA-3) and the lipoarabinomannan polysaccharide antigen (LAM) cross-reacted but the *M. paratuberculosis* strain VRI 316/102-2 crude protoplasmic antigen (CTL) and LAM did not (284). The cross-reactivity between the PPA-3 and LAM antigens indicated that these tests have at least some antigens in common, but the lack of cross-reactivity between the CTL and LAM ELISAs suggest the absence of shared antigens in these preparations. Therefore, it is possible that, individually, these commercially available ELISAs can identify only a subset of an infected population of animals.

Preabsorption of test sera with *M. phlei* antigens is another significant modification proven to increase ELISA sensitivity (16–18, 75, 81, 85, 327). This step removes nonspecific antibodies against environmental mycobacteria that could potentially cross-react with *M. paratuberculosis* antigens. Antigen preparation of the *M. phlei* absorption antigens also influences the sensitivity of the ELISA (81). As expected, the most reproducible results are obtained when the same method is used to prepare both the test antigen and antigens used for preabsorption.

TABLE 4. Summary of *M. paratuberculosis* serology studies in the last decade

Test[a]	Animal species	Type of immune response[b]	Infection status[c]	Summary of results	Reference
Absorbed ELISA	Cattle	Humoral	NA[d]	Absorption of sera with *M. phlei* significantly increased the sensitivity of the test by reducing false-positives results	81
AGID, CF, LAM-ELISA	Cattle	Humoral	NA	LAM-ELISA was more accurate in predicting the infection status of an animal than either the CF or AGID test	199
Absorbed ELISA	Cattle	Humoral	Not infected; clinical, shedding; subclinical, shedding; subclinical, low shedding	A significant increase in ELISA response was noted for animals shedding *M. paratuberculosis*; absorption of sera with *M. phlei* increased the ELISA sensitivity for all groups of animals	16
Absorbed ELISA nonabsorbed ELISA	Cattle	Humoral	Clinical, shedding; subclinical, shedding; subclinical, low shedding	Both ELISAs were significantly more sensitive for heavy shedders; absorption of sera with *M. phlei* decreased sensitivity and increased specificity for both groups of animals	17
IFN-γ ELISA, IFN-γ bioassay, absorbed ELISA	Cattle	Humoral and cellular	Clinical, shedding; subclinical, shedding; subclinical, low shedding	IFN-γ ELISA was better at detecting animals in the subclinical stage of disease	23
AGID, CF, IDEXX ELISA, CSL ELISA	Cattle	Humoral	Shedders; nonshedders	Overall, ELISA test was more sensitive than either the CF or AGID test; all tests performed better at detecting antibodies from animals shedding bacteria	270
Absorbed ELISA, nonabsorbed ELISA	Cattle	Humoral	Clinical, shedding; subclinical, shedding; subclinical, low shedding	Both ELISAs were significantly more sensitive for heavy shedders; absorption of sera with *M. phlei* decreased sensitivity and increased specificity for both groups of animals	18
AGID	Sheep	Humoral	Diffuse histologic lesions; discrete histologic lesions	Sheep with diffuse lesions tended to have higher AGID scores than did sheep with discrete lesions	262
IDEXX ELISA	Goats	Humoral	Infected animals; noninfected animals	The ELISA was comparable in sensitivity (54%) and specificity (99%) to similar tests done in cattle	30
AGID, CF, CSL ELISA, non-absorbed ELISA	Sheep	Humoral	Grossly detectable lesions; histologically detectable lesions; no histological lesions	There were no differences among the tests in their ability to identify clinical or subclinical stages of disease (grossly detectable vs histologically detectable lesions); all tests performed better for animals in the later stages of disease	153
PPA-3 AGID, IFN-γ test	Sheep	Humoral and cellular	Diffuse histologic lesions; discrete histologic lesions	The IFN-γ test detected infected animals sooner than did the AGID test; no positive animals were detected until 120 days postinfection; diffuse lesions were correlated with positive AGID and negative IFN-γ results	234
