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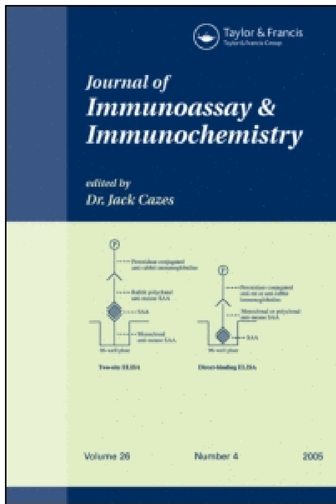

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B-CELL EPITOPE SPECIFICITY OF CARBOXY TERMINUS OF *MYCOBACTERIUM PARATUBERCULOSIS* ModD

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B-CELL EPITOPE SPECIFICITY OF CARBOXY TERMINUS OF *MYCOBACTERIUM PARATUBERCULOSIS* ModD

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□ *Epitope mapping of ModD of* Mycobacterium paratuberculosis *was performed using overlapping peptides. In total, 80 overlapping peptides, covering the entire mature ModD, were commercially synthesized. Each peptide spanned 14 amino acids with an offset of 4 amino acids, i.e., with an overlap of 10 amino acids. Synthetic peptide antigenicity was evaluated by enzyme-linked immunosorbent assay (ELISA) using rabbit antisera to culture filtrate (CF) of* M. avium *or* M. paratuberculosis *or recombinant ModD (rModD). The peptides o f ModD reacting most strongly (ELISA OD > 1.0) were clustered near the N- and C-terminal ends. The peptides around the C-terminal end only showed the greatest specificity for* M. paratuberculosis*, yielding high ELISA OD values with rabbit anti-*M. paratuberculosis *CF serum and low ELISA OD values with rabbit anti-*M. avium *CF serum. Sera from naturally* M. paratuberculosis*-infected cattle, however, bound poorly to the short, 14-amino-acid peptides. Thus, two longer peptides covering amino acids 100 to 125 and 328 to 353 were synthesized based on their broad reactivity to rabbit serum against* M. paratuberculosis *CF. The peptide covering amino acids 328 to 353 showed the highest level of specific bovine antibody binding.*

Keywords culture filtrate, epitope, ModD, *Mycobacterium avium*, *Mycobacterium paratuberculosis*, synthesized peptide

INTRODUCTION

Johne's disease is caused by *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*), a member of the *Mycobacterium avium* complex. This slowly growing bacterium causes chronic inflammatory bowel disease in a variety of ruminant hosts,[1] while *Mycobacterium avium* subspecies

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avium (*M. avium*) is relatively fast-growing opportunistic pathogen of lower virulence that is common in soil and water.[2] Despite their several phenotypic differences, nucleotide similarity between *M. avium* and *M. paratuberculosis* is greater than 97%.[3] This has led to designation of *M. paratuberculosis* as a subspecies of *M. avium*.[4]

The close genetic and proteomic relationship of *M. paratuberculosis* with the other mycobacteria leads to extensive antigen or epitope sharing, hindering efficient serologic diagnosis of Johne's disease. Absorption of clinical serum samples with *Mycobacterium phlei* has helped to circumvent cross-reactivity problems.[5] Most commercial enzyme-linked immunosorbent assay (ELISA) kits in use today employ *M. phlei* serum absorption, and such assays have high diagnostic specificity for paratuberculosis.[6] However, this absorption step also compromises assay sensitivity. Taken together, this information indicates the necessity of identifying specific epitopes that can be applied to paratuberculosis serodiagnosis.

