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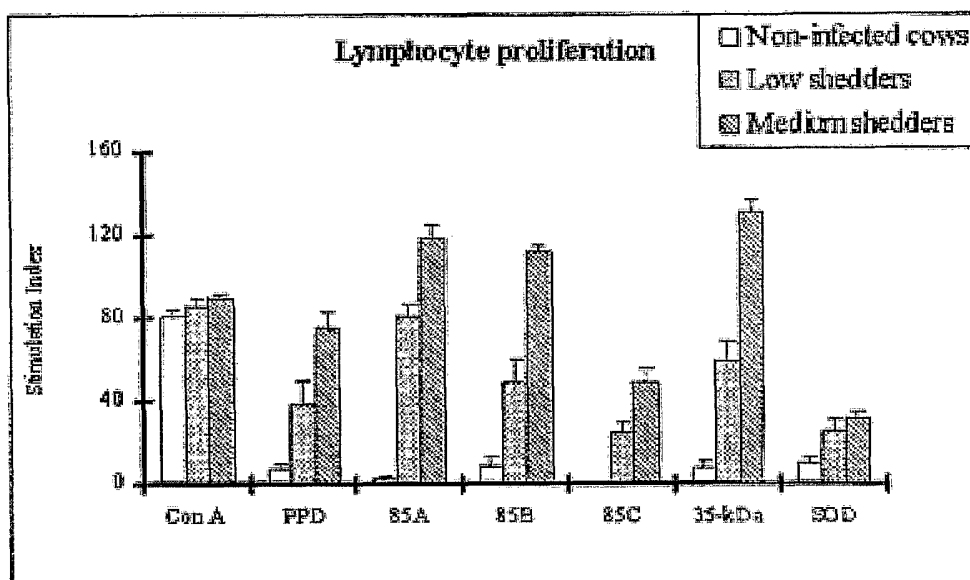
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(54) Title: COMPOSITIONS FOR ELICITING AN IMMUNE RESPONSE AGAINST MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS



(57) Abstract: The invention provides compositions and method for stimulating an immunological response against Mycobacterium avium subspecies paratuberculosis (MPT). The compositions comprise at least five recombinant immunogenic components. The immunogenic components can be MPT antigens or DNA polynucleotides encoding MPT antigens, or combinations thereof. MPT antigens used in the invention include MPT 85A, 85B, 85C, 35kDa, super oxide dismutase (SOD), MptC, MptD and ESAT-6 protein. The method comprises administering the composition to an animal in an amount effective to stimulate an immunological response against MPT bacteria. The method is of benefit to any animal susceptible to MPT infection, but is particularly beneficial for ruminants.



RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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COMPOSITIONS FOR ELICITING AN IMMUNE RESPONSE AGAINST  
MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS

5 This application claims priority to U.S. provisional patent application serial no. 60/653,536, filed February 16, 2005, the disclosure of which is incorporated herein in its entirety by reference.

FIELD OF THE INVENTION

10 The present invention relates generally to stimulation of immunological responses, and more specifically to compositions and methods for stimulating prophylactic or and therapeutic immunological responses against *Mycobacterium avium* subspecies *paratuberculosis*.

15 BACKGROUND OF THE INVENTION

*Mycobacterium avium* subspecies *paratuberculosis* (MPT) is the causative agent of Johne's disease (JD), which causes chronic granulomatous enteritis in ruminants. Clinically affected animals develop chronic diarrhea and progressive weight loss that eventually results in death, while subclinically infected animals mainly have decreased production of milk. JD is of tremendous economic importance to the worldwide dairy industry, causing major losses due to reduced production and early culling of animals with estimates of 20% of U.S. dairy herds affected and costs of \$220 million per year to the dairy industry (Wells, et al. 2000. J. Am. Vet. Med. Assoc. 216:1450–1457). Cattle are most susceptible to infection with this organism within the first 6 months of life, but disease typically does not become evident until 3 to 5 years of age. Infection occurs by ingestion of contaminated manure, colostrum, or milk from infected cows (Sweeney, 1996. Vet. Clin. N. Am. Food Anim. Pract. 12:305–312). Fetal infection also occurs, particularly in pregnant cows with advanced disease (Sweeney, et al. 1992. Am. J. Vet. Res. 53:477–480). Although JD is an important infectious disease of ruminants, there is no effective vaccine against this disease. The only currently available vaccine in the United States consists of killed *M. avium* subsp. *paratuberculosis* in an oil adjuvant (Kormendy, B. 1992. Acta Vet. Hung. 40:171–184; Larsen, et al., 1978. Am. J. Vet. Res. 39:65–69). However, such vaccination programs have raised serious public health concerns. For example, at least one veterinarian was accidentally inoculated in the hand during vaccination of animals (Patterson, et al., (1988) J. Am. Vet. Med. Assoc. 192:1197-

1199). Further, studies have demonstrated that there is a strong reaction at the injection sites after vaccination with this killed bacteria (Kormendy, B. 1992. Acta Vet. Hung. 40:171–184; Larsen, et al., 1978. Am. J. Vet. Res. 39:65–69). Another drawback of this vaccine is that the vaccinated animals become tuberculin skin test positive (Kormendy, B. 1992. Acta Vet. Hung. 40:171–184; Larsen, et al., 1978. Am. J. Vet. Res. 39:65–69). Thus, there is a need for the development of more effective vaccines against JD that can be used as safe and effective prophylactic and/or therapeutic compositions for MPT infection.

#### SUMMARY OF THE INVENTION

The present invention provides compositions and methods for stimulating an immunological response in animals against MPT. The compositions comprise immunogenic components which can be MPT antigens or polynucleotides encoding for MPT antigens, or combinations thereof. In one embodiment, the compositions comprise at least five recombinant immunogenic components. Exemplary MPT antigens include MPT 85A, 85B, 85C, 35kDa, super oxide dismutase (SOD), MptC, MptD and ESAT-6 like protein.

The method comprises administering the composition to an animal in an amount effective to stimulate an immunological response against MPT bacteria. The method is of benefit to any animal susceptible to MPT infection, but is particularly beneficial for ruminants.

Compositions comprising recombinant MPT protein antigens, DNA polynucleotides encoding MPT antigens, or combinations thereof can be formulated with standard pharmaceutical carriers and can be administered via any of a variety of conventional routes. The compositions can be administered at any time to an animal susceptible to contracting MPT infection or an animal that is infected with MPT. However, it is preferable to administer the compositions of the invention prior to MPT infection, such as by administration to pregnant animals who can transfer prophylactic immunologic components to their newborns via colostrum, or by administration during the period from one to five weeks after birth.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graphical representation of data from analysis of proliferative responses of peripheral blood mononuclear cells from infected and healthy control cows stimulated *in vitro* with 5 MPT recombinant proteins. The results are expressed as a stimulation index and the error bars represent standard deviation from the mean. No significant proliferation was

noted to any antigen by PBMCS from non-infected cows ( $P>0.05$ ). 85A and the 35-kDa protein showed most proliferative activity in low and medium shedders, respectively.

Fig. 2. is a graphical representation of data from analysis of interferon- $\gamma$  production in response to individual antigens in related to MPT shedding levels. The results are given as O.D. values in stimulated wells – O.D. values in control (naturally produced IFN- $\gamma$ ) wells. Error bars represent standard deviations from the means. 85A and 85B were most inducible antigens to produce IFN- $\gamma$  in bovine peripheral blood mononuclear cells from both shedders.

Fig. 3. is a graphical representation of data from analysis of antibody responses to individual antigens in relation to MPT shedding levels. Bars represent the means O.D. values at 405 nm. Error bars represent standard deviations from the means. All recombinant antigens showed increases of antibody responses according to shedding levels and antibody responses to the 35-kDa protein were positively separated between non-infected healthy cows and both shedders ( $P<0.01$ ).

Figs. 4A-4D are graphical representations of data from analysis of changes in T cell subset distribution in bovine peripheral blood lymphocytes after stimulation with recombinant antigens, as determined by FACS analysis. Fig. 4A. CD4; Ag 85A and Ag 85B induced a higher proportion of CD4<sup>+</sup> T lymphocytes in medium shedders compared to low shedders while the percentage of CD4<sup>+</sup> lymphocytes was unchanged in non-infected control cattle. Fig. 4B. CD8; Ag 85A increased the proportion of CD8<sup>+</sup> T lymphocytes in medium shedders, while the increased percentage of CD8<sup>+</sup> lymphocytes was very low in non-infected cattle. Fig. 4C. CD25; Ag 85A and Ag85B increased the proportion of CD25<sup>+</sup> T cells in both shedder groups while they had little effect in non-infected cattle. In contrast, Ag 85C and the 35-kDa protein significantly increased the proportion of CD25<sup>+</sup> T cells only in the medium shedders ( $P<0.05$ ). Fig. 4D.  $\gamma\delta$ <sup>+</sup> T-cells; all antigens resulted in significantly lower increases in all cell subsets in both the low and medium shedder groups except SOD for  $\gamma\delta$ <sup>+</sup> T cells in medium shedders.

Fig. 5 is a graphical representation of data from analysis of differential changes of CD3<sup>+</sup> T lymphocytes in response to stimulation with recombinant proteins and two controls (ConA and PPD). Data are expressed as the average of cells staining positive for CD3 (1 standard error of the mean) in response to each recombinant antigen relative to the shedding level.

Fig. 6 is a graphical representation of data from analysis of increased CD21<sup>+</sup> B lymphocyte subsets in bovine peripheral blood lymphocytes after stimulation with recombinant antigens, as determined by FACS analysis. The results are reported as the

average percent increase in positive-staining cells and the error bars represent 1 standard error of the mean (SEM). Recombinant 35-kDa protein induced the largest increase in CD21<sup>+</sup> B lymphocytes in medium shedders. No significant increase in the proportion of B lymphocytes was observed in response to the other antigens regardless of bacterial shedding levels ( $P>0.05$ ).

Fig. 7. is a graphical representation of data from analysis of IL-2 profiles of bovine PBMCs from non-infected cattle, low and medium shedders after stimulation with recombinant antigens for 24 hrs. Results represent the mean fold increases of IL-4 over unstimulated PBMCs, which served as calibrators. Ags 85 A and B most strongly stimulated medium shedders while the 35 kDa protein and SOD had lesser effects ( $p < 0.05$ ).

Figs. 8A-8C are graphical representations of data from analysis of comparison of cytokine mRNA profiles for IFN- $\gamma$  (Fig. 8A), IL-12p40 (Fig. 8B) and TNF- $\alpha$  (Fig. 8C) of bovine PBMCs from non-infected cattle, low and medium shedders after stimulation with recombinant antigens for 24 hrs. Results represent the mean fold increase over un-stimulated PBMCs, which served as calibrators. The results are similar with Ags 85 A and B most strongly stimulating medium shedders while the 35 kDa protein and SOD had lesser effects.

Fig. 9. is a graphical representation of data from analysis of IL-4 mRNA profiles of bovine PBMCs from non-infected cattle, low and medium shedders after stimulation with recombinant antigens for 24 hrs. Results represent the mean fold increases of IL-4 over unstimulated PBMCs, which served as calibrators. The 35-kDa protein strongly stimulated IL-4 mRNA expression in both low and medium shedders.

Fig. 10 is a graphical representation of bacterial recovery from spleen and liver after administration of the indicated DNA constructs and controls and subsequent challenge with MPT.

Fig. 11 is a graphical representation of the mean number of granulomas in spleen and liver after administration of the indicated DNA constructs and controls and subsequent challenge with MPT.

Figs. 12A and 12B are photographic representations of Ziehl-Neelsen staining of tissues revealing numerous acid-fast bacilli (Fig. 12A). In contrast, the infection was much less severe in the mice vaccinated with the MPT DNA constructs encoding all five antigens (Fig. 12B).

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions and methods for stimulating an immunological response against MPT in an animal. The compositions comprise DNA polynucleotides encoding MPT antigens, MPT antigens, or combinations thereof. In one embodiment, at least five immunogenic components are selected from recombinant MPT antigens and DNA polynucleotides. The method comprises administering the composition to an animal in an amount effective to stimulate an immunological response against MPT bacteria.

As used herein, an "immunological component" is a component of the composition that can directly or indirectly stimulate an immunological response. Accordingly, when introduced into an animal, expression vectors comprising DNA polynucleotides encoding MPT antigens enter cells of the animal and express MPT antigens. The expressed MPT antigens in turn stimulate an immunological response. Thus, a DNA polynucleotide encoding an MPT antigen is considered an immunogenic component which indirectly stimulates an immunological response. In respect of an administered MPT protein antigen, since the antigen is recognized directly by the immune system, the antigen is considered an immunogenic component which directly stimulates an immunological response.

The method can provide benefit to any animal susceptible to MPT infection, where infection is considered to mean colonization of the intestinal mucosa of the animal by MPT. However, the compositions and method are particularly well suited for prophylaxis or therapy for MPT infection of ruminants, including but not limited to cattle, sheep, goats, deer and elk, antelope, and buffalo.

Thus, the compositions can be administered to any MPT infected or non-infected animal. Administration of the compositions to infected animals according to the method of the invention is considered to stimulate a therapeutic immunological response. However, it is preferable to administer the compositions prior to MPT infection to stimulate a prophylactic response. For example, the compositions can be administered to a pregnant animal who can transfer prophylactic immunologic components to their non-infected newborns via colostrum. Alternatively, the compositions can be administered during the period from one to five weeks after birth to provide a prophylactic effect which can prevent infection or reduce the severity of disease if infection occurs. Thus, in one embodiment, the method of the invention is prophylactic for MPT infection, while in another embodiment, the method is therapeutic for MPT infection. The method can also be used for prophylaxis or therapy of Johne's Disease.

Suitable MPT antigens for use in the invention include but are not limited to MPT proteins 85A, 85B, 85C, 35kDa, SOD, MptC, MptD and ESAT-6 like protein. Recombinant MPT protein antigens can be obtained for use in the invention by techniques known to those skilled in the art, such as by conventional recombinant cloning methods. Suitable DNA cloning procedures and methods for expressing and purifying recombinant proteins are known. (See, for example, Sambrook et al., 2001, *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, New York, NY). In general, to obtain recombinant MPT protein antigens, MPT genomic DNA can be obtained from an MPT culture according to standard methods, such as by the well known alkaline lysis procedure. The DNA encoding the antigens can be amplified, such as by the polymerase chain reaction, from the genomic DNA and the amplification products can be cloned individually or in various combinations into a one or more suitable expression vectors. Appropriate host cells can be transfected with the expression vector and the transfected cells can be cultured under appropriate conditions for expression of the antigens. The antigens can be subsequently extracted and purified from the culture according to standard techniques.