LAM-ELISA	Cattle	Humoral	Infected animals; noninfected animals	Milk and serum samples from the same animals had comparable LAM-ELISA results	288
AGID, IDEXX ELISA	Cattle	Humoral	Clinical symptoms; no clinical symptoms	The ELISA was more sensitive than the AGID test; the ELISA did not detect some animals that were positive by the AGID test	84
IDEXX ELISA	Sheep	Humoral	Clinical; subclinical; not infected	ELISA was more sensitive in detecting clinically infected than subclinically infected sheep	96
IDEXX ELISA	Cattle	Humoral	Clinical, shedding; subclinical, shedding; subclinical, low shedding; not infected	The sensitivity of the test was highest for clinical cases and lowest for subclinically infected animals shedding small numbers of bacteria	289
AGID, absorbed ELISA	Sheep	Humoral	Diffuse histologic lesions; discrete histologic lesions	Both ELISA and the AGID test were sensitive for detecting animals with diffuse lesions; the sensitivity of both tests was significantly lower for animals with discrete lesions	57
AGID	Sheep	Humoral	Infected animals; noninfected animals	The commercially available AGID test approved for use in cattle was able to identify 25 of 27 infected sheep, indicating that it is useful for diagnosing paratuberculosis in sheep	97
LAM-ELISA	Cattle	Humoral	Infected animals; noninfected animals	The LAM preparation used as antigens for the ELISA was standardized; the standardized test had a positive predictive value of 74%	160
PPA-3 AGID, PPA-3 ELISA	Sheep	Humoral	Diffuse histologic lesions; discrete histologic lesions	The serology test results corresponded to the type of histological lesion; all animals that were positive by AGID serology had diffuse lesions; all animals with acid-fast bacilli in the lesions were positive by the AGID test and ELISA	235
PPA-3 ELISA, LAM-ELISA, CSL ELISA	Cattle	Humoral	NA	Inhibition ELISA studies demonstrated that PPA-3 and LAM antigens cross-react but that CSL and LAM antigens do not	284
IDEXX ELISA, LAM-ELISA, Absorbed LAM-ELISA, HSP-ELISA	Cattle	Humoral	NA	Preabsorption of the LAM-ELISA with *M. phlei* decreased the number of positive results; not all animals were classified as positive by the different tests; the HSP-ELISA was the least sensitive of all the tests	85
CSL IFN-γ ELISA	Cattle	Cellular	Infected animals; noninfected animals; vaccinated animals	2 of 10 infected calves had positive IFN-γ results; 3 of 9 vaccinated calves had positive IFN-γ results; all four noninfected calves were positive by IFN-γ ELISA	197
CSL IFN-γ ELISA, DTH	Sheep	Cellular	Diffuse lesions, shedders; borderline discrete lesions, low shedders; discrete lesions, low shedders	Sheep with discrete lesions were positive by IFN-γ EIA and DTH; sheep with diffuse lesions and shedding large numbers of bacteria were negative	236
CF, DTH, IDEXX ELISA	Goats	Humoral and cellular	Experimentally infected animals	Infection could not be confirmed histologically until at least 17 wk postinfection, and all serology tests were unable to detect infection until 37 wk postinfection	263

[a] Absorbed ELISA, test serum absorbed with *M. phlei* antigens to remove cross-reacting antibodies. AGID, agar gel immunodiffusion test; CF, complement fixation test; LAM-ELISA, lipoarabinomannin antigen ELISA; nonabsorbed ELISA, test serum is not preabsorbed with other mycobacterial antigens; IFN-γ-ELISA, gamma interferon ELISA; PPA-3 ELISA, protoplasmic peptide antigen ELISA; CSL ELISA, commercially available ELISA kit (PARACHEK^{T}) from CSL Ltd., Parkville, Australia; IDEXX ELISA, commercially available ELISA test kit from IDEXX Laboratories Inc., Westbrook, Me; absorbed LAM ELISA, absorbed ELISA using lipoarabinomannin extracts as capture antigens; HSP-ELISA, ELISA using the 65-kDa heat shock protein as a capture antigen; CSL IFN-γ ELISA, commercially available IFN-γ test kit (BOVIGAM) from CSL Ltd.; DTH, delayed-type hypersensitivity reaction.