Epitope mapping is particularly important for the highly conserved mycobacterial antigens because the immunodominant antigens commonly share epitopes.[7,8]

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The complete genome sequences of several mycobacteria are available, including those of *Mycobacterium tuberculosis, Mycobacterium leprae, Mycobacterium bovis, Mycobacterium smegmatis, M. avium,* and *M. paratuberculosis*.[9] Using these genomic data, together with bioinformatics tools, epitopes specific to each species can be predicted by *in silico* analysis. This approach assumes the epitopes are linear, not conformational, and relies on physicochemical parameters such as hydrophilicity, accessibility, flexibility of short segments, and various elements of secondary structure of other well-characterized protein antigens.[10] *In silico* analysis, however, has its limitations. Homologous genes can be differentially expressed by different mycobacterial species, allowing for diagnostic specificity in the absence of analytical specificity.[11] Second, there are no unique physicochemical parameters that can specifically be attributed to epitopes.[10,12] Hence *in silico* epitope predictions are only as good as the genomic and physicochemical databases from which they derive and must be accepted simply as predictions.

In previous work, we identified ModD as one of 14 proteins having potential diagnostic value for bovine pratuberculosis. It was both antigenic and actively secreted in vitro by *M. paratuberculosis*.[13,14] However, non-infected cattle showed unacceptably high levels of naturally occurring antibodies to ModD in serum; i.e., as a diagnostic reagent it lacked specificity for bovine paratuberculosis.[15] We hypothesized that some portions of ModD are common with other mycobacteria and others are *M. paratuberculosis*-specific. To explore this hypothesis, epitope mapping of *M. paratuberculosis* ModD was conducted using overlapping synthetic

peptides, rabbit antibodies and bovine sera from paratuberculosis cases and controls. Results are contrasted with those of *in silico* analysis.

EXPERIMENTAL

Bacterial Strains and Preparation of Immunogens

Mycobacterium paratuberculosis JTC303 and *M. avium* ATCC 11758 were grown at 37°C in modified Watson–Reid (WR) medium supplemented with mycobactin J (Allied Monitor, Fayetteville, MO).[16,17] Bacterial cells were removed from culture filtrates (CF) by centrifugation at 30,000 × g for 10 min, then further clarified by filtration (0.2 μm pore size of filter, Nalge Nunc, Rochester, NY). The filtrate was then concentrated roughly100-fold using Centricon Plus-80 (molecular weight cutoff 5000; Amicon, Beverly, MA) and dialyzed in 10 m*M* phosphate-buffered saline (PBS), pH 6.8. The protein concentration was measured by BCA protein assay kit (Pierce, Rockford, IL).

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Peptide Synthesis

The 80 peptides covering the mature secreted form of ModD were synthesized commercially (JPT Peptide Technologies GmbH, Berlin, Germany). Each peptide spanned 14 amino acids with an offset of 4 amino acids, i.e., with an overlap of 10 amino acids. Two longer peptides covering amino acids 100 to 125 and 328 to 353 were additionally synthesized after seeing results from screening the first eighty 14-amino-acid peptides. A biotin-TTDS was attached to the N-terminus of each peptide, and the C-terminal was blocked with glycine amide. Each peptide was identified based on the amino acid position in ModD (with signal sequence) of its first (N-terminal) amino acid residue.

Expression of ModD

Preparation of recombinant ModD was described previously.[15] Briefly, genomic DNA was isolated from *M. paratuberculosis* K10 strain, and the DNA encoding ModD, without its signal sequence, was amplified by polymerase chain reaction (PCR). After purification of PCR products, it was ligated with pET-22b(+) vector after digestion with *Nde*I and *Xho*I enzymes. Expression of ModD in transformed BL21(DE3) cells was induced by isopropyl-beta-D-thiogalactopyranoside (IPTG, Promega, Madison, WI). The soluble ModD protein was extracted after cell disruption by sonication. Recombinant ModD (rModD) containing a C-terminal histidine tag was purified using Ni-nitrilotriacetic acid (NTA) resin (Qiagen, Chatsworth, CA).