For administration to animals, suitably purified recombinant MPT antigens can be combined with standard pharmaceutical carriers. Acceptable pharmaceutical carriers for use with proteins are described in Remington's Pharmaceutical Sciences (18th Edition, A. R. Gennaro et al. Eds., Mack Publishing Co., Easton, Pa., 1990). Further, the antigens may be provided in conventional liposomal or microsomal formulations.

Compositions comprising the MPT antigens for use in stimulating an immunological response can be administered by any acceptable route. Suitable routes of administration include oral, mucosal and parenteral (e.g., intravascular, intramuscular, and subcutaneous injection). Those skilled in the art will recognize that the amount of antigens administered to a particular animal will depend on a number of factors such as the route of administration, and the size, physical condition and the MPT status of the animal. The relative amounts of each antigen in a formulation can be adjusted according to known parameters, such as to provide molar equivalents or other ratios of the antigens. Further, the compositions can be used in a single administration or in a series of administrations to boost the immunological response to the MPT antigens. In general, a total dosage of between 10-200  $\mu$ g of protein can be administered. When DNA encoding MPT antigens is administered, in general, between 30-500  $\mu$ g of DNA can be administered.



In one embodiment, the method of the invention comprises administration of a composition comprising at least five recombinant MPT antigens. For example, MPT antigens 85A, 85B, 85C, 35 kDa antigen and superoxide dismutase (SOD) can be administered in a single formulation. 85A, 85B and 85C are fibronectin-binding proteins; the 35 Kda and SOD proteins are outer surface proteins. The DNA sequence encoding the MTP 85A gene and the amino acid sequence of the 85A gene, is provided in GenBank accession no. AF280067 (October 10, 2003, entry). The DNA sequence encoding the MTP 85B gene and the amino acid sequence of the 85A gene is provided in GenBank accession no. AF219121 85B gene (November 21, 2002 entry). The DNA sequence encoding the MTP 85C gene and the amino acid sequence of the 85C gene is provided in GenBank accession no. AF280068 (November 21, 2002 entry). The DNA sequence encoding the MTP SOD gene and the amino acid sequence of the SOD gene is provided in GenBank accession no. AF180816 (November 30, 2001 entry). The DNA sequence encoding the 35 kDa protein is provided herein as SEQ ID NO:1. The amino acid sequence of the 35 kDa protein is provided herein as SEQ ID NO:2. Additional MPT include MptC, MptD and ESAT-6 like protein. The DNA sequence encoding the MptC protein is provided herein as SEQ ID NO:3. The amino acid sequence of the MptC protein is provided herein as SEQ ID NO:4. The DNA sequence encoding the ESAT-6 like protein is provided herein as SEQ ID NO:5. The amino acid sequence of the ESAT-6 like protein is provided herein as SEQ ID NO:6. The DNA sequence encoding the MptD sequence is provided herein as SEQ ID NO:7. The DNA sequence encoding the MptD amino acid sequence is provided herein as SEQ ID NO:8.

The DNA sequences of primers used to amplify DNA encoding the MPT antigens used herein from genomic MPT DNA are provided in Table 1.

**Table 1.**

| Gene/<br>primer name | SEQ<br>ID:<br>NO | Primer Sequence<br>(5' -> 3') | Length<br>of DNA<br>product<br>(bp) | Accession No. of<br>ss amplification<br>product and amino<br>acid sequence of<br>antigen |
|----------------------|------------------|-------------------------------|-------------------------------------|------------------------------------------------------------------------------------------|
| 85A<br>pVR85AF       | 9                | CGGGATCCATGATGACGCTTGTCGACA   | 1050                                | AF280067                                                                                 |
| pVR85AR              | 10               | CGGGATCCTTAGGTGCCCTGG         |                                     |                                                                                          |

|                                         |    |                            |      |          |
|-----------------------------------------|----|----------------------------|------|----------|
| 85B<br>pVR85BF                          | 11 | CGGGATCCATGACAGATCTG       | 1000 | AF219121 |
| pVR85BR                                 | 12 | CGGGATCCTTATCCGCCGCC       |      |          |
| 85C<br>pVR85CF                          | 13 | CGGGATCCATGTCGTTTCATCGAA   | 1100 | AF280068 |
| pVR85CR                                 | 14 | CGGGATCCTCAGGTGGCGGGC      |      |          |
| SOD<br>pVRSODF                          | 15 | GGATCCTGGGACTATGCAGC       | 590  | AF180816 |
| pVRSODR                                 | 16 | AGATCTTCAGCCGAAGATCAGGC    |      |          |
| 35Kda<br>(MAP2121c)<br>pVR35KDF         | 17 | GGATCCCCACTTGGTGATCT       | 910  |          |
| pVR35KDR                                | 18 | AGATCTTCACTTGTACTCATGGAACT |      |          |
| MptC (MAP<br>3734)<br>pVRMPTCF          | 19 | GGATCCCGCGGTCGGCGT         | 1750 |          |
| pVRMPTCR                                | 20 | AGATCTTCATGGTCGAGGTGCCT    |      |          |
| MptD (MAP<br>3733)<br>pVRMPTDF          | 21 | GGATCCCGCCGCATCGAC         | 600  |          |
| pVRMPTDR                                | 22 | AGATCTTCAAGCTAGGCCGGC      |      |          |
| ESAT 6<br>Like(MAP<br>0161)<br>pVRESATF | 23 | GGATCCCGGGCGCGGTG          | 270  |          |
| pVRESATR                                | 24 | AGATCTTCAGAACAGGCCG        |      |          |

In another embodiment, compositions comprising DNA polynucleotides which encode five or more MPT antigens can be prepared. MPT antigen encoding sequences can be obtained by amplification of MPT genomic DNA using appropriate primers and inserting the amplification products into expression vectors in the same manner as described for preparation of recombinant antigen proteins.

Suitable expression vectors contain appropriate eukaryotic transcription and translation signals, and may contain additional elements, such as polyadenylation and/or protein trafficking signals. One example of a suitable expression vector is pVR1020

(available from Vical, Inc., San Diego, Calif.), which contains an immediate-early

cytomegalovirus promoter to promote efficient expression in a eukaryotic host, as well as a plasminogen activator secretion signal to facilitate secretion of the antigens from the cells of the eukaryotic host.

It will be recognized by those skilled in the art that one or more distinct expression vectors, as distinguished from one another by the MPT antigens they encode and/or by their regulatory or other elements, such as polycloning sites, will be used in the instant method. Thus, a single expression vector encoding at least five MPT antigens, or at least five expression vectors each encoding a different MPT antigen, or combinations of expression vectors each encoding at least one MPT antigen, can be used in the present method to deliver polynucleotides encoding at least five MPT antigens.

In one embodiment, DNA polynucleotide sequences encoding MPT antigens 85A, 85B, 85C, SOD, MptC, MptD, 35kDa, and ESAT6-like proteins can be provided in separate expression vectors that can be used for protein expression and purification and for administration in various combinations to animals for stimulating an immune response.

The expression vectors encoding the MPT antigens may be formulated in any pharmaceutically effective preparation for administration to the animals. Such formulations may be, for example, a saline solution such as phosphate buffered saline (PBS). It is preferred to utilize pharmaceutically acceptable formulations which also provide long-term stability of the DNA. Thus, it is preferable to remove and/or chelation trace metal ions from the formulation buffers or from vials and closures in which the DNA is stored to stabilize and protect the DNA during storage. In addition, inclusion of non-reducing free radical scavengers, such as ethanol or glycerol, is useful to prevent damage of the DNA from free radical production that may still occur, even in apparently demetalated solutions. Further, the DNA may be provided in conventional liposomal or microsomal formulations.

There is no limitation to the route that the DNA polynucleotides of the invention can be delivered, so long as their delivery stimulates an immunological response against MPT in the recipient animals. Accordingly, the DNA polynucleotides of the present invention can be administered to the animal by any means known in the art, such as enteral and parenteral routes. These routes of delivery include but are not limited to intramuscular injection, intraperitoneal injection, intravenous injection, and oral delivery. A preferred route is intramuscular.

In another embodiment, the composition of the invention comprises at least five immunogenic components which are provided as a combination of MPT protein antigens and DNA polynucleotides encoding MPT antigens. Such compositions can be obtained by

combining the recombinant MPT antigens described herein and the polynucleotides encoding MPT proteins described herein. In this regard, the polynucleotide sequences can be present in one or more expression vectors and the MPT antigens can be provided as recombinant proteins. Compositions comprising recombinant MPT antigens and polynucleotides encoding MPT antigens can be combined with conventional pharmaceutical carriers and administered as described herein and/or according to standard techniques. Conventional liposomal or microsomal preparations of the recombinant MPT antigens and polynucleotides encoding MPT antigens can be provided. Further, and as will be recognized by those skilled in the art, whether or not the compositions comprise recombinant antigens alone as the immunogenic components, DNA polynucleotides alone as the immunogenic components, or combinations of recombinant antigens and DNA polynucleotides encoding the recombinant antigens as the immunogenic components, the compositions of the invention may further comprise a suitable adjuvant.

Thus, and without intending to be bound by any particular theory, administration of the recombinant MPT antigens, polynucleotides encoding recombinant antigens, and/or combinations thereof according to the method of the invention is believed to stimulate an immunological response that can be prophylactic or therapeutic with respect to MPT infection.

The following examples describe the various embodiments of this invention. These examples are illustrative and are not intended to be restrictive.

### Example 1

This Example provides a comparison of distinct lymphoproliferation effects in response to stimulation with individual antigens.

To examine lymphoproliferative responses, five MPT recombinant antigens, 85A, 85B, 85C, 35 kDa antigen and superoxide dismutase (SOD), were analyzed for their ability to elicit proliferative responses in PBMCs obtained from cows with different MPT shedding levels. For this and other Examples as indicated herein, a total of 38 Holstein cows, 2 to 3 years old, were divided into 3 groups. Healthy controls (n=18) were negative for MPT infection as determined by negative fecal culture and negative IS900 PCR testing. The healthy controls came from a farm that has been fecal culture and IS900 PCR negative for the past ten years. Positive animals were subdivided into low shedders (n=16) and medium shedders based on the number of colony forming units (CFU)/gram of feces (n=4). Low

shedders are considered animals with 1-30 CFU/gram of feces. Medium shedders are considered animals with between 31-300 CFU/gram of feces. Heavy shedders (>300 CFU/gm feces) were unavailable since they are culled immediately from farms once they are identified. Fecal cultures and IS900 PCR testing to determine MPT infection status were preformed as previously described (Shin et al, (2004) J. Vet. Diagn. Invest. 16:116-120).

For use in lymphproliferations assays, recombinant antigens 85A, 85B, 85C, 35 kDa antigen and SOD were cloned and expressed using standard techniques and as previously described. (Dheenadhayalan et al., (2002) DNA Seq. 13:287-294; Shin et al., (2004) J. Vet. Sci. 5:111-117) and purified as previously described (Skeikyet al., (1998) J. Immunol. 161:6171-6179). The antigens used in these Examples had negligible (10 pg/ml) endotoxin in a Limulus amebocyte assay.

For isolation and culture of bovine peripheral blood mononuclear cells, peripheral blood (20 ml) of all cows was collected from the tail vein with heparinized vacuum tubes. Isolation of lymphocytes from heparinized blood was performed by differential centrifugation using Histopaque 1.077 (Sigma). Twenty ml of heparinized whole blood was layered over 15 ml Histopaque in a 50-ml sterile polypropylene tube (Falcon) and then centrifuged at 1000 X g for 30 min at room temperature. The plasma layer was discarded and the mononuclear cell layer was carefully collected and washed three times with phosphate-buffered saline (PBS, pH 7.2). Contaminating red blood cells were lysed with 0.87% ammonium KCl buffer by inverting for 2min at room temperature, then immediately adding 30 ml PBS.

The washed cell pellets were suspended in PBS and counted using a hemacytometer and trypan blue to determine percent viability. Differential cell counts consistently showed greater than 96 % lymphocytes, 1 % monocytes and less than 3 % granulocytes in the cell suspension.

The lymphocytes were resuspended at  $2 \times 10^6$ /ml in RPMI 1640 containing 10 % endotoxin free FCS (Celect Gold; ICN Biomedicals, Inc., Costa Mesa, CA), 2 mM L-glutamine, 10 mM HEPES, 100 IU/ml Penicillin, 100  $\mu$ g/mL streptomycin and 50  $\mu$ g/mL gentamycin (Sigma) and 250ul were added to either 96-well round-bottomed plates or flat-bottomed plates, depending on the purposes of the experiment.

To investigate lymphocyte proliferation in response to the individual antigens, a blastogenesis assay was performed. Briefly, PBMCs were initially incubated in a 96-well flat-bottomed microplate for 3 days at 37C in a humidified atmosphere with 5% CO<sub>2</sub>. Cultures were then stimulated with Con A (10  $\mu$ g/mL), purified protein derivative (PPD) (each positive controls) (10  $\mu$ g/mL) or each purified recombinant protein (10  $\mu$ g/mL) and 40

$\mu$ l (1.0  $\mu$ Ci) of methyl-3H-thymidine (PerkinElmer Life Science Inc, MA, USA) in culture medium were added to each well. The cells were incubated for an additional 18h in the same conditions, and the cells were then harvested using a semi automatic cell harvester (Skatronas Liter Norway). Blastogenic activity was recorded as counts per minute (cpm) of radioactivity based on liquid scintillation counting. Results were expressed as stimulation indices (SI) calculated as follows:

$$\text{SI (stimulation Index)} = \frac{(\text{CPM of antigen stimulated positive culture}) - (\text{CPM of background})}{(\text{CPM of control culture or Negative}) - (\text{CPM of background})}$$

For this and other Examples herein, statistical analysis of data was performed in Excel and GraPad Prism software package version 2.0. Differences between individual groups, antigens and cytokine gene expression were analyzed with the Student's *t*-test. Differences were considered significant if probability values of  $P < 0.05$  were obtained.

As demonstrated in Fig. 1, proliferative activities of bovine PBMCs from medium shedder cows were higher than other groups in response to all recombinant proteins and two positive controls ( $P < 0.05$ ) although there was variation among individual cows. PBMCs from medium shedder cows treated with 85A, 85B and the 35-kDa protein antigens demonstrated a stimulation index (SI) significantly higher ( $P < 0.005$ ) than that of PBMCs treated with other recombinant antigens. In addition, proliferative responses to the 35kDa protein in low shedders were even greater than those of medium shedders in response to 85C and SOD (Fig. 1). Thus, this Example indicates that the 85A, 85B and the 35-kDa protein antigens may be important in affecting lymphocyte proliferation in animals previously exposed to MPT.