[b] Type of immune response detected by the test.

[c] Description of infection status of study animals, as described in the reference.

[d] NA, not applicable.

DISEASE CONTROL

Natural Reservoirs

Effective disease control programs depend on addressing potential sources of infection and routes of transmission. Studies have demonstrated that *M. paratuberculosis* is viable for up to 250 days in water, feces, and cattle slurry (20, 179, 188). Consequently, contamination of an animal's environment by manure from infected animals is the most common mode of transmission. Vertical transmission during pregnancy has also been implicated since *M. paratuberculosis* has been isolated from the uterus (173, 230), fetal tissues (183), and semen (180).

Another factor affecting the control of Johne's disease is its ability to infect many different animal species. The host range of *M. paratuberculosis* is not limited to ruminants, since isolated reports of paratuberculosis in swine (295) and rabbits (6, 140) appear in the literature. Additionally, spontaneous cases of Johne's disease have been found in many species of wild animals. In North America, *M. paratuberculosis* has been isolated from white-tailed deer (44, 261), mule deer (322, 323), bighorn sheep (322, 323), Rocky Mountain goats (322, 323), bison (28), and elk (323). Similarly, *M. paratuberculosis* has been found in wild red deer from the Italian Alps (220) and wild rabbits in Scotland (6, 140, 141).

Management of wild-animal herds for either profit or preservation of endangered species has exacerbated certain diseases that are normally uncommon in the wild-animal population, including Johne's disease. Paratuberculosis has been diagnosed in farmed deer (90, 108, 128, 242, 294) alpacas (249), and elk (161, 194, 252). Furthermore, it appears that an infection can persist in certain populations without causing overt disease (79), which prevents both the relocation of these animals to the wild and the introduction of other livestock onto land formerly inhabited by these herds. Of greater concern is the ability of wild animals to infect domestic livestock with *M. paratuberculosis*. An *M. paratuberculosis* strain originally isolated from a bighorn sheep was able to infect other species of wild animals as well as domestic ruminants, implicating wild-animal populations as a natural reservoir for this pathogen (322, 323). In these same studies, either direct contact with diseased animals or exposure to pen effluent from diseased animals caused healthy, noninfected elk and sheep to become infected with *M. paratuberculosis*, confirming that transmission of *M. paratuberculosis* can occur via both of these routes. These data are supported by a study by Cetinkaya et al. (37), which demonstrated that the presence of farmed deer on land concurrently inhabited by dairy cattle increased the risk of Johne's disease in the dairy cattle population.

Management

Since Johne's disease is so insidious within an animal population, management is considered to be the most useful tool for controlling paratuberculosis within domestic livestock herds. However, management control recommendations often fail because they do not take into account the unique circumstances of individual farms (254). Specific management practices are reviewed in detail elsewhere (46, 254) and demonstrate the necessity for good animal husbandry. The most important management practices that have been identified are overall cleanliness of the farm, manure handling, newborn-calf care, and restriction of contact between calves and mature animals (77, 132). Other identified risk factors include the breed of cattle raised on the farm, with Guernsey and Jersey dairy cattle being associated with a higher risk factor, and the presence of farmed deer (37). The association between a high prevalence of clinical paratuberculosis and acidic soils has also been implicated as a risk factor for Johne's disease (162–164, 248). However, as discussed in a recent review on this topic (162), no experimental evidence exists to corroborate this relationship.

Another critical management tool is herd testing. Presently, ELISA serology and fecal culture are the most commonly used methods (254). Because these two tests measure different aspects of the disease (indirect antibody response to infection and shedding of live bacteria), a combination of the tests is more likely to detect infected animals (254). Therefore, infected animals can be removed (culled) from a herd, reducing the exposure of uninfected animals to the disease. Statistically, a significant drawback to testing entire herds lies in the poor positive predictive value of the tests, caused by the greater likelihood of a false-positive result in a truly negative herd when large numbers of animals are tested. In contrast, diagnostic testing of individual animals with a low pretest probability of infection is correlated with a poor negative predictive value (D. R. Smith, personal communication).