Production of Rabbit Antisera

Rabbit antisera were prepared commercially (Panigen Corporation, Inc., Madison, WI). Two New Zealand White rabbits were first immunized with 500 μg of CF from *M. avium* or *M. paratuberculosis*. Each rabbit then received three more inoculations of 250 μg of relevant CF at 2-week intervals. At every immunization the CF was combined with 0.5 ml of incomplete Freund's adjuvant. The rabbits were sacrificed 2 weeks after the fourth immunization. Serum was harvested and stored in small aliquots at −20°C. One additional rabbit was immunized with rModD following the same protocol.

Peptide ELISA

Streptavidin-coated 96-well microtiter plates (Pierce Biotechnology, Inc., Rockford, IL) were washed using buffer (KPL number 50-63-00); then the biotinylated peptides were applied (1 nmol per well) and incubated for 1 h at room temperature. After washing, the plates were air dried, sealed, and stored at 4°C until use. Rabbit serum diluted 1:5000 (100 μl) was added to each well and incubated 30 min at room temperature. Wells were washed three times with buffer (KPL number 50-63-00) followed by addition of horseradish peroxidase (HRP)-conjugated antibodies to rabbit immunoglobulin (Ig) G (Vector Laboratories, Inc., Burlingame, CA). Incubation and washing were carried out as previously described. For peptide ELISAs using bovine sera, a serum dilution of 1:100 was used with an incubation period for antibody binding of 3 h. Then HRP-conjugated antibodies to bovine IgG (KPL, Gaithersburg, MD) were added to detect antibody binding. Finally, each well received 100 μl of TMB solution (Moss, Inc., TMBE-500). After incubation at room temperature for 10 min, stop solution was added. The optical density (OD) of the contents of each well was measured at 450 nm.

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In Silico Analysis

B-cell epitope prediction was performed using Web server-based software (Saha, 2004; accessible at http://www.imtech.res.in/raghava/bcepred). Alignment of amino acid sequence was conducted with CLUSTALW, available at http://clustalw.genome.ad.jp.

RESULTS

Binding of Rabbit Antiserum to ModD Peptides

Rabbit serum anti-rModD bound to both native and rModD by immunoblot (data not shown). This antiserum reacted strongly (ELISA

OD > 1.0) with 12 peptides clustered near the N- and C-terminal ends of ModD (Figure 1C). The amino acid sequences of the most reactive peptides in their local region (ELISA OD > 0.3) are listed in Table 1.

Binding of rabbit anti-*M. avium* CF and anti-*M. paratuberculosis* CF to ModD peptides near the N-terminal ends of the protein was similar to that of rabbit anti-rModD (Figure 1). However, these two anti-CF antisera reacted differently to peptides near the C-terminal end of ModD

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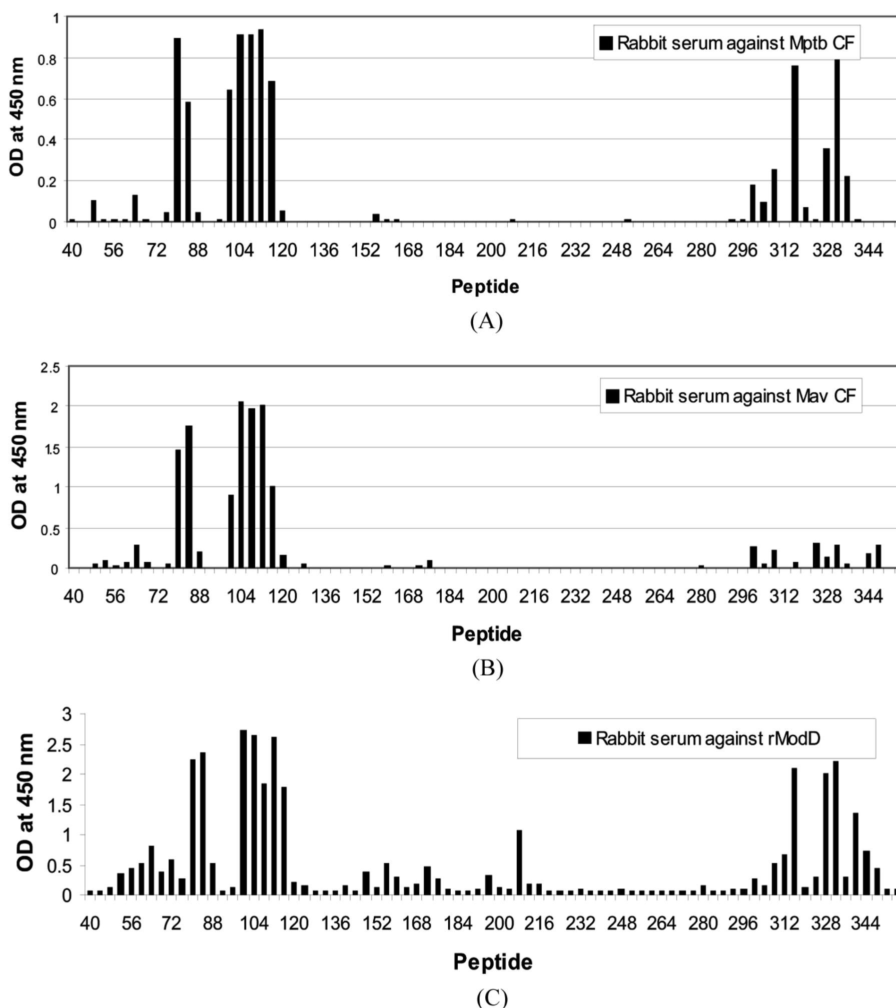