## Example 2

This Example demonstrates the effects of recombinant antigens on IFN- $\gamma$  production. To analyze IFN- $\gamma$  production, IFN- $\gamma$  levels were measured in culture supernatants using a commercial kit specific for bovine IFN- $\gamma$  following the manufacturer's instructions (Biosource Int. Camarillo, CA). The plates were read at 450 nm in a Bio-Tek 312E ELISA reader (BioTEK Instruments, Inc, Winooski, VT 05404-0998), using any reference filter from 630 nm to 750 nm. The results were calculated based on comparison of negative and positive control optional density (O.D). Results were determined as either negative ( $< \text{OD of positive}$

control) or positive ( $>$  OD of positive control) relative to the cut-off value according to the manufacturer's instructions.

IFN- $\gamma$  production after stimulation with the five recombinant antigens or two positive controls was measured in PBMCs from infected and uninfected control cows. The results are presented in Fig. 2 as corrected OD values (OD of antigen stimulated minus OD of control) representing the elevation of IFN- $\gamma$  production by the various antigens.

As can be seen from Fig. 2, all recombinant antigens tested induced significant release of IFN-  $\gamma$  in cultures of bovine PBMCs from infected cattle compared to uninfected controls ( $P < 0.05$ ), and IFN-g levels were consistently higher in medium shedders than in low shedders ( $P < 0.05$ ). The recombinant antigens 85A and 85B induced significantly higher levels of IFN-  $\gamma$  in the low shedders than the other recombinant antigens tested and as compared to the two positive controls ( $P < 0.05$ ). Thus, this Example indicates the recombinant antigens 85A and 85B may be important in stimulating a cell-mediated response against MPT.

### Example 3

This Example provides a comparison of antibodies in sera isolated from non-infected and infected cows which recognize the recombinant antigens of the invention.

Enzyme-linked immunosorbent assays (ELISA) were performed to evaluate the seroreactivity of the recombinant antigens following steps as previously described (Shin , et al., (2004) J. Vet. Sci. 5:111-117). Briefly, an indirect ELISA was optimized using 2.5, 5 or 10  $\mu$ g/mL of each antigen and 1:100 diluted serum by checkerboard titration. Flat-bottomed 96-well plates (Maxisorp, Nunc, Denmark) were coated with 100  $\mu$ L of each antigen in carbonate-bicarbonate buffer (14.2 mM Na<sub>2</sub>CO<sub>3</sub>, 34.9 mM NaHCO<sub>3</sub>, 3.1 mM NaN<sub>3</sub>, pH 9.5) at 4°C overnight, followed by washing three times with PBS containing 0.05 % Tween 20 (PBST, washing buffer) using a microwell plate washer Bio-Tek ELx405 (BioTEK Instruments, Inc, Winooski, VT). Uncoated sites in the wells were blocked with 5% skim milk in PBST at 37 °C for 1h. The plates were washed twice with PBST and 100  $\mu$ L of optimally diluted (1:25,000) conjugated anti-bovine IgG-HRP (Sigma) was added to all wells and incubated at 37 °C for 1h. The plates were washed three times in PBST and 200  $\mu$ L of 2'-Azino-Bis-Thiazoline-6-Sulfonic acid (Sigma) was added to each well. The plates were incubated at 37°C in the dark. After 30min incubation, stop solution (1M HCl) was added and the plates were read 3 times at 405 nm at 2-minute intervals in a Bio-Tek 312 ELISA reader

(BioTEK Instruments, Inc, Winooski, VT 05404-0998). Positive and negative sera and antigen and antibody controls were included in each plate.

The results from measuring levels of IgG antibodies to the recombinant antigens in sera from both shedder groups and healthy controls are depicted in Fig. 3. Although there was a wide variation in antibody content in sera from individual cows, the mean IgG antibody responses against all recombinant antigens increased significantly in both the low and medium shedder groups. No significant differences were observed among the mean levels of antibody of the low shedder group to any of the antigens tested (Fig. 3). Strikingly, antibody responses to the 35-kDa protein were significantly higher in the medium shedder group than those to the other antigens ( $P<0.05$ ), which may be important because the 35-kDa protein is also effective at stimulating lymphocyte proliferation, and thus may stimulate both cell-mediated and humoral immune responses.

#### Example 4

This Example demonstrates changes in lymphocyte subset distribution in PBMCs obtained from non-infected, low shedder and medium shedder cows in response to stimulation with the recombinant antigens.

To perform this analysis, a single-color flow cytometric analysis was performed with monoclonal antibodies against bovine lymphocyte markers (Table 2). Briefly, cells were washed three times in FACS buffer, incubated with the first

**Table 2.**

| Monoclonal antibody | Isotype | Antigen identified | Ab Reference                                                   |
|---------------------|---------|--------------------|----------------------------------------------------------------|
| IL-A11              | IgG2a   | CD4                | (Brodersen, et al., 1998. Vet. Immunol. Immunopathol. 64:1-13) |
| CACT80C             | IgG1    | CD8 $\alpha$       | (Davis, et al. 1989. Am. Fish. Soc. Symp. 7:521-540.)          |
| MM1A                | IgG1    | CD3                | (Rhodes, et al. 2001. J. Immunol. 166:5604-5610)               |
| BAQ15A              | IgM     | CD21 B cells       | (Mukwedeya, et al. 1993. Vet Immunol Immunopathol 39:177-186)  |
| CACT116A            | IgG1    | CD25 (IL-2Ra)      | (Naessens et al. 1992.                                         |



CACT63A

IgG1

 $\gamma\delta$  T cells

Immunology 76:305-309.)

(Davis, et al. 1996. Vet. Immunol. Immunopathol. 52:301-311)

antibody (Table 1) for 30min at 4 C, washed three times, subsequently incubated with a fluorescein isothiocyanate-labeled horse anti-mouse immunoglobulin antibody (Vector) for 30 min at 4C, washed twice, and collected in 200  $\mu$ l of FACS fixer buffer prior to analysis.

5 Analysis was done on a flow cytometer (FACSCalibur; Becton Dickson). A forward-scatter-side-scatter live gate was used to measure 5,000 to 10,000 lymphocytes per sample. Based on the florescence data of the lymphocytes, the results were expressed as the percentage of cells with positive staining relative to a sample stained with an irrelevant isotype control antibody.

10 As depicted in Figs. 4A-4D, antigen-stimulated T cell and/or B cell subsets were examined by single color flow cytometry for differences in the percentage of  $CD4^+$ ,  $CD8^+$ ,  $CD3^+$  ( $CD3^+$  is depicted in Fig. 5),  $CD21^+$  and  $CD25^+$  lymphocyte subsets, as well as  $\gamma\delta^+$  T cells in PBMC cultures from both shedder groups and healthy controls after stimulation with each recombinant antigen (Figs. 4 and 5). All lymphocyte subsets investigated in this study  
15 increased but, depending on bacterial shedding levels, there were slight differences ( $P < 0.05$ ) between non-infected cattle and low shedders according to recombinant antigens.

CD3 is a pan T-cell marker that is expressed by  $CD4^+$  and  $CD8^+$  cells as well as  $\gamma\sigma^+$  T cells. The proportion of  $CD25^+$  T cells increased in culture regardless of the recombinant antigen used ( $P < 0.05$ ) (Fig. 4C). These results suggest that all antigens used in this study are  
20 able to stimulate sensitized T cells.

While all recombinant antigens tested increased the proportion of  $CD4^+$  cells in cultures of bovine PBMCs from infected cattle compared to uninfected controls ( $P < 0.05$ ), 85A and 85B increased the proportion of  $CD4^+$  T cells to significantly higher levels than 85C, the 35-kDa protein, and SOD ( $P < 0.05$ ) (Fig. 4A). The proportion of  $CD4^+$  T cells was  
25 also greater in cultures treated with 85A and 85B among PBMCs from medium shedders than from low shedders ( $P < 0.05$ ) (Fig. 4A). Further, 85A and 85B did not increase  $CD4^+$  T cells in noninfected cattle. While not intending to be bound by any particular theory, these results may indicate that 85A and 85B antigens induce  $CD4^+$  T cells specifically sensitized by MTP and provide protective immunity against MTP infection by maintaining circulating  $CD4^+$  T-

cell populations in the early infectious phase, i.e., at the time mucosal colonization by MPT is first occurring.

In contrast to the increase of CD4<sup>+</sup> cells induced by all the antigens relative to uninfected controls, a significant increase in the proportion of CD8<sup>+</sup> T cells was found only in cultures treated with 85A, 85B, and 85C and the proportion of CD8<sup>+</sup> cells was also greater in cultures treated with 85A and 85B among PBMCs from medium shedders than from low shedders ( $P < 0.05$ ) (Fig. 4B). Thus, these antigens may preferentially stimulate cell mediated responses.

Only SOD was able to significantly increase the proportion of  $\gamma\sigma^+$  T cells in the cultures of medium shedders ( $P < 0.05$ ) (Fig. 4D). However, SOD stimulated lymphocytes to a lesser degree than the other antigens tested, except for  $\gamma\sigma^+$  T lymphocytes, as the number of  $\gamma\sigma^+$  T cells was significantly higher in PBMC cultures treated with SOD in noninfected cattle, as well as in both shedder groups (Fig. 4D). Thus, SOD may preferentially stimulate  $\gamma\sigma^+$  T lymphocytes compared to the other antigens. Further, because  $\gamma\sigma^+$  T cells are numerous in mucosal tissues, which is the point of entry for mycobacterial pathogens, the SOD antigen may be important in the earlier stages of infection via its preferential stimulation of  $\gamma\sigma^+$  T cells.

All recombinant antigens tested significantly increased the proportion of CD21<sup>+</sup> B cells in cultures of bovine PBMCs from both low and medium shedders compared to uninfected controls ( $P < 0.05$ ) (Fig. 6). Interestingly, the proportion of CD21<sup>+</sup> B cells was significantly higher in cultures of bovine PBMCs from medium shedders than that of the other recombinant antigens tested ( $P < 0.05$ ).

### Example 5

This Example provides a comparison of stimulation of cytokine mRNA production in bovine PBMC's after stimulation with recombinant antigens.

For preparation of RNA and DNase I treatment of cells, PBMC cell pellets were obtained and washed twice in 50 mL phosphate buffered saline (PBS), pelleted and  $5 \times 10^6$  cells were lysed with 350  $\mu$ L lysis buffer according to the manufacturer's recommendations (RNeasy mini kit, Qiagen, CA) and kept at -80 C until RNA extraction and complementary DNA (cDNA) synthesis. Total RNA (tRNA) was extracted from lysed cells or PMBCs using the RNeasy mini kit (Qiagen). The extracted tRNA was treated with 10 U/ $\mu$ l of RNase-free

DNase I at 37 C for 10 min followed by heat inactivation at 95 C for 5 min and then chilled on ice.

For cDNA synthesis, reverse transcription (RT) was performed in a 20  $\mu$ L final volume containing 1.6  $\mu$ L total RNA, 200 U Superscript II RT (GibcoBRL), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 0.01 M DTT, and 0.5 mM dNTPs. The reaction mix was subjected to 42 C for 50 min and inactivated at 70 C for 15 min. The cDNA was analyzed immediately or stored at -20 C until use.

To perform real-time quantitative (RT-PCR), approximately 1 to 5  $\mu$ g of total RNA from each treatment group was reverse transcribed by using Superscript reverse transcriptase, Random Hexamers, and reverse transcriptase reagents (Gibco BRL). Real-time primers and probes were designed by Primer Express software (Applied Biosystems) using the sequences for bovine GAPDH, cytokines and growth factors obtained from Genbank. The internal probes were labeled with the florescent reporter dye 5-carboxyfluorescein (FAM) on the 5' end and the quencher dye N', N', N', N', N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end. The PCR mixture consisted of 400 nM primers, 80 nM Taqman probe and commercially available PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 10  $\mu$ L of the diluted cDNA sample in a final volume of 25  $\mu$ L. The samples were placed in 96-well plates and amplified in an automated flurometer (ABI Prism 7700 Sequence Detection System, Applied Biosystems). Amplicon conditions were 2 min at 50C, 10 min at 95C, followed by 40 cycles at 95C for 15 s and 60C for 1 min. Final quantitation was done using the comparative cycle threshold (C<sub>T</sub>) method and is reported as relative transcription or the *n*-fold difference relative to a calibrator cDNA.

As can be seen from Fig. 7, all recombinant antigens stimulated high levels of IL-2 mRNA from PBMCs of medium shedders, with the antigen 85 complex having a greater effect than either the 35-kDa protein or SOD ( $P < 0.05$ ) (Fig. 7). 85A also induced a high level of IFN- $\gamma$ , IL-12p40, and TNF- $\alpha$  mRNA (Fig. 8A, B, and C, respectively) in medium shedders ( $P < 0.05$ ). Strikingly, PBMCs stimulated with the 35-kDa protein antigen highly expressed IL-4 mRNA in both low and medium shedders ( $P < 0.05$ ) (Fig. 9). This induction of IL-4 mRNA by the 35-kDa protein significantly increased depending on shedding levels ( $P < 0.001$ ). In contrast, no significant differences were observed among the other antigens ( $P > 0.05$ ). These studies are in agreement with the results presented in Example 3 which

indicate immune responses to the 35-kDa protein may be more important in the later stage of disease since flow cytometric analysis showed that the 35-kDa protein strongly induced proliferation of B lymphocytes, especially in medium shedders (Fig. 3). Thus, this Example demonstrates that all of the recombinant antigens tested may stimulate a cell-mediated  
5 immunological response.

### Example 6

This Example demonstrates the effect of vaccinating mice with DNA polynucleotides encoding MPT antigens and subsequent challenge with MPT.

10 For demonstrating of the effect of the DNA constructs, specific-pathogen-free C57/BL6 female mice were obtained from the Harlan Sprague Dawley Inc (Indianapolis, IN). The mice were ~8 weeks old at the time of vaccination. There were five groups of mice and each vaccine group consists of 25 animals during this experiment. The animals were fed commercial mouse chow and water *ad libitum*, and maintained on a 12/12-hour light/dark  
15 cycle.