Vaccination

For the dairy industry, economic losses from Johne's disease are primarily due to premature disposal of animals and reduced milk production (76, 304, 325). Therefore, a vaccine that prevents animals from becoming infected would be an ideal goal. Vaccines for paratuberculosis have been commercially available for many years but unfortunately are not completely effective in preventing disease (46, 61, 175, 304, 314). Most studies conducted on paratuberculosis vaccines have been field trials in which a herd or geographical region has experienced a high burden of Johne's disease and a vaccination strategy was implemented in an attempt to reduce economic losses. In these studies, design parameters and/or experimental conditions were not fully described or controlled. Therefore, only general conclusions can be drawn regarding the efficacy of the vaccine in preventing disease. Nonetheless, Johne's disease vaccines appear to provide partial protection, since they reduce fecal shedding in cattle (46, 175, 176), the number of clinically affected cows (46, 304, 314), sheep (82), and goats (258), and the number of animals testing positive histologically or bacteriologically (Table 5) (304, 314). This partial protection has been confirmed in more controlled experimental trials using vaccinated and experimentally challenged ruminants. Like the field trials, these studies demonstrate a significant reduction in the number and severity of intestinal lesions, the number of positive fecal cultures, and the severity of clinical symptoms in vaccinated animals (145, 166, 281). These results contrast with former studies (125, 126, 182). Thus, further clinical trials using well-defined parameters are needed to confirm the more recent positive findings of the effect of vaccination.

From the controlled experimental vaccine trials performed with ruminants with Johne's disease, certain conclusions can be

TABLE 5. Summary of *M. paratuberculosis* vaccination studies in the last decade

Type of vaccine (vaccine strain[a])	Animal species (no. in study)	Route of inoculation and dose given	Effect of *M. paratuberculosis* vaccine on:		Reference
			Clinical signs	Evidence of immune response	
Heat killed (strain 18)	Cattle (20)	NR[c]	NT[c]	Antibody titers in vaccinated animals were detectable by 2 m postvaccination and were maintained for 15 m	271
Heat killed (5889 Bergey)	Cattle (866)	25–100 mg (dry wt), intramuscular	Partial protection; vaccination reduced fecal shedding	Vaccinated animals had increased antibody titers during the first 3 yr which declined by yr 5	174
Live attenuated (316 F)	Sheep (580)	5 mg (wet wt), subcutaneous	Partial protection; no. of animals with clinical symptoms decreased over 3 yr.	NT	82
Heat killed (Lelystad[b])	Cattle (176)	5 mg, subcutaneous	36/176 cows had both a positive fecal culture and histological lesions, whereas only 2 had histological lesions without concurrent positive cultures	Vaccinated, noninfected animals (126/176) tended to have a positive DTH response	313
Live attenuated (316 F)	Sheep (17)	10^3 CFU, subcutaneous	Partial protection; vaccination reduced the no. and severity of bacteriological isolations and histological lesions in the intestine	Vaccinated animals displayed a strong and immediate increase in antibody titers within 1 wk, but infected animals delayed this response until 6 wk postinfection	166
Heat killed (5889 Bergey)	Cattle (866)	25–100 mg (dry wt), intramuscular	Partial protection; vaccination reduced fecal shedding	Vaccinated animals had increased antibody titers during the first 3 yr, which declined by yr 5	175
Heat killed (Lelystad)	Cattle (499)	5 mg, subcutaneous	Partial protection; vaccination decreased both the no. of animals culled for clinical paratuberulosis and the percentage of animals with positive histology	NT	314
Live attenuated (316 F)	Goats (20)	NR	NT	Vaccination induced a cellular immune response as measured by a lymphocyte proliferation assay	207
Heat killed (not reported)	Cattle (652)	NR	Partial protection; vaccination reduced the no. of clinically infected animals by almost 90%	NT	304
Heat killed (Field strain) and live attenuated (316 F)	Cattle (28)	1.5 ml of live vaccine or 1 ml of heat-killed vaccine, subcutaneous	Partial protection; infection was confirmed in 1 of 9 vaccinated calves, 0 vaccinated calves had positive fecal culture results	Three vaccinated calves had a positive ELISA, indicating antibody production; all calves had at least one positive IFN-γ ELISA	197
Live attenuated (316 F)	Sheep (41)	1 ml, subcutaneous, given 2 wk after oral inoculation with ca. 10^7 CFU of a virulent sheep strain of *M. paratuberculosis*	Partial protection; 7/14 unvaccinated versus 2/14 vaccinated sheep developed clinical signs, and 11/14 unvaccinated and 8/14 vaccinated animals were positive for *M. paratuberculosis* by either histology or PCR testing (severity of lesions for the vaccinated group lower than for the unvaccinated group)	Vaccinated sheep had higher IFN-γ and antibody production than did nonvaccinated sheep	145