FIGURE 1 Reactivity of rabbit polyclonal sera to synthetic peptides of ModD in ELISA. The rabbit polyclonal sera were prepared against (A) *M. paratuberculosis*, (B) *M. avium* culture filtrate, and (C) recombinant ModD. The ELISA OD shown in (A) and (B) was subtracted from the corresponding OD of the negative control serum. The ModD peptides were identified by number corresponding to the amino-terminal amino acid of the complete ModD protein of *M. paratuberculosis* K10. The peptides corresponding to signal sequence were not included.

TABLE 1 Peptide Sequences Covering the Dominant Antigenic Determinant

Peptide Number[a]	Sequence[b]	Residues[a]
64	QPAPNAQPAPGAPA	64–77
72	APGAPAPNGQPAPA	72–85
84	PAAPAPNDPNAAPP	84–97
100	GAPPNGAPPPPVDP	100–113
112	DPNAPPPPPADPNA	112–125
148	ASHLDYGSALLSKV	148–161
156	ALLSKVTGPPPMPD	156–169
172	PPVANDTRIVMGRL	172–185
196	NNAKAAVRLGSDMG	196–209
208	MGEFFMPYPGTRIN	208–221
300	PAAPPAAPGGPGAP	300–313
316	GAPGTPAAPGAPAA	316–329
332	PAAPGAPAAPGAPA	332–345
340	APGAPAPGQAPAVE	340–353

[a]Each peptide was 14 amino acids with an overlap of ten amino acids. It covers only the mature form of ModD.

[b]Residues are listed in single-letter code starting from the N-terminal end.

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(Figure 1, A and B). In particular, peptide 316 reacted significantly more with rabbit anti-*M. paratuberculosis* CF than with rabbit anti-*M. avium* CF. The specificity of ModD peptides in this region, in contrast with crude CF and rModD, was demonstrated by ELISA using the three different rabbit antisera (Figure 2). The probable basis for *M. paratuberculosis* specificity of peptide 316 was found by alignment of ModD from *M. paratuberculosis* and *M. avium*, which showed that the latter has seven additional amino acids in the region corresponding to ModD peptide 316 of *M. paratuberculosis* (Figure 3).