The commercially available eukaryotic expression plasmid pVR1020 (Vical, Inc., San Diego, Calif.) was used for the DNA vaccine. This plasmid contains an immediate-early cytomegalovirus promoter to ensure efficient expression in a eukaryotic host as well as the human tissue plasminogen activator (hTPA) secretion signal to facilitate secretion of the  
20 target antigen from the eukaryotic cell (Brandt, et al. 2000. Infect. Immun. 68:791-795). DNA encoding MPT 85A, 85B, 85C, SOD, 35kDa, 35kDa(li), MptC, MptD, and ESAT-6 like genes was amplified by polymerase chain reaction from MPT genomic DNA using the gene specific primers listed in Table 1. Briefly, the primers used for amplification of MPT 85A, 85B and 85C coding sequences each included a BamHI site. One primer for  
25 amplification of SOD, MptC, MptD, 35kDa, and ESAT6-like coding sequences included a BamHI site while the other included a BglII site. The amplification products were digested using the indicated restriction enzymes and cloned into the commercially available pCR2.1 TOPO cloning vector (Invitrogen, CA) using standard techniques. The MPT genes were then subcloned downstream of the human tissue plasminogen activator (hTPA) secretion signal in  
30 the pVR1020 plasmid (Vical, Inc., San Diego, Calif.) using standard techniques and essentially as previously described (Dheenadhayalan et al., (2002) DNA Seq. 13:287-294; Shin et al., (2004) J. Vet. Sci. 5:111-117) (Skeikyet al., (1998) J. Immunol. 161:6171-6179). These recombinant constructs were transfected into HEK-293 (human embryonic kidney)

cells with using lipofectamin<sup>TM</sup> 2000 transfection reagent (Invitrogen, CA) and the expression of the antigen genes was confirmed at the transcription level using RT-PCR.

For immunization and MPT challenge, mice were divided into five different groups (Table 3). The animals were administered with 50  $\mu$ g of each DNA in 50  $\mu$ l

Table 3.

| Groups      | 1                                 | 2                    | 3                                        | 4                    | 5                           |
|-------------|-----------------------------------|----------------------|------------------------------------------|----------------------|-----------------------------|
| DNA Vaccine | 85A, 85B<br>85C, 35kDa<br>and SOD | Group1<br>+<br>IL-12 | MptC, MptD<br>ESAT6like<br>and 35kDa(Li) | Group3<br>+<br>IL-12 | Vector Control<br>(pVR1020) |

PBS per dose via intramuscular injection. Mice were immunized three times at 3-week intervals. IL-12 genes were additionally injected as indicated in Table 3. Three weeks after the second boosters, the animals were challenged by intraperitoneal injection of  $10^9$  CFU units of (MPT). Six animals in each group were sacrificed at 4<sup>th</sup>, 8<sup>th</sup>, 12<sup>th</sup> and 16<sup>th</sup> week after challenge and the recovery of bacteria from organs (liver, spleen, mesenteric lymph node, lung) was enumerated on Herald's EggYolk (HEY) slant agar supplemented with Mycobactin J and antibiotics as previously described (Kamath, et al. Infect. Immun. 67:1702–1707). After challenge, feces were also collected every week from mouse cages and cultured using the same agar. Tissues including liver, spleen, lung, intestine and mesenteric lymph node were fixed by immersion in 10% buffered formalin and processed for histopathological examination using standard histotechnology techniques. The presence of MPT (acid-fast bacteria) in the liver and spleen of each mouse was assessed by Ziehl-Neelsen staining.

Fig. 10 shows the decreased mycobacterial burden in the livers and spleens of vaccinated mice relative to controls at 4, 8 and 12 weeks post-challenge and demonstrates an approximately 90% reduction (1 log<sub>10</sub>) in the bacterial burden in the spleens and livers for mice vaccinated with the MPT DNA vaccine cocktail compared to non-immunized controls. The relative liver and spleen histopathology data at 4, 8 and 12 weeks post-challenge paralleled the bacterial growth results. Substantive differences were seen in liver and spleen tissues taken from animals immunized with the plasmid cocktail compared to nonimmunized mice (Fig. 11). MPT infected nonvaccinated controls had numerous randomly dispersed granulomas with central epithelioid macrophages surrounded by small lymphocytes. Ziehl-Neelsen staining revealed numerous acid-fast bacilli were seen (Fig. 12A and inset). In

contrast, the infection was much less severe in the mice vaccinated with the MPT DNA vaccine cocktail (Fig. 12B).

Thus, this Example demonstrates that administration with a DNA expression vectors encoding at least five MPT antigens can provide significant protection from MPT infection.

We claim:

1. A method for stimulating an immune response in an animal comprising administering an effective amount of a composition comprising at least five immunogenic components,  
5 wherein the immunogenic components are selected from the group consisting of:
  - a) isolated DNA sequences encoding MPT antigens;
  - b) recombinant MPT antigens; or
  - c) combinations of a) and b).
- 10 2. The method of claim 1, wherein the MPT antigens are selected from the group consisting of 85A, 85B, 85C, 35kDa, 35kDa(Li), SOD, MptC, MptD and ESAT-6.
3. The method of claim 1, wherein the polynucleotide sequences encoding MPT antigens encode an MPT antigen selected from the group consisting of 85A, 85B, 85C, 35kDa, SOD,  
15 MptC, MptD and ESAT-6.
4. The method of claim 3, wherein the composition comprises polynucleotide sequences encoding MPT antigens 85A, 85B, 85C, MPT 35kDa protein, and superoxide dismutase (SOD).  
20
5. The method of claim 1, wherein the composition is administered to a ruminant.
6. The method of claim 5, wherein the ruminant is a bovine, a sheep, a goat, a deer or an elk.  
25
7. The method of claim 6, wherein the ruminant is a bovine.
8. The method of claim 5, wherein the ruminant is not infected with MPT.
- 30 9. The method of claim 5, wherein the ruminant is infected with MPT.
10. The method of claim 6, wherein the bovine has Johne's Disease.

11. The method of claim 1, wherein the composition further comprises a pharmaceutically acceptable carrier.

12. The method of claim 1, wherein the animal is pregnant.

13. The method of claim 11, wherein the composition further comprises an adjuvant.

14. A composition for use in stimulating an immune response against MPT in an animal comprising at least five immunogenic components, wherein the immunogenic components are selected from the group consisting of:

- a) recombinant MPT antigens;
- b) isolated polynucleotide sequences encoding MPT antigens; or
- c) combinations of a) and b).

15. The composition of claim 14, wherein the MPT antigens are selected from the group consisting of 85A, 85B, 85C, 35kDa, SOD, MptC, MptD and ESAT-6.

16. The method of claim 15, wherein the polynucleotide sequences encoding MPT antigens encode an MPT antigen selected from the group consisting of 85A, 85B, 85C, 35kDa, SOD, MptC, MptD and ESAT-6.

17. The composition of claim 14, wherein the composition comprises polynucleotide sequences encoding MPT antigens 85A, 85B, 85C, MPT 35kDa protein, and superoxide dismutase (SOD).