[a] Mycobacterial strain used for vaccine preparation, as described in the reference.
[b] Vaccine prepared by the Central Veterinary Institute, Lelystad, The Netherlands.
[c] NR, not reported; NT, not tested.

drawn. Stuart (282) used dairy calves inoculated subcutaneously with 5 mg (wet weight) (ca. 5×10^4 CFU) of a live attenuated vaccine at 1 week of age and exposed 1 month later, for a duration of 6 months, to infected calves actively shedding *M. paratuberculosis*. A subset of the vaccinated calves were revaccinated at 1, 21/2, and 4 years after exposure. All animals were monitored for 5 years, and surviving animals were sacrificed at the termination of the study. Statistically, fewer animals in the once-vaccinated group died of Johne's disease (8 of 28) than did the nonvaccinated animals (17 of 28) or the revaccinated animals (13 of 28). However, only five cows in this group were not infected with *M. paratuberculosis* after 5 years, compared to three cows for the revaccinated group and one animal for the nonvaccinated group. Thus, it appears that revaccination, or boosting, does not improve the ability of an animal to resist infection. Similarly, the vaccine does not prevent infection from occurring.

Vaccination and boosting was also tested in sheep (125). In this study, animals were vaccinated subcutaneously at 4 months of age with 5 mg (wet weight) of a heat-killed strain of *M. paratuberculosis*. Oral challenges of these animals were performed with a virulent bovine clinical strain of *M. paratuberculosis* 1 month after vaccination and again by incorporating *M. paratuberculosis* in the drinking water for 9 to 14 months after vaccination. Revaccination was performed in a subset of sheep at 11 months with either the same heat-killed vaccine or a fractionated extract containing peptides and glycolipids from three vaccine strains of *M. paratuberculosis*. Animals were sacrificed at approximately 20 months of age. At this time, all animals were infected with *M. paratuberculosis* and no significant differences in gross lesions and numbers of viable bacteria recovered from tissues were observed between the different experimental groups, substantiating the previous experiment that vaccination does not prevent subsequent infection by a virulent strain of *M. paratuberculosis*.

In the sole report on oral vaccination against Johne's disease

in sheep, protective immunity was not elicited (126). Three-month-old animals were dosed with 5×10^6 CFU of *M. paratuberculosis* strain 316F weekly for 10 weeks and challenged 1 month later with 10^8 CFU of a clinical *M. paratuberculosis* isolate per week for an additional 10 weeks. At the termination of this study (1 month after the last challenge), vaccinated sheep displayed no differences from control animals in DTH responses or the number of viable bacilli recovered from either the mesenteric lymph nodes or small intestine. Mild lesions consistent with a *M. paratuberculosis* infection were observed in vaccinated, nonchallenged sheep, but viable bacilli were not recovered from these tissues. The main conclusion from this study was that oral vaccination was not effective in protecting against a challenge with a pathogenic strain. However, high doses of the vaccine strain were given over several weeks, conditions that that may not elicit the correct type of protective immune response. In this context, Andersen (5) induced a dose-dependent immunity in mice with a vaccine based on short-term culture filtrate proteins of *M. tuberculosis*. At high vaccine doses, a nonprotective humoral response occurred, but lower doses induced a Th1 response that was protective. Another problem of the oral vaccination trial in sheep is that the study was terminated after only 1 month postchallenge. Related studies have demonstrated a significant difference in the number and severity of lesions in vaccinated animals compared with nonvaccinated animals, but only after 6 months or more (145, 166, 282).