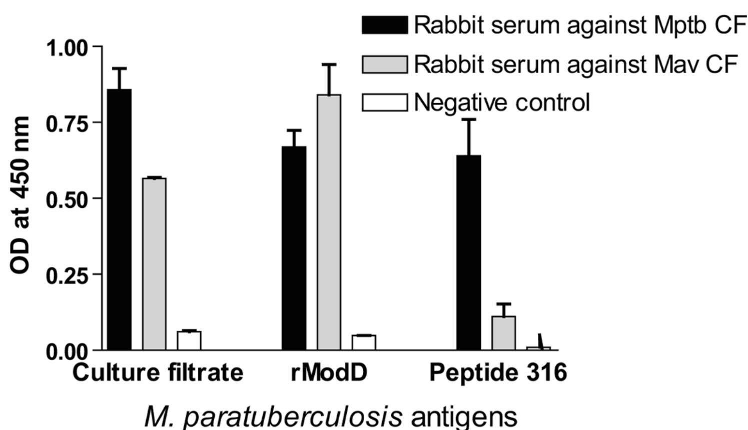

FIGURE 2 Reactivity of culture filtrate, recombinant ModD, and peptide 316 to rabbit polyclonal sera against culture filtrate from *M. avium* and *M. paratuberculosis*.

```
                      316          320            329
M. paratuberculosis   GAP - - - - - GTPAAPG - - APAA
M. avium              GAPGAPAPGAPAAPGVTAPAA
                      ***          * *****      ****
```

FIGURE 3 Alignment of ModD peptide 316 of *M. paratuberculosis* and *M. avium*. Asterisks indicate identical amino acid residues.

Reactivity of Bovine Serum from Naturally Infected Cattle to ModD Peptides

Initially the peptide ELISAs using bovine sera were conducted as described for rabbit antiserum. However, no measurable ELISA OD to any synthetic peptide was observed, even though the same bovine sera reacted strongly by ELISA to rModD. By extending the assay reaction time to 3 h, low-level ELISA reactivity (ODs 0.05 to 0.10) to a few of the peptides was seen. However, binding of negative control bovine serum to peptides also was observed supporting the previously observed non-*M. paratuberculosis* specificity of rModD. To explain the low reactivity of peptide to bovine sera, two longer, 26-amino-acid peptides covering the two main rabbit B-cell epitope regions, peptide 100 and 328, were synthesized for evaluation. The peptide 328 was preferred to peptide 312 because of its broad reactivity to rabbit serum as shown in Figure 1C. Bovine sera from infected cattle ($n = 6$) reacted significantly more to the longer ModD peptide covering amino acids 328 to 353 (Figure 4).

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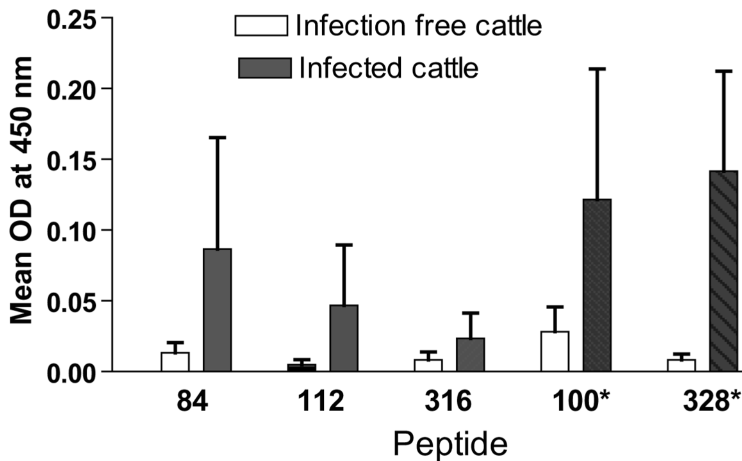

FIGURE 4 Mean ELISA OD of ModD synthetic peptides to bovine sera. The difference of mean OD was analyzed by Student's *t*-test and the asterisks indicates difference at 95% confidence level. Peptides 100 and 328 are marked with asterisks to indicate they are longer, 26 instead of 14 amino acids.