18. The composition of claim 14, further comprising a pharmaceutically acceptable carrier.

19. The composition of claim 14, further comprising an adjuvant.



Fig. 1

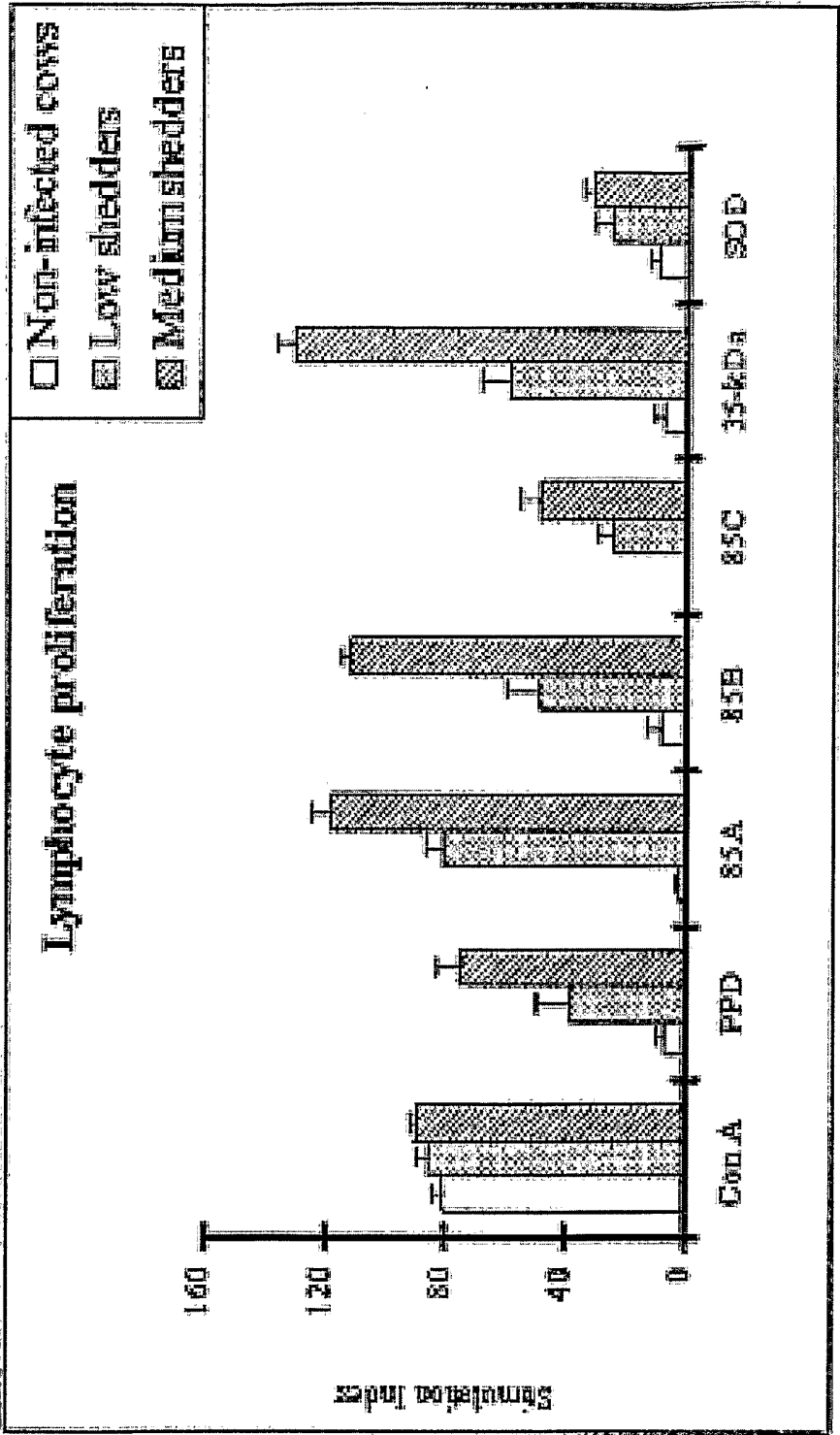


Fig. 2

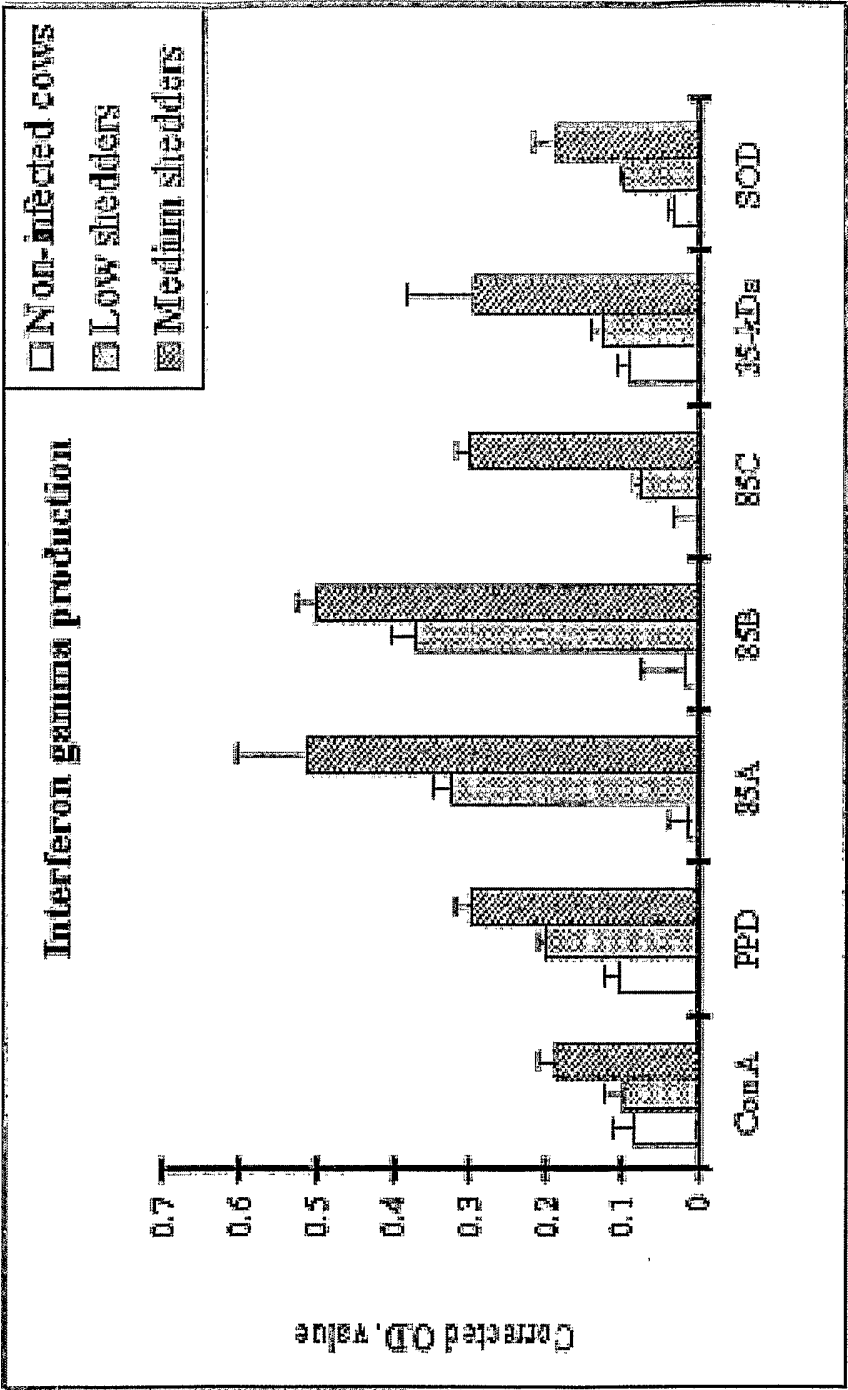


Fig. 3

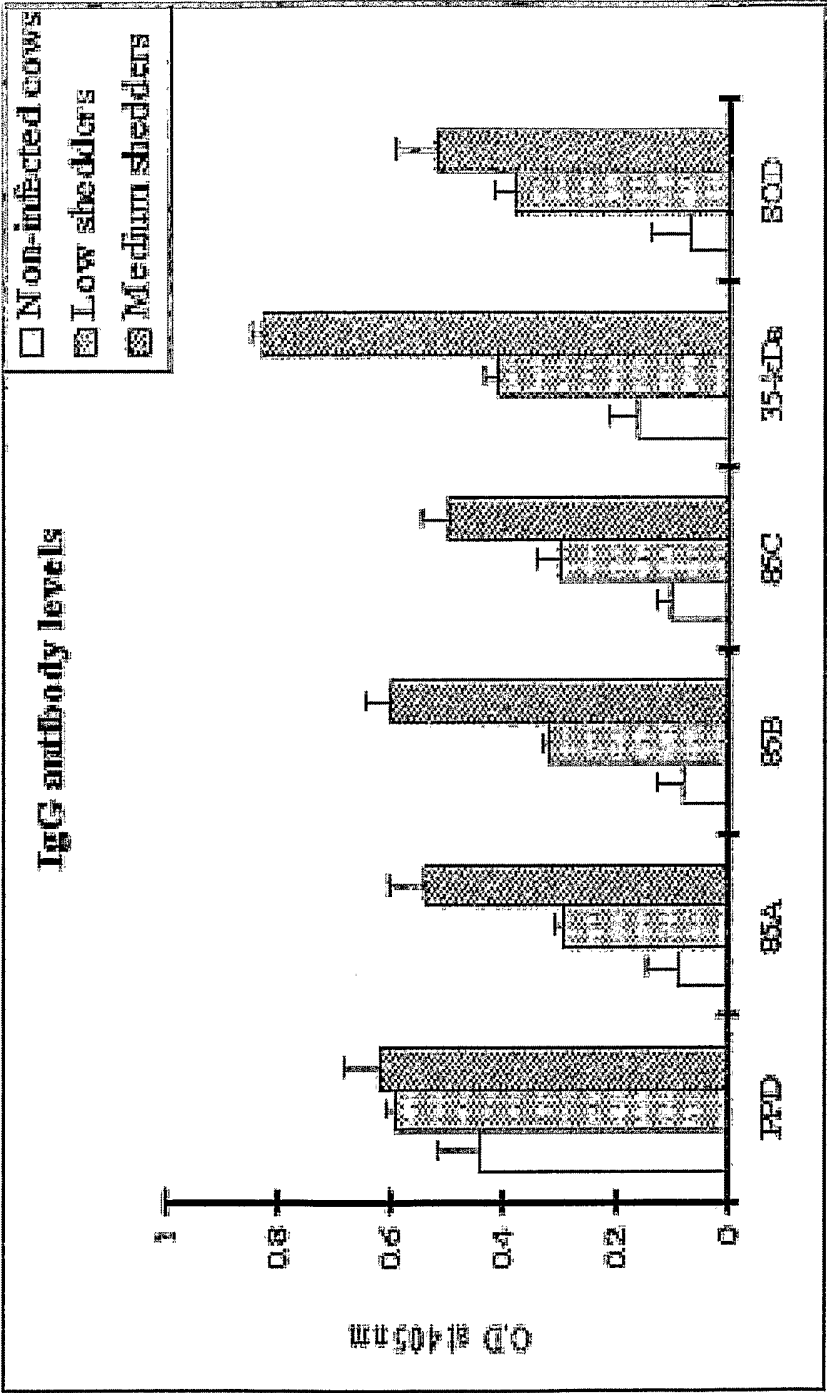


Fig. 4A

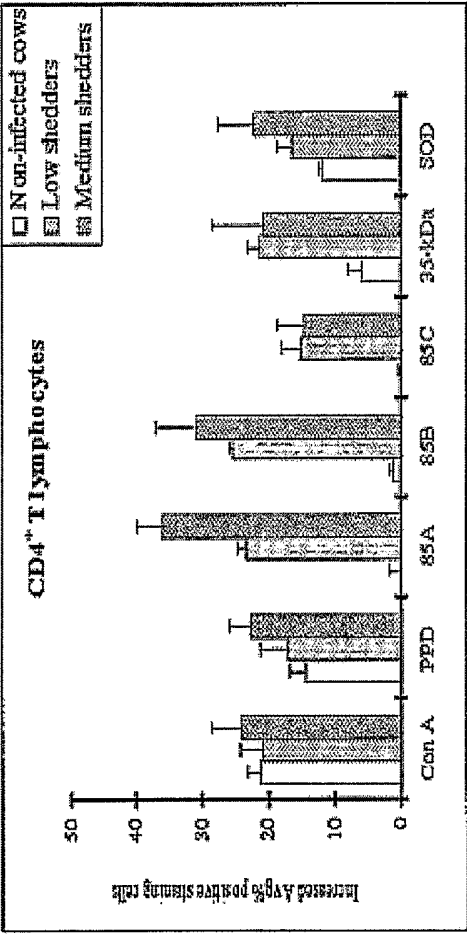


Fig. 4B

B.

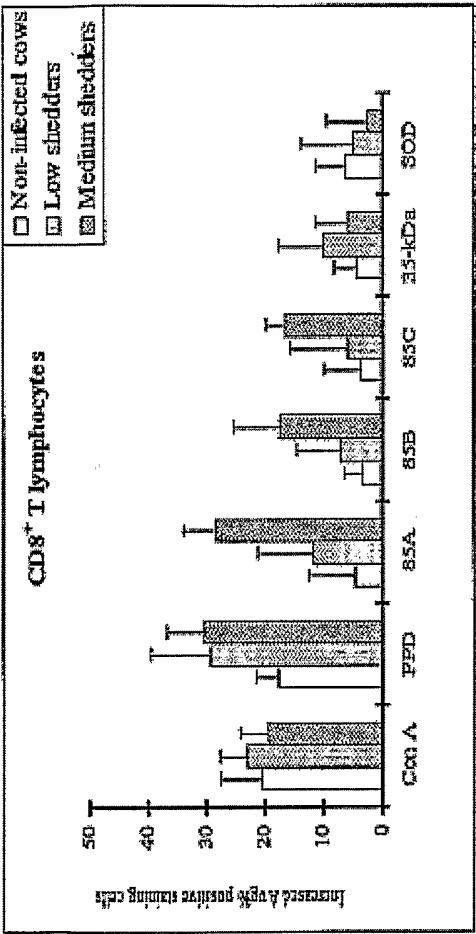


Fig. 4C

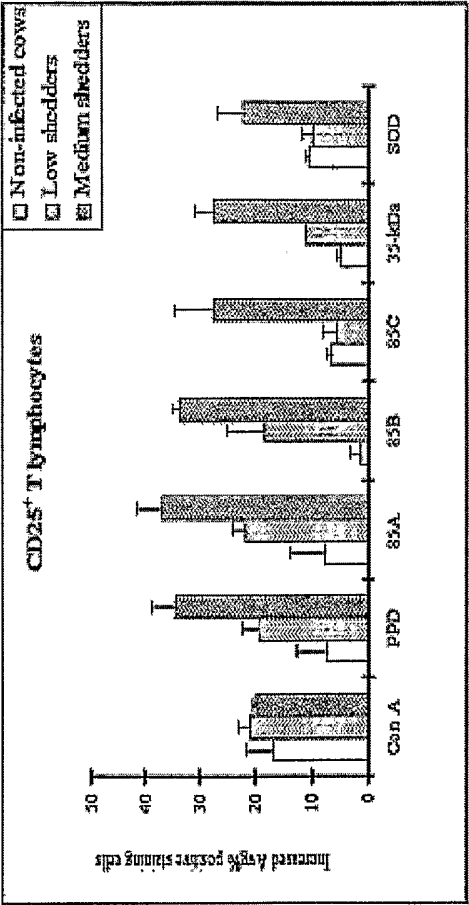


Fig. 4D

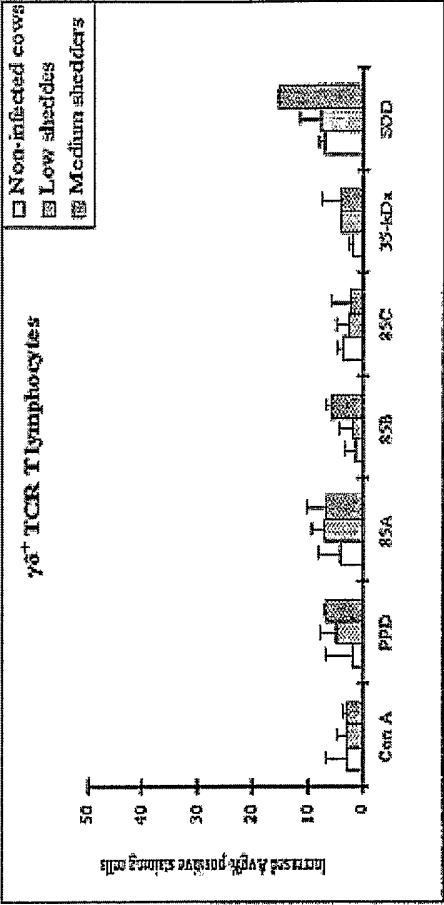


FIG. 4—Continued.