Protective immunity against mycobacterial diseases, especially paratuberculosis in ruminants, is poorly understood. It is assumed that vaccination stimulates a protective CMI response. *M. paratuberculosis* directly suppresses a murine T-cell DTH response to injections of sheep red blood cells (171), implying that this may be another method used by this bacterium to evade the host immune response. As stated above, a strong CMI response to *M. paratuberculosis* infections is associated with the formation of granulomatous tuberculoid lesions and containment of the disease by the host whereas a weak CMI response is associated with diffuse lepromatous lesions and disease progression. Furthermore, the strength of this response can be directly correlated with the presence and severity of tuberculoid lesions and the bacterial load in the tissues (31, 145, 236). In paratuberculosis, a strong DTH response, indicative of a strong CMI response, has been correlated with the ability of an animal to contain the infection (145, 313). This situation may be correlated with some observations in human tuberculosis, where a vaccine-induced low level of hypersensitivity was associated with protection but a persistent vaccine-associated hypersensitivity was not (111). Several groups have demonstrated the association of tuberculoid-type lesions with the elimination of an *M. paratuberculosis* infection in both sheep (145, 166, 236) and mice (50). Therefore, it can be hypothesized that the Johne's disease vaccines stimulate an initial strong cellular immune response, partially overcoming the suppressive effect of pathogenic *M. paratuberculosis* on the host immune system. To this end, vaccinated animals have higher IFN-γ and serum antibody responses than do naturally infected animals (145, 166).

In the United States, vaccination is still considered to be a controversial management tool (76). In general, vaccination can interfere with diagnostic testing, can cause observable granulomas at the vaccination site, and may present a health risk to veterinarians administering the vaccine due to accidental self-injection (226). However, a study in The Netherlands showed that vaccination against paratuberculosis was profitable for herd owners, reducing the number of clinically infected cattle by approximately 90% (304). It should be noted that in the United States, heat-killed *M. avium* strain 18 is used, while in Europe, a modified live vaccine is marketed. As reviewed previously (46, 61), both live attenuated and killed whole-cell (bacterin) vaccines are somewhat successful in controlling clinical paratuberculosis. Table 5 lists studies from the last 10 years that have used either the live attenuated (82, 145, 166, 197, 207) or killed (174, 175, 197, 271, 304, 313, 314) *M. paratuberculosis* or *M. avium* vaccines. Comprehensive studies on the most effective strain(s) of *M. paratuberculosis* for use in vaccine production are also needed. Since various strains of *M. bovis* BCG differ in their ability to elicit experimentally induced immune reactions (134), it is possible that a similar phenomenon may occur with *M. paratuberculosis*. A number of *M. paratuberculosis* strains have been reported in the literature as being used for both experimental and commercial vaccine production (Table 5). These include strain 316F (61, 82, 166), strain 5889 Bergey (175), *M. avium* strain 18 (46, 271), field isolates (197), and other unidentified reference strains (304, 313, 314).

Research on *M. tuberculosis* secreted antigens suggests that a subunit vaccine may be developed against mycobacterial infections. For example, Horwitz et al. (158) have demonstrated the potential use of *M. tuberculosis* antigen 85B as a subunit vaccine against tuberculosis. A similar approach may be feasible for *M. paratuberculosis*, and a subunit vaccine of this kind would be readily compatible with current diagnostic tests. This type of vaccine may benefit from the use of an immunoadjuvant, such as the genetically detoxified derivative of either the *Escherichia coli* heat-labile enterotoxin or the closely related *Vibrio cholerae* cholera toxin. Mutant heat-labile enterotoxin and cholera toxin both elicit a protective humoral and cellular host immune response when used as a mucosal adjuvant in conjunction with killed bacteria (117), suggesting new possibilities for vaccine development for *M. paratuberculosis*.