TABLE 2 Prediction Rate of B-Cell Epitope on ModD

Physicochemical Properties[a]	Number of Predicted B-cell Epitopes	Number of Observed Reactive Peptides[b]
Hydrophilicity (1.9)	10	4
Flexibility (2)	4	0
Accessibility (1.9)	12	6
Turns (2.4)	2	1
Exposed surface (2.3)	0	0
Polarity (1.8)	0	0
Antigenic propensity (1.9)	5	1

[a]Thresholds of each parameter are indicated within parentheses.
[b]Number of peptide matching more than 50% of each open reading frame.

Accuracy of *In Silico* B-Cell Epitope Prediction

As shown in Table 1, 14 ModD peptides reacted with rabbit anti-CF antisera, indicating they contain B-cell epitopes. These experimentally identified epitopes were compared with ModD epitopes predicted by BcePred. In general, epitope prediction was poor with only 0 to 43% of the observed epitopes being predicted, depending on the prediction parameter selected (Table 2). The best predictions were obtained with the "Accessibility" parameter. This correctly identified six of 12 observed ModD epitopes. The combination of multiple parameters only resulted in worse epitope prediction.

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DISCUSSION

Ratliff et al. found that supernatant from proliferating *Mycobacterium vaccae* contained a protein that bound to fibronection, thus called fibronectin attachment protein (FAP; synonymous with ModD), and that polyclonal and monoclonal antibodies to FAP of *M. vaccae* bound to both FAP and *M. vaccae* cells. In addition, monoclonal antibodies to FAP cross-reacted with a cell-wall component in BCG, *M. tuberculosis, M. kansasii,* and *M. avium* showing inhibition of bacterial cell binding to fibronectin.[18] This indicated that FAP of *M. vaccae* exists both on the bacterial cell surface and in culture supernatants, and is conserved among mycobacteria. Conservation of FAP among mycobacteria was also demonstrated by showing that FAP of *M. avium* inhibited the binding of FAP of *M. leprae* to fibronectin.[19] Amino acid alignment of the FAP of *M. avium, M. leprae*, and *M. tuberculosis* revealed that all three mycobacteria have conserved regions. However, FAP of *M. avium* has additional amino acid insertions near its N-terminus and its C-terminus. In the conserved regions the three FAP proteins are 50% identical.[19]. Our data extend these observations to show that the FAP of *M. avium* and *M. paratuberculosis* are 85% identical, and as in *M. tuberculosis,*

highly antigenic.[20–22] However, our data also suggest that there is a high probability that FAP (ModD) of *M. paratuberculosis* has linear B-cell epitopes specific to *M. paratuberculosis,* in spite of the highly conserved nature of this protein.

Previously we described five proteins, including ModD, that are secreted by *M. paratuberculosis* during in vitro culture in Watson–Reid medium.[14] Recombinant forms of these proteins reacted with rabbit antisera to CF but not antisera to heat-treated whole cells. In the present study, the existence of ModD in CF was again confirmed by peptide ELISAs using rabbit anti-*M. paratuberculosis* CF as well as rabbit anti-rModD. Only one peptide, covering ModD amino acids 208 to 221, showed significant reactivity with rabbit anti-rModD but not the other rabbit antisera to CF. Though we can not explain this difference, recent studies from other laboratories have also shown that several proteins of *M. tuberculosis*, when expressed in *Escherichia coli*, were unable to completely mimic their native counterparts in structure and antigenicity.[23,24] We speculate that a structural change in rModD may have affected antibody binding, i.e., epitope structure.

We demonstrated that ModD is immunogenic in cattle as well as in rabbits, consistent with the observations of Beatty et al. with *M. bovis* BCG.[20] Visualization of live infected macrophages showed release of ModD by *M. bovis* and the association of ModD with MHC class II molecules in late enodsomal/lysosomal compartments, suggesting that these proteins are prime candidates for host cell processing and presentation by MHC class II molecules.