Fig. 5

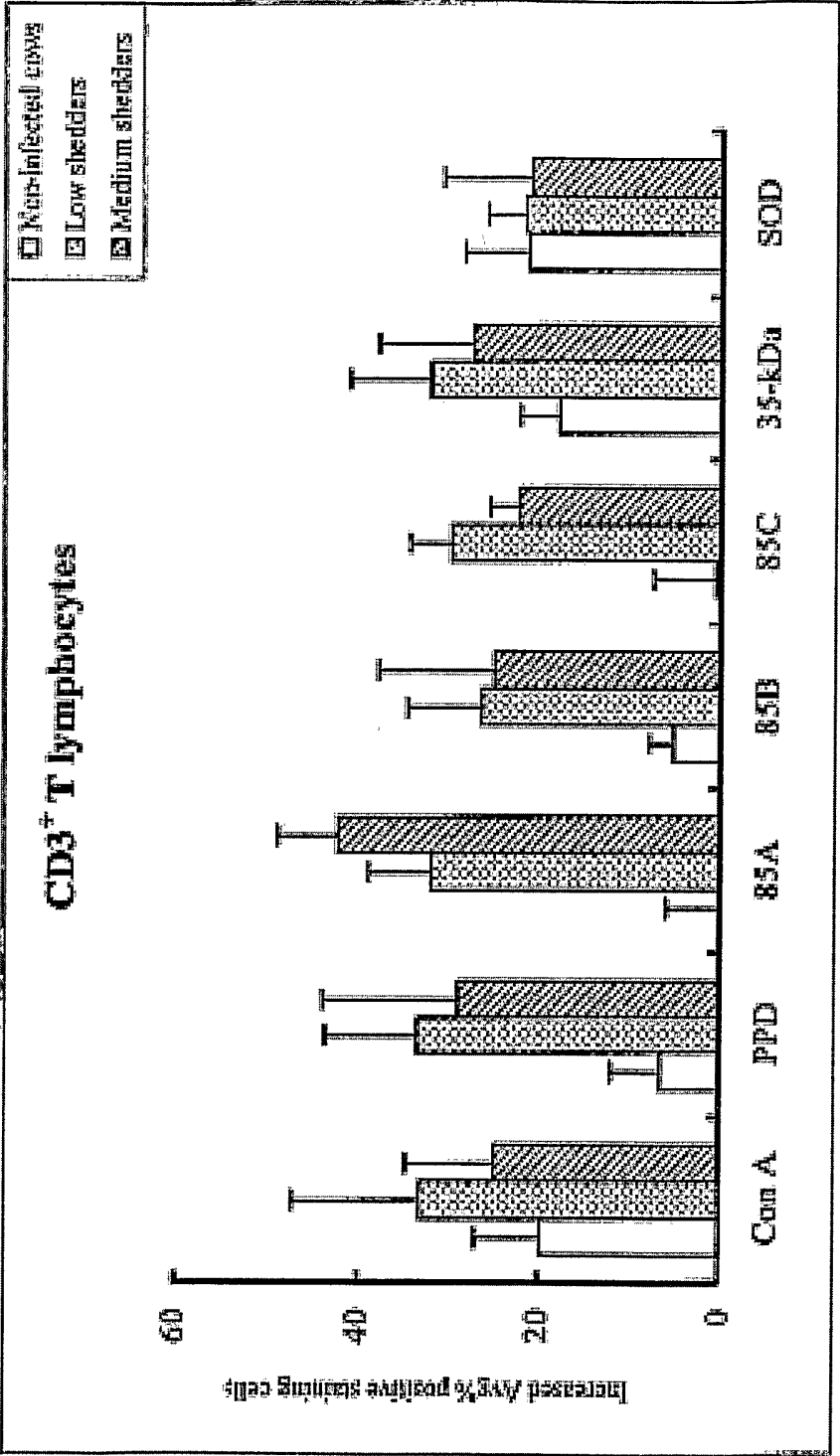


Fig. 6

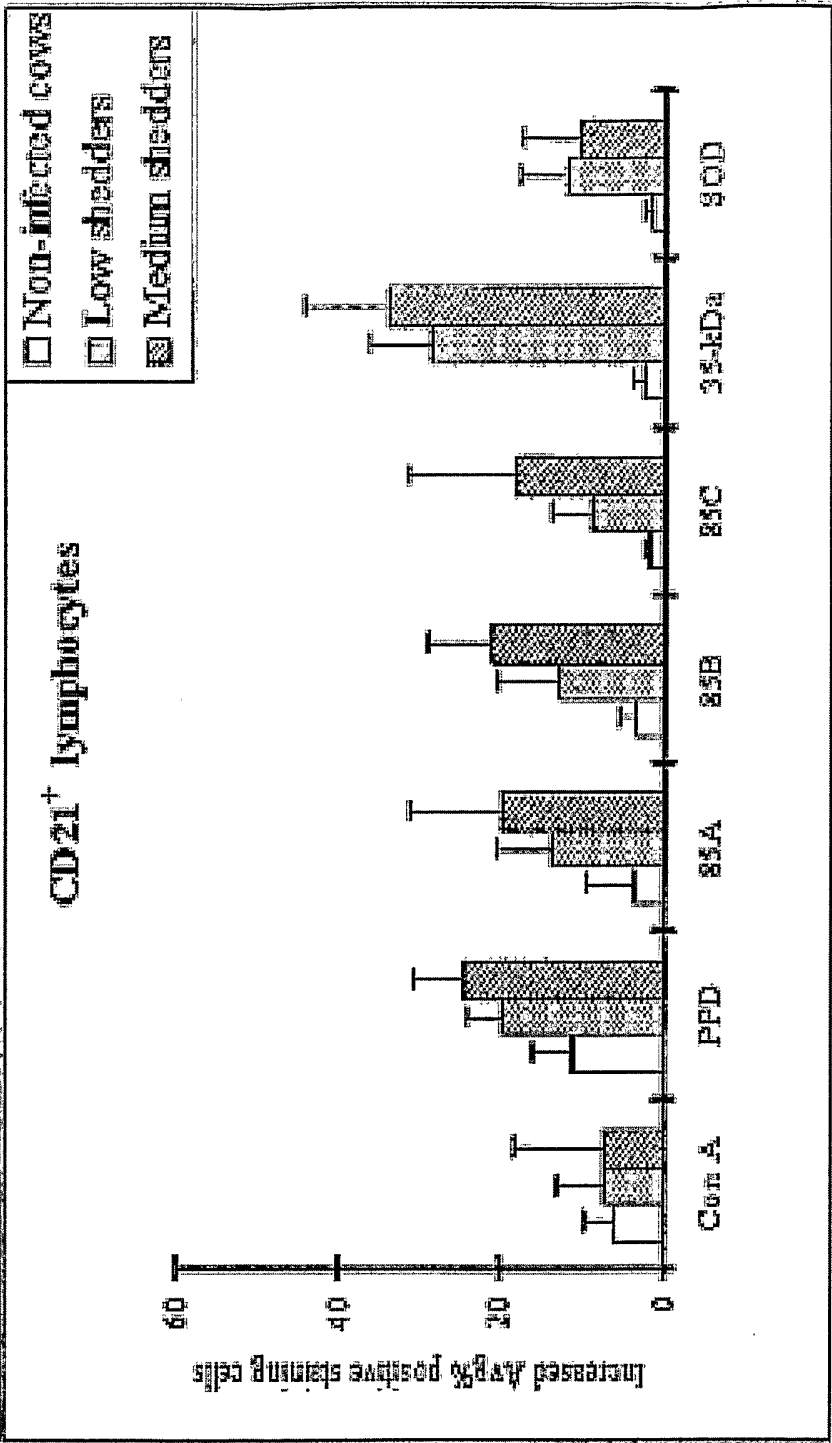


Fig. 7

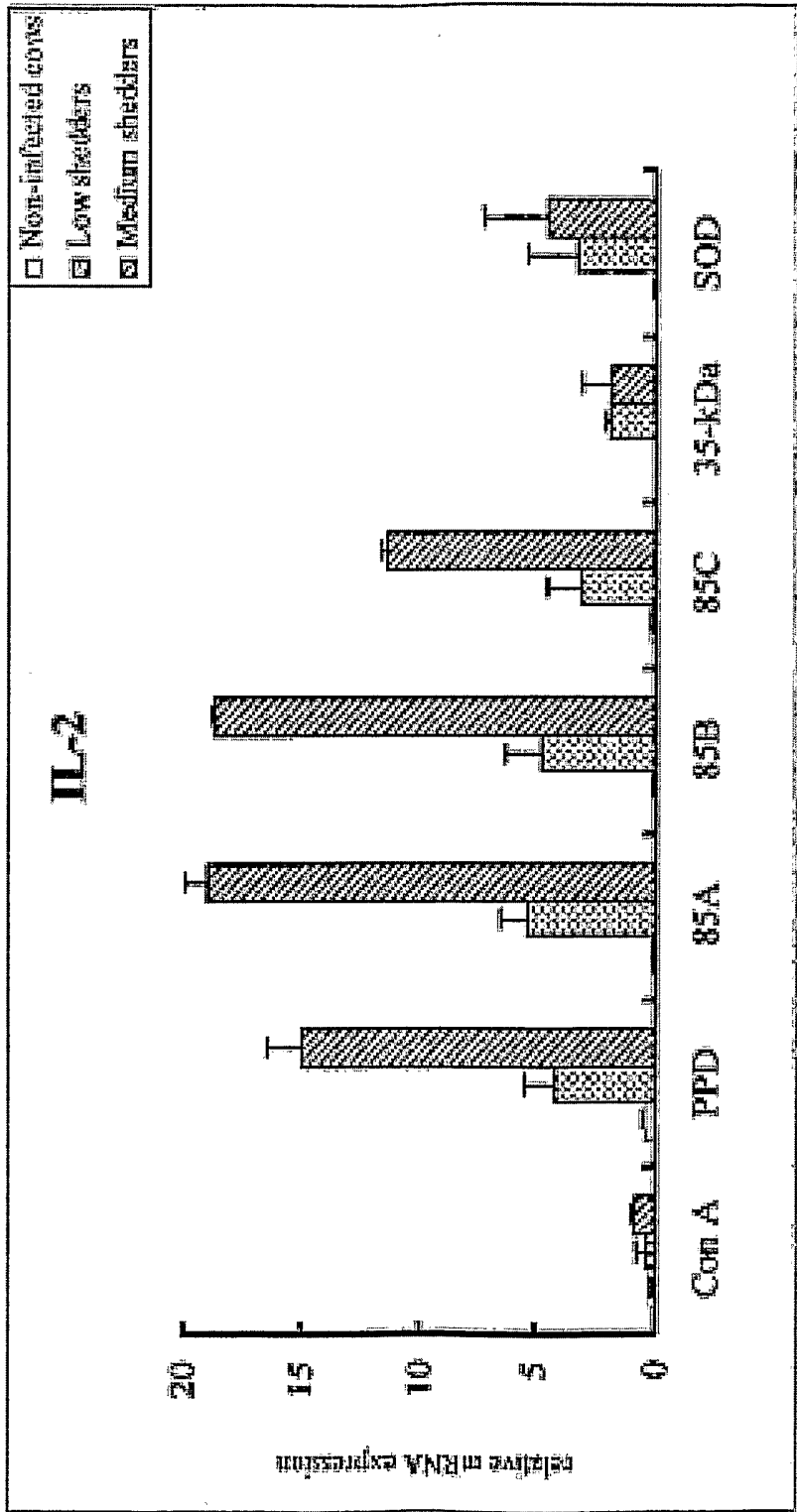




Fig. 8A

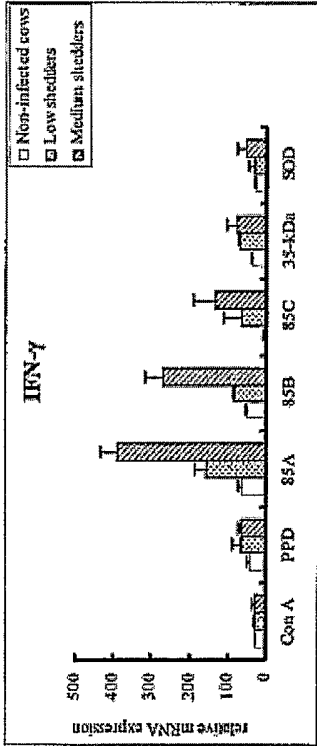


Fig. 8B

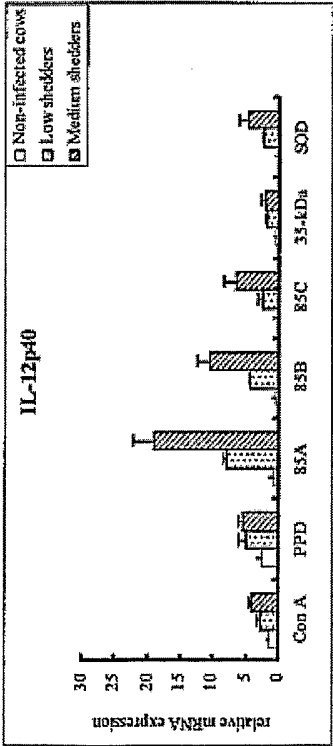


Fig. 8C

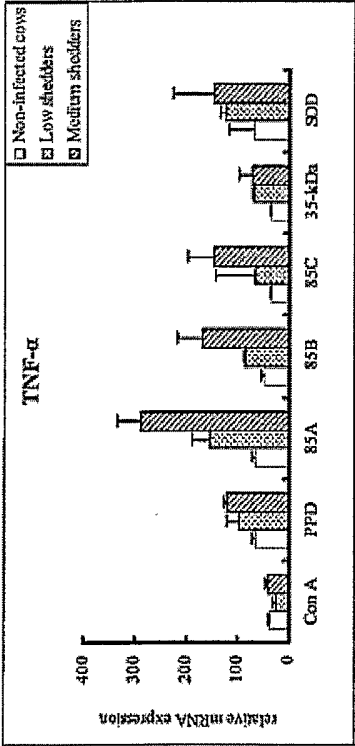


Fig. 9

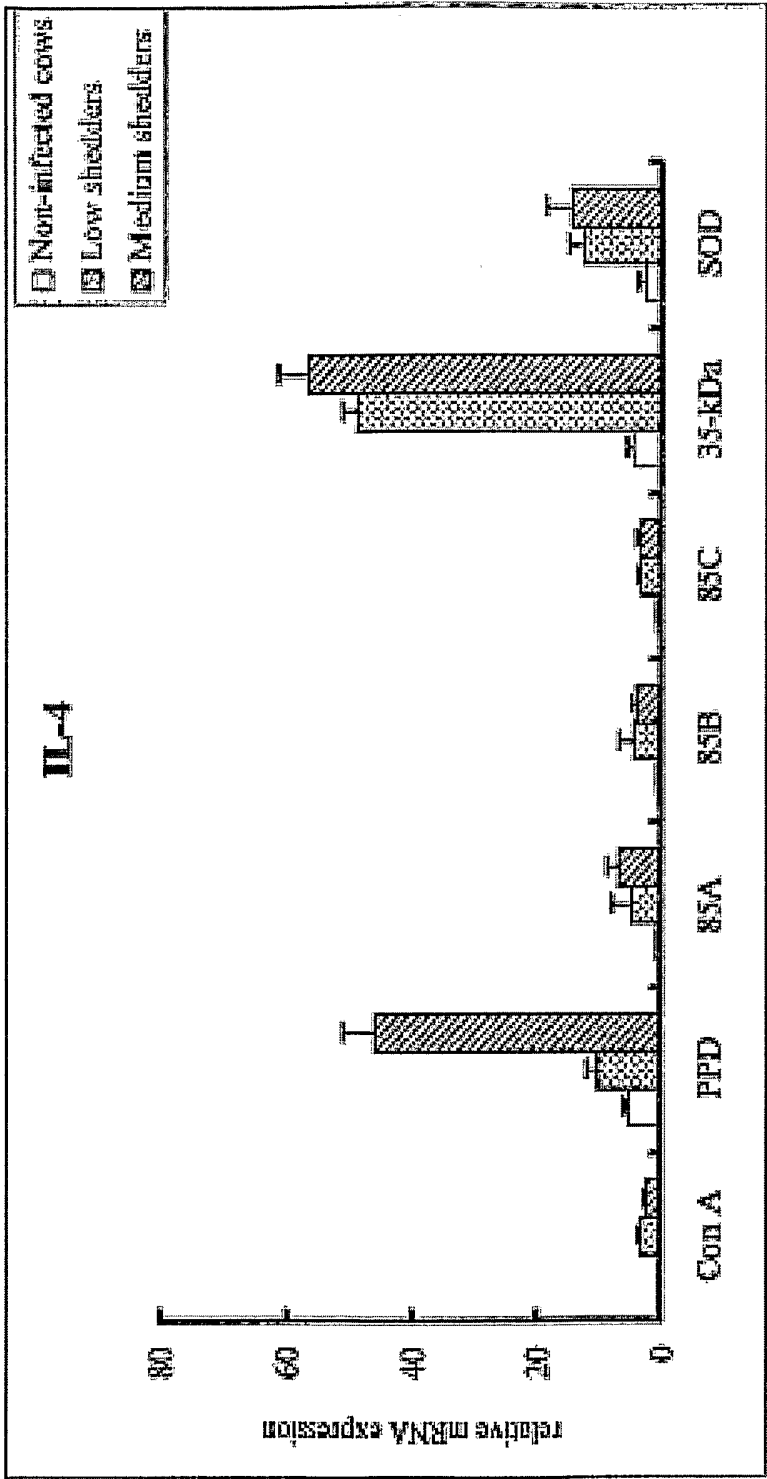


Fig. 10A

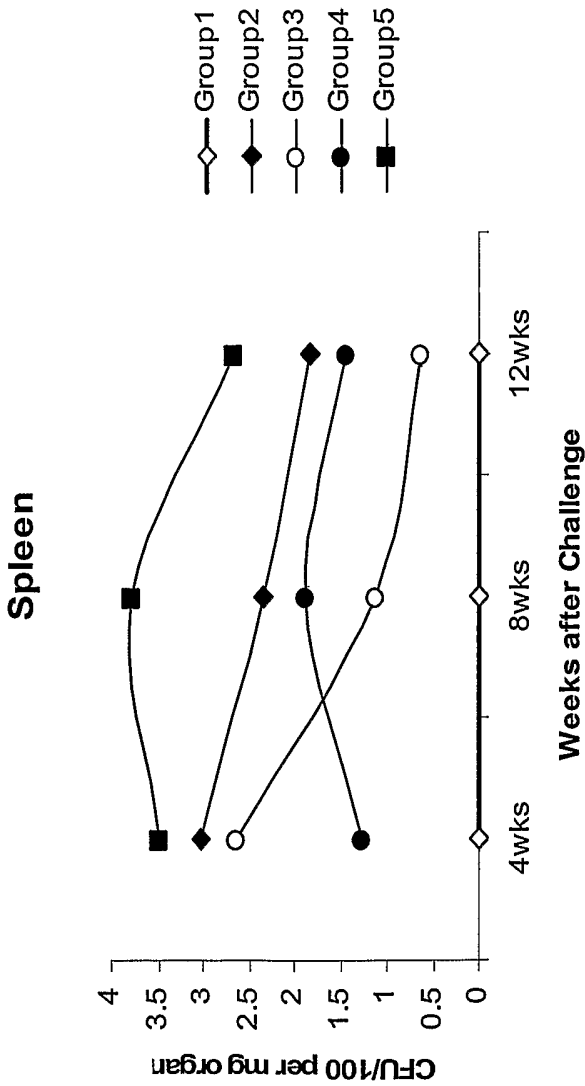


Fig. 10B

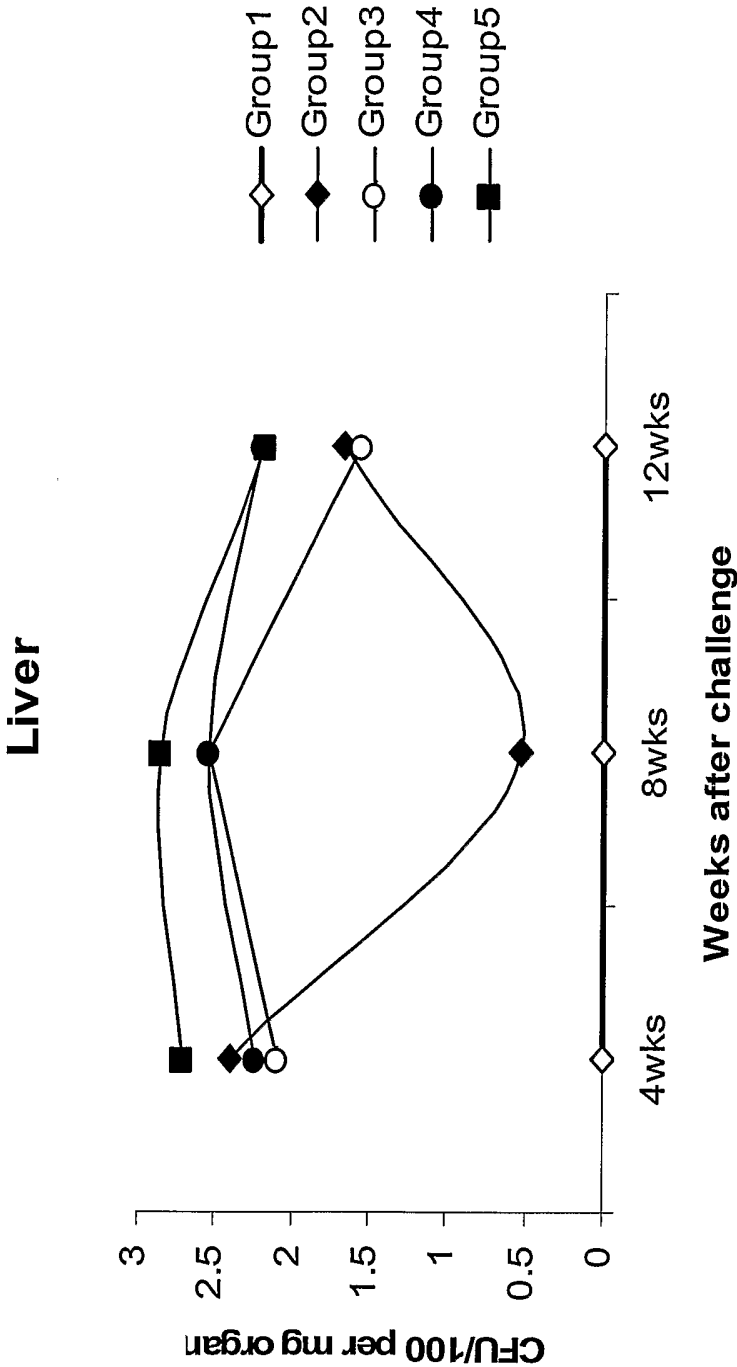


Fig. 11A

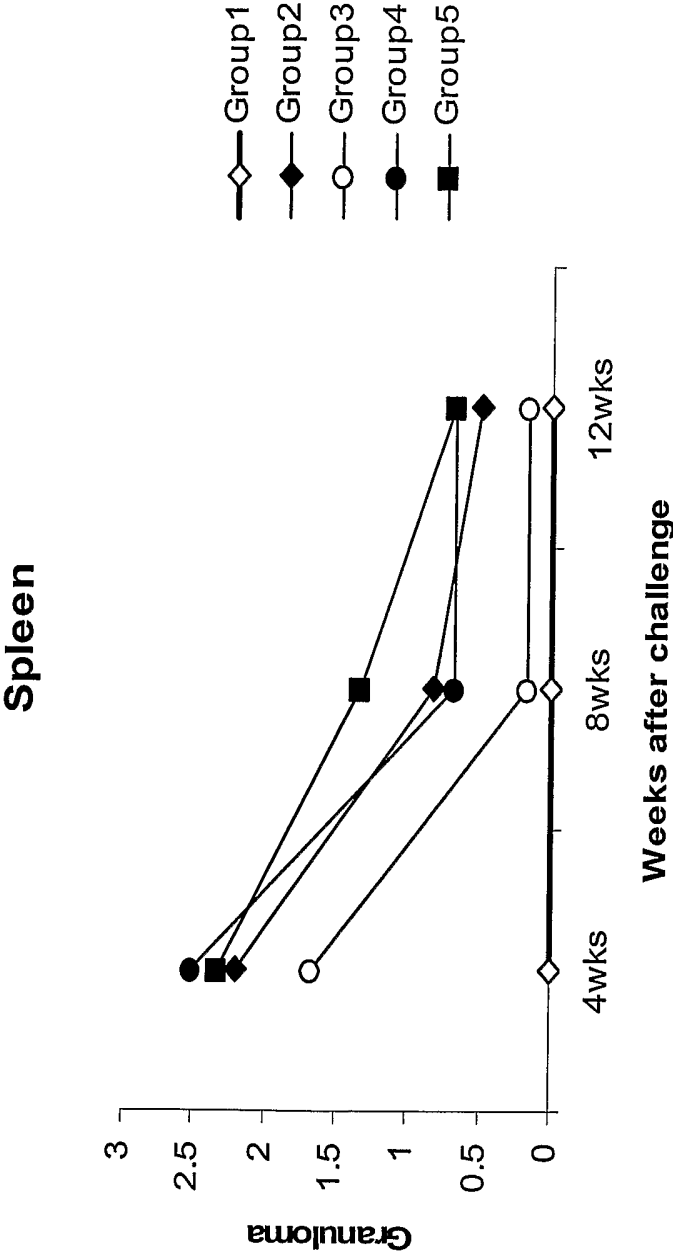
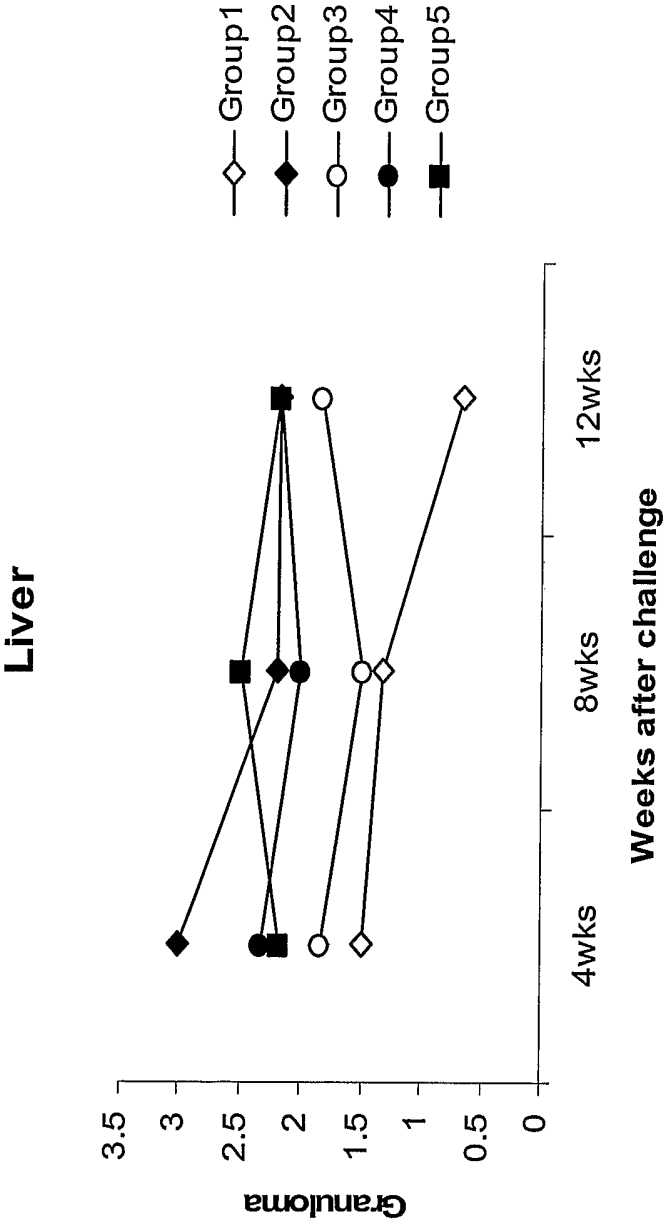
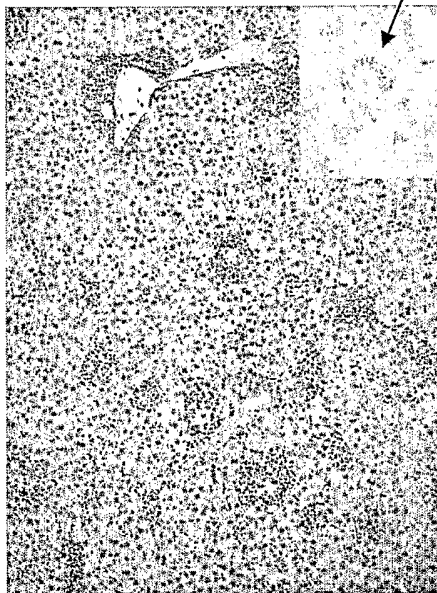
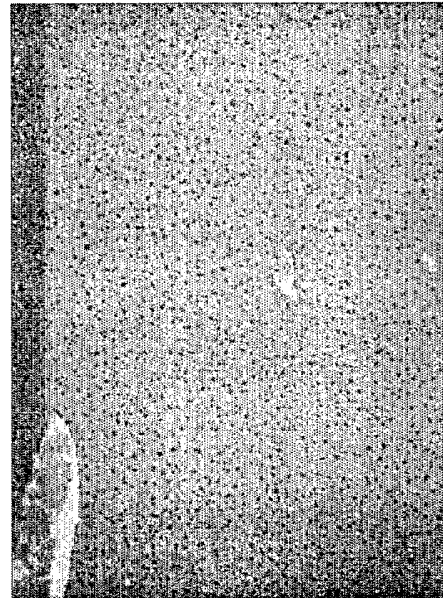


Fig. 11B





Magnified  
acid fast  
bacterium



**Fig. 12A**

**Fig. 12B**

## Chang Sequence Listing

## SEQUENCE LISTING

&lt;110&gt; Chang, Y.F.

&lt;120&gt; COMPOSITIONS FOR ELICITING AN IMMUNE RESPONSE AGAINST MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS

&lt;130&gt; 018617.00120

&lt;150&gt; US 60/653,536

&lt;151&gt; 2005-02-16

&lt;160&gt; 24

&lt;210&gt; 1

&lt;211&gt; 828

&lt;212&gt; DNA

&lt;213&gt; Mycobacterium avium subspecies paratuberculosis

&lt;220&gt;

&lt;223&gt; MPT 35 kDa protein DNA encoding sequence

&lt;400&gt; 1

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&lt;210&gt; 2

&lt;211&gt; 275

&lt;212&gt; PRT

&lt;213&gt; Mycobacterium avium subspecies paratuberculosis

&lt;220&gt;

&lt;223&gt; amino acid sequence of the 35 kDa protein

&lt;400&gt; 2

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Ala | Asn | Pro | Phe | Val | Lys | Ala | Trp | Lys | Tyr | Leu | Met | Ala | Lys |
|     |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |
| Phe | Asn | Ala | Thr | Ile | Asp | Glu | Arg | Ala | Asp | Pro | Lys | Val | Gln | Ile |
|     |     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |
| Gln | Gln | Ala | Ile | Glu | Glu | Ala | Gln | Arg | Thr | His | Gln | Ala | Leu | Thr |
|     |     |     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |



| Chang Sequence Listing |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gln                    | Gln | Ala | Ala | Gln | Val | Ile | Gly | Asn | Gln | Arg | Gln | Leu | Glu | Met |
| Arg                    | Leu | Asn | Arg | Gln | Leu | Ala | Asp | Val | Glu | Lys | Leu | Gln | Val | Asn |
| Val                    | Arg | Gln | Ala | Leu | Thr | Leu | Ala | Asp | Gln | Ala | Thr | Ala | Ala | Gly |
| Asp                    | Thr | Ala | Lys | Ala | Thr | Glu | Tyr | Asn | Asn | Ala | Ala | Glu | Ala | Phe |
| Ala                    | Ala | Gln | Leu | Val | Thr | Ala | Glu | Gln | Ser | Val | Glu | Asp | Leu | Lys |
| Thr                    | Leu | His | Asp | Gln | Ala | Leu | Asn | Ala | Ala | Ala | Gln | Ala | Lys | Lys |
| Ala                    | Val | Glu | Gln | Asn | Ala | Met | Val | Leu | Gln | Gln | Lys | Ile | Ala | Glu |
| Arg                    | Thr | Lys | Leu | Leu | Ser | Gln | Leu | Glu | Gln | Ala | Lys | Met | Gln | Glu |
| Gln                    | Val | Ser | Ala | Ser | Leu | Gln | Ser | Met | Ser | Glu | Leu | Ala | Ala | Pro |
| Gly                    | Asn | Val | Pro | Ser | Leu | Asp | Glu | Val | Arg | Asp | Lys | Ile | Glu | Arg |
| Arg                    | Tyr | Ala | Asn | Ala | Ile | Gly | Ala | Ala | Glu | Leu | Ala | Gln | Gly | Ser |
| Val                    | Gln | Gly | Arg | Met | Leu | Glu | Val | Glu | Gln | Ala | Gly | Val | Gln | Met |
| Ala                    | Gly | His | Ser | Arg | Leu | Glu | Gln | Ile | Arg | Ala | Ser | Met | Arg | Asp |
| Glu                    | Ala | Leu | Pro | Thr | Gly | Gly | Thr | Pro | Ala | Ala | Gly | Gly | Thr | Gln |
| Ala                    | Ala | Pro | Ala | Pro | Gly | Gln | Gly | Ala | Gly | Asp | Ala | Val | Ser | Glu |
| Lys                    | Pro | Leu | Gly | Gln |     |     |     |     | 265 |     |     |     |     | 270 |

&lt;210&gt; 3

&lt;211&gt; 1782

&lt;212&gt; DNA

&lt;213&gt; Mycobacterium avium subspecies paratuberculosis

&lt;220&gt;