It would be extremely useful to develop methods to differentiate vaccinated from infected animals. The time frame for a vaccinated animal to demonstrate detectable antibody levels can vary from 2 weeks to 6 months after vaccination (166, 271). Once vaccinated, animals appear to maintain these levels for at least 1 year (271) and up to 3 years (174, 175) afterward, which interferes with the assays currently used for diagnostic purposes and export requirements. Although the development of a live attenuated vaccine compatible with diagnostics is difficult, Cirillo et al. (55) have described a recombinant DNA technology approach to generate this type of vaccine. It is our conviction that either this type of a recombinant live attenuated vaccine or a subunit vaccine coupled with the proper use of adjuvants has the greater promise for a more effective vaccine against paratuberculosis.

Treatment

Presently, no drugs are approved for the treatment of Johne's disease in livestock, and the rare instances where antibiotic therapy is attempted is limited to the extralabel use of

standard antimicrobial agents. Since treatment of paratuberculosis is expensive and unrewarding, it is only used in extreme efforts to prolong the life of very valuable animals for breeding purposes. The treatment regimens most commonly used for Johne's disease are either clofazimine (61), or isoniazid and either rifabutin or ethambutol, followed by a daily dose of isoniazid for the duration of the animal's life (124, 281). Although isoniazid is prescribed for the treatment of *M. tuberculosis* and *M. bovis* infections in cattle (243), both *M. paratuberculosis* and *M. avium* are resistant to isoniazid in vitro (157, 201, 324), and therefore it may not be effective in vivo.

M. paratuberculosis is susceptible to many antibiotics in vitro. These include D-cycloserine (45, 324), ethambutol (49, 246, 324), amikacin (49, 324), clarithromycin (246, 324), and rifabutin (324). The fluoroquinolone classes of antibiotics may also merit further investigation, since the experimental fluoroquinolone Bay y 3118 is highly effective against *M. paratuberculosis* (324). It has been suggested that antibiotic therapy does not result in a complete cure, possibly due to the inaccessibility of mycobacteria to the drugs in vivo (61). Alternatively, some of the drugs used in the in vivo studies (e.g., isoniazid) may not have been the most appropriate choices for treatment, since the corresponding in vitro drug susceptibility patterns of the *M. paratuberculosis* strains causing the infection were not reported. Progress in the development of antibiotics to treat paratuberculosis infections will require the rational design of studies that identify appropriate bacterial targets, utilize drugs that are capable of penetrating macrophages, and focus on cost-effective chemotherapies that are approved for use in food animals. Until these considerations are addressed, chemotherapy will not be a practical alternative for treatment of Johne's disease.

RESEARCH PRIORITIES AND CONCLUSION

The knowledge of how *M. paratuberculosis* causes disease still lags behind that for other pathogenic mycobacteria. Like other pathogens, the interactions between *M. paratuberculosis* and its host are complex. The progression of our understanding of the basic physiology of *M. paratuberculosis* is hampered by the difficulty of manipulating it in a laboratory setting. However, there is hope that as more of the *M. paratuberculosis* genome is sequenced and characterized, differences in the genetic structure and physiology between *M. paratuberculosis* and other mycobacteria will become evident. This will expand our understanding of what makes *M. paratuberculosis* unique and will further our repertoire of specific targets that can be exploited for diagnostic testing and treatment of Johne's disease. However, there are important gaps in this knowledge. Regarding the basic biology of the microorganism, it would be most important to determine the molecular basis of mycobactin dependency, which is the most prominent diagnostic feature of the subspecies *paratuberculosis*. Another important aspect would be to ascertain the role of *M. paratuberculosis* species-specific genes encoding virulence determinants associated with macrophage survival, host range, tissue specificity, and pathogenesis. In addition, to develop new live attenuated vaccines, it would be important to define mutations in virulence determinants or housekeeping functions, leading to an attenuated phenotype. In this context, a functional genomic approach based on the screening of mutant banks in combination with microarray technology offers the greatest promise.