Consistent with our previous ELISA results using *M. paratuberculosis* rModD, this protein shares many epitopes in common with *M. avium* ModD. Amino acid homology is greatest between amino acids 76 and 130. Although clinically evident infection of cattle with *M. avium* is not commonly reported, the existence of common epitopes likely explains the lack of specificity of the *M. paratuberculosis* rModD ELISA. However, mapping of ModD using overlapping peptides in ELISAs demonstrated the existence of *M. paratuberculolsis*-specific epitopes. Peptides covering ModD amino acids 316 to 354 strongly reacted with rabbit anti-*M. paratuberculosis* CF but weakly reacted with rabbit anti-*M. avium* CF. Peptides covering amino acids 316 to 329 had differences in antisera reactivity, and amino acid alignment of *M. paratuberculosis* and *M. avium* ModD revealed that seven amino acids of *M. paratuberculosis* ModD are deleted in comparison with *M. avium* ModD, thus explaining linear epitope specificity.

Epitope mapping by ELISA with rabbit sera identified epitopes not predicted by in silico analysis. B-cell epitope predictions using BcePred showed that none of the seven parameters tried predicted more than 50% of observed epitopes (Table 2). This suggests that the database of physicochemical parameters for prediction of B-cell epitopes requires

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strengthening, at least as related to mycobacteria. Reliable prediction of species-specific epitopes by amino acid alignment of *M. paratuberculosis* ModD and *M. avium* ModD also was not feasible due to the limited number of strains this far sequenced. For example, the ModD amino acid alignment of *M. paratuberculosis* with that of *M. avium* 101 (NCBI accession number Q48919) indicate this region might be species-specific, but the peptide ELISA revealed that this area of ModD is highly cross-reactive. This discrepancy could be due to the different *M. avium* strain used to produce CF for rabbit immunization, *M. avium* ATCC 35712. Although the specific amino acid sequence of ModD from this strain has not been reported, a high rate of variability in *M. avium* subspecies *avium* ModD has been described.[25]

Epitope mapping of ModD using sera from naturally infected cattle revealed that technical modifications to the assay are required to produce significant ELISA OD values. Using the same 30-min incubation period of serum with peptides as for rabbit serum, no detectable ELISA OD was found with bovine serum for any of the 80 ModD peptides. Extending the serum-peptide incubation time to 3 h produced weak but significant differences in bovine antibody binding to the peptides among sera. One possible explanation for this difference in rabbit versus bovine peptide ELISA reactivity is differences in mode of exposure, i.e., natural infection with native protein versus experimental immunization with recombinant protein. Conformational changes or deglycosylation of rModD may have occurred as has been shown for other mycobacterial proteins expressed in *E. coli.*[22–24] Another explanation is different animal species or individuals may simply respond differently and generate a different repertoire of antibodies to ModD epitopes.[26,27]

We postulate that cattle may require a longer peptide for reactivity in ELISA. Although the size of most known B-cell epitopes is relatively small, usually six to nine amino acids,[28–30] the report of Welling et al. supported the necessity of longer peptides to illicit a stronger immune response.[31] In fact, we found that peptide ELISAs using longer, 26- versus 14-amino-acid, peptides produced higher ELISA OD values with serum from *M. paratuberculosis*-infected cattle. The longer peptide might facilitate folding to produce a secondary structure more similar to the native protein. Alternatively, the ModD epitope recognized by cattle may simply be longer than the minimal length of continuous epitope shown in other studies typically done with laboratory animals.

CONCLUSIONS

The reported data revealed that ModD is an immunogenic secreted protein of *M. paratuberculosis.* It has both cross-reactive and species-specific

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epitopes. The peptides around the C-terminal end only showed the greatest specificity for *M. paratuberculosis*, yielding high ELISA OD values with rabbit anti-*M. paratuberculosis* CF serum and low ELISA OD values with rabbit anti-*M. avium* CF serum. The peptide covering amino acids 328 to 353 showed the highest level of specific bovine antibody binding. These novel epitopes represent excellent targets for new paratuberculosis diagnostics based on antibody detection.

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