These microbial studies will need to be complemented with studies using appropriate in vitro and in vivo systems. The in vitro models for Johne's disease would include the use of bovine cell lines, such as the Madin-Darby bovine kidney (MDBK) epithelial cell line (American Type Culture Collection), which could be useful to study the interaction of *M. paratuberculosis* with the mucosal epithelium, and the BoMac cell line, which could be used in the study of macrophage survival. Experiments performed with these cell lines may have to be validated with primary epithelial and macrophage cells. There are advantages to each of these approaches, as well as some distinctive differences between the two. Primary cells are untransformed, but they vary in phenotypic properties from one donor to the next. The cell lines will offer consistent results on a day-to-day basis, but since they are transformed, they may not accurately represent the mature primary cells in culture. In particular, epithelial cells are highly differentiated in vivo, unlike the MDBK cells. Therefore, the calf ileal loop assay, which has been successfully used to study *Salmonella enterica* serovar Typhimurium interactions with the cells of the mucosal epithelium (119), may be valuable for similar studies of *M. paratuberculosis*. In the immune response of the ruminant host, it would be important to elucidate the various immune cell interactions, such as the potential regulatory effects of γ/δ T cells on $CD8^+$ cell function (51), found in greatest proportions in the neonatal calf (150). This issue is also important in the context of eliciting a protective immune response by vaccination and could have bearing on the future development of more effective live attenuated or subunit vaccines.

Since ruminant hosts require specialized containment facilities and are expensive to maintain, prior research needs to be performed to establish the more suitable small-animal model that best parallels Johne's disease. It seems that transgenic and immunologically chimeric mice offer the greatest possibilities for meaningful experiments. These models would be important to test potential vaccine strains and the elicitation of protective immunity prior to conducting controlled experiments in ruminants and, finally, large-scale field trials. The most rigorously designed parameters must be established prior to conducting these field trials so that accurate and meaningful data are obtained.

The potential role of *M. paratuberculosis* in the etiology of Crohn's disease, or its potential to serve as an opportunistic pathogen in patients with inflammatory bowel disease, deserves substantial future investigation. In this context, the recommendations of a panel from a workshop conducted at the National Institutes of Health (http://www.niaid.nth.gov/dmid/meetings/crohns) have established several research priorities to address potential *M. paratuberculosis* infections of humans. These guidelines parallel the recommendations on Johne's disease research provided above and include the establishment of cell or organ culture models, new small- and large-animal models of infections, and basic aspects of gene expression and differential genomics. In addition, the panel recommended to optimize diagnostics of *M. paratuberculosis* antigens, and drug susceptibility testing. Some progress has been made in this context, but substantial funding from this agency would be required to meet these goals in a timely fashion. In conclusion, progress

on the understanding of *M. paratuberculosis* infections will require a combination of molecular genetic studies with appropriate in vitro and in vivo models to evaluate pathogenesis.

ACKNOWLEDGMENTS

Research in our laboratory is supported by NRI CGP-USDA grant 99-35204-7789 and BARD-USDA grant IS2564-95C. Support for N.B.H. was provided by the Department of Veterinary and Biomedical Sciences at the University of Nebraska, Lincoln.

We acknowledge Z. Feng for assistance with computer analysis and graphics preparation. Additionally, we thank our associates N. E. Cáceres, O. Chacon, Z. Feng, X. Liu, J. Zabaleta, and D. Zinniel for their helpful disscussions. J. Bannantine, H. Bercovier, L. E. Bermudez, J. D. Cirillo, C. Czuprynski, R. Donis, V. Kapur, R. A. Moxley, D. Smith, and D. J. Steffen are gratefully acknowledged for their critical review of the manuscript, and J. Hermon-Taylor and T. J. Bull are acknowledged for their insightful comments on *M. paratuberculosis*.

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