&lt;223&gt; DNA sequence encoding the MptC protein

&lt;400&gt; 3

atgagcacgatgattcgttattttgcttgaattacttgacgaacgcggtcggcggtcacctt 60  
cgcgatgatgcttgccttgcaagccgcacaggaattctgcagggactcggtttccttttc 120  
gtcgtaccgctgatgggtgtgctcatccacaagccggttgatacgcacgccctttggtgc 180  
tggattgtggcgatcgccgtcgctgtcgctcgctcaccacggactgcttgctggagcacc 240  
tcgctgggttacctggctggcacagatgtgctgaccagctttcacacccgcatcggcaac 300  
catctcgccacgctgccaatcggttggttccgcaccgatcgcaccggaccgatcgggcgt 360  
ctgctcagcaaggggaccatggacgtcgcgaccttcccgcgacactgctgcggcatgtc 420  
atcgtaggtgttagccgcgctgccaccatgatcatcggaagctatgtgctggactggcgg 480  
gtcgggctcgcatccaccgcccgcgctgctatgcgcactgacgcttcgactactcatg 540  
gtcgtcatccaccgcaacgacgaccagtagatcacgacatcggagtgaccgcgtcacgc 600  
atcgctcgagtacgcccgtcagcagccgacactgctgcatacggcgtcctgaaccggccc 660  
ggactgggaacgctagaggaatcgcttgcggcaacagacttcgcagtcctcgcttgacc 720

## Chang Sequence Listing

attcgcggcgcggtgggcgctcatcggattcttcggaaccgtccaactgggtggtgaccgcc 780  
 atcatcgcgctcaccgtcgctctcgcagtaagcggcagccttgcgctttcgacgatggtc 840  
 ggtttgctgatcatcaccttgcgcatgatcgacccgatttctcagctcggcgatctcgcc 900  
 ggccatgtccagggtcaacgccgacgccatccgccgagtgcgtgaactgttgacgggtaccc 960  
 ccgttgccccgaaccacccatccagggtcaagccacgggtgccgacatcgaattgcagggg 1020  
 gttcgtttcgcttacgacggaggtcaaccacccatcgatgggtatcgacgccgtctttcca 1080  
 caaggtagcctgactgccgtcgctcgccccgtctggcgcgggaaaaagcacgctgctcaaa 1140  
 ctaatcgggcggttcttcgacgtcgacgaaggcagcatcaccatcgggcggtcgacatc 1200  
 cgccaactcggcaccgcccggcggtctcacatctcacggcgcaagttttccaagacgtgtac 1260  
 ctctttgaaggcacaattgccgacaacctgcggatggccgatccgcaagccaccgatgat 1320  
 gatctccgccacgtcgccacgatcagtcgcctcgacgaggtagtcgatcgcttcccaat 1380  
 ggggtgggaaacccgcgttggtgaggcggggtcgacgctgtccggcggggaaaagcacgct 1440  
 gtctccatagcgcgcgccctgtcaaagacgcaccggtagtactgctggacgaggcgacc 1500  
 gcggcgctggatgccgccaatgagaccgccgtttccgacgctatccacgaactagcgct 1560  
 gaccgcacagtcacgttgctgcacaccgcctttcgaccgtggcgggcccgatcagata 1620  
 ctggtgctcgacggcgccggatcaccgagcgtggccgacgacacactggtgggtgctg 1680  
 ggcggcacctacgcccgttctgggaagaacgaactcgcgcgcgaggggtggcaactgctgc 1740  
 caaattgcgcccactcctgaactgcaggcacctcgaccatga 1782

&lt;210&gt; 4

&lt;211&gt; 593

&lt;212&gt; PRT

<213> *Mycobacterium avium* subspecies *paratuberculosis*

&lt;220&gt;

&lt;223&gt; amino acid sequence of the MptC protein

&lt;400&gt; 4

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Ser | Thr | Met | Ile | Arg | Tyr | Leu | Leu | Glu | Leu | Leu | Asp | Glu | Arg |
|     |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |
| Gly | Arg | Arg | His | Leu | Arg | Val | Met | Leu | Ala | Leu | Gln | Ala | Ala | Gln |
|     |     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |
| Gly | Ile | Leu | Gln | Gly | Leu | Gly | Phe | Leu | Phe | Val | Val | Pro | Leu | Met |
|     |     |     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |
| Gly | Val | Leu | Ile | His | Lys | Pro | Val | Asp | Thr | His | Ala | Leu | Trp | Cys |
|     |     |     |     | 50  |     |     |     |     | 55  |     |     |     |     | 60  |
| Trp | Ile | Val | Ala | Ile | Ala | Val | Ala | Val | Val | Ala | His | His | Gly | Leu |
|     |     |     |     | 65  |     |     |     |     | 70  |     |     |     |     | 75  |
| Leu | Ala | Trp | Ser | Thr | Ser | Leu | Gly | Tyr | Leu | Val | Gly | Thr | Asp | Val |
|     |     |     |     | 80  |     |     |     |     | 85  |     |     |     |     | 90  |
| Leu | Thr | Ser | Phe | His | Thr | Arg | Ile | Gly | Asn | His | Leu | Ala | Thr | Leu |
|     |     |     |     | 95  |     |     |     |     | 100 |     |     |     |     | 105 |
| Pro | Ile | Gly | Trp | Phe | Arg | Thr | Asp | Arg | Thr | Gly | Pro | Ile | Gly | Arg |
|     |     |     |     | 110 |     |     |     |     | 115 |     |     |     |     | 120 |
| Leu | Leu | Ser | Lys | Gly | Thr | Met | Asp | Val | Ala | Asp | Leu | Pro | Ala | His |
|     |     |     |     | 125 |     |     |     |     | 130 |     |     |     |     | 135 |
| Leu | Leu | Arg | His | Val | Ile | Val | Gly | Val | Ala | Ala | Pro | Ala | Thr | Met |
|     |     |     |     | 140 |     |     |     |     | 145 |     |     |     |     | 150 |
| Ile | Ile | Gly | Ser | Tyr | Val | Leu | Asp | Trp | Arg | Val | Gly | Leu | Ala | Phe |
|     |     |     |     | 155 |     |     |     |     | 160 |     |     |     |     | 165 |

|     |     |     |     |     | Chang Sequence |     |     |     |     | Listing |     |     |     |     |
|-----|-----|-----|-----|-----|----------------|-----|-----|-----|-----|---------|-----|-----|-----|-----|
| Thr | Ala | Gly | Ala | Leu | Leu            | Cys | Ala | Leu | Thr | Leu     | Arg | Leu | Leu | Met |
| Val | Val | Ile | His | Arg | Asn            | Asp | Asp | Gln | Tyr | Asp     | His | Asp | Ile | Gly |
| Val | Thr | Ala | Ser | Arg | Ile            | Val | Glu | Tyr | Ala | Arg     | Gln | Gln | Pro | Thr |
| Leu | Arg | Ala | Tyr | Gly | Val            | Leu | Asn | Arg | Pro | Gly     | Leu | Gly | Thr | Leu |
| Glu | Glu | Ser | Leu | Val | Arg            | Gln | Gln | Thr | Ser | Gln     | Ser | Arg | Leu | Thr |
| Ile | Arg | Gly | Ala | Trp | Ala            | Leu | Ile | Gly | Phe | Phe     | Gly | Thr | Val | Gln |
| Leu | Val | Val | Thr | Ala | Ile            | Ile | Ala | Leu | Thr | Val     | Ala | Leu | Ala | Val |
| Ser | Gly | Ser | Leu | Ala | Leu            | Ser | Thr | Met | Val | Gly     | Leu | Leu | Ile | Ile |
| Thr | Leu | Arg | Met | Ile | Asp            | Pro | Ile | Ser | Gln | Leu     | Gly | Asp | Leu | Ala |
| Gly | His | Val | Gln | Val | Asn            | Ala | Asp | Ala | Ile | Arg     | Arg | Val | Arg | Glu |
| Leu | Leu | Thr | Val | Pro | Pro            | Leu | Pro | Glu | Pro | Thr     | His | Pro | Gly | Gln |
| Ala | Thr | Gly | Ala | Asp | Ile            | Glu | Leu | Gln | Gly | Val     | Arg | Phe | Ala | Tyr |
| Asp | Gly | Gly | Gln | Pro | Thr            | Ile | Asp | Gly | Ile | Asp     | Ala | Val | Phe | Pro |
| Gln | Gly | Ser | Leu | Thr | Ala            | Val | Val | Gly | Pro | Ser     | Gly | Ala | Gly | Lys |
| Ser | Thr | Leu | Leu | Lys | Leu            | Ile | Gly | Arg | Phe | Phe     | Asp | Val | Asp | Glu |
| Gly | Ser | Ile | Thr | Ile | Gly            | Gly | Cys | Asp | Ile | Arg     | Gln | Leu | Gly | Thr |
| Ala | Gly | Val | Ser | His | Leu            | Thr | Ala | Gln | Val | Phe     | Gln | Asp | Val | Tyr |
| Leu | Phe | Glu | Gly | Thr | Ile            | Ala | Asp | Asn | Leu | Arg     | Met | Ala | Asp | Pro |
| Gln | Ala | Thr | Asp | Asp | Asp            | Leu | Arg | His | Val | Ala     | Thr | Ile | Ser | Arg |
| Leu | Asp | Glu | Val | Val | Asp            | Arg | Leu | Pro | Asn | Gly     | Trp | Glu | Thr | Arg |
| Val | Gly | Glu | Ala | Gly | Ser            | Thr | Leu | Ser | Gly | Gly     | Glu | Lys | Gln | Arg |
| Val | Ser | Ile | Ala | Arg | Ala            | Leu | Leu | Lys | Asp | Ala     | Pro | Val | Val | Leu |
| Leu | Asp | Glu | Ala | Thr | Ala            | Ala | Leu | Asp | Ala | Ala     | Asn | Glu | Thr | Ala |
| Val | Ser | Asp | Ala | Ile | His            | Glu | Leu | Ala | Arg | Asp     | Arg | Thr | Val | Ile |
| Val | Val | Ala | His | Arg | Leu            | Ser | Thr | Val | Ala | Ala     | Ala | Asp | Gln | Ile |
| Leu | Val | Leu | Asp | Gly | Gly            | Arg | Ile | Thr | Glu | Arg     | Gly | Arg | His | Asp |
| Thr | Leu | Val | Gly | Ala | Gly            | Gly | Thr | Tyr | Ala | Arg     | Phe | Trp | Glu | Glu |
| Arg | Thr | Arg | Ala | Arg | Gly            | Trp | Gln | Leu | Arg | Gln     | Ile | Ala | Pro | Thr |
| Pro | Glu | Leu | Gln | Ala | Pro            | Arg | Pro |     | 580 |         |     |     |     | 585 |

&lt;210&gt; 5

&lt;211&gt; 288

&lt;212&gt; DNA

&lt;213&gt; Mycobacterium avium subspecies paratuberculosis

&lt;220&gt;

&lt;223&gt; DNA sequence encoding the ESAT-6 like protein

## Chang Sequence Listing

<400> 5  
 atgtccgacccgatcacttacaacccgggcgcggtggccgacttcgccaccgacgtcgcc 60  
 tcccgcgcggccgagttgcagttccattttcgacgacacctccaaccgcacgcacgccctg 120  
 caggaattcttcgccggggcacggcgcgctcgggctttttcgaggcgaggcccagatgctg 180  
 tccgggctgcaggggctcatcgacacgatccgccagcacgggcagaccacctcgacgtg 240  
 ctggacagcgcgatcagcacggaccagcacatcgccggcctgttctga 288

<210> 6  
 <211> 95  
 <212> PRT  
 <213> Mycobacterium avium subspecies paratuberculosis

<220>  
 <223> amino acid sequence of the ESAT-6 like protein

<400> 6  
 Met Ser Asp Pro Ile Thr Tyr Asn Pro Gly Ala Val Ala Asp Phe  
           5          10  
 Ala Thr Asp Val Ala Ser Arg Ala Gly Gln Leu Gln Ser Ile Phe  
           20          25  
 Asp Asp Thr Ser Asn Arg Thr His Ala Leu Gln Glu Phe Phe Ala  
           35          40  
 Gly His Gly Ala Ser Gly Phe Phe Glu Ala Gln Ala Gln Met Leu  
           50          55  
 Ser Gly Leu Gln Gly Leu Ile Asp Thr Ile Arg Gln His Gly Gln  
           65          70  
 Thr Thr Ser His Val Leu Asp Ser Ala Ile Ser Thr Asp Gln His  
           80          85  
 Ile Ala Gly Leu Phe  
           95

<210> 7  
 <211> 627  
 <212> DNA  
 <213> Mycobacterium avium subspecies paratuberculosis

<220>  
 <223> DNA sequence encoding the MptD protein

<400> 7  
 atgacggccactagctcgacgacccagtcagtcgccgcatcgacgtccggatgagcgcc 60  
 agggatctcatcaacatcggggctttcggcgctctctacatcgccactgtgttcgcatc 120  
 aacgtgttcgctttcatcaatccgctcgtcatgttggtcgccctggcggtcagcatgatc 180  
 gccggcgggcggtgccgttcattgtttcctcaccggggtgcgacatgcgggcatggtgacg 240  
 gtgttttgcgattatcacggccggactgctcgactgaccgggcacccccgatctgcttc 300  
 gtgatcacagttgctgctgcggttggtggccgaagtcgtcctgtggctgggacgctatcgc 360  
 tcccgcacccatgggtgtactggcgtagcaatctacggcggtggtacatcggggccgctg 420  
 ctgcccattcttctacgctcgcgatgaatatttctccagtcgccggcatggcacagatgggt 480  
 ccgcgctacctcgaagagatggaacgggttgttgcgccagccgtgctaatacgattcgac 540  
 ctgtccacgggtggtattcgggctgatcggcggactgctcggagtaaggttgctgcgcaag 600  
 cattttcagaggggccgcctagcttga 627

## Chang Sequence Listing

<210> 8  
 <211> 208  
 <212> PRT  
 <213> Mycobacterium avium subspecies paratuberculosis

<220>  
 <223> MptD amino acid sequence

<400> 8  
 Met Thr Ala Thr Ser Ser Thr Thr Gln Ser Ser Arg Arg Ile Asp  
           5          10  
 Val Arg Met Ser Ala Arg Asp Leu Ile Asn Ile Gly Val Phe Gly  
           20          25  
 Ala Leu Tyr Ile Ala Thr Val Phe Ala Ile Asn Val Phe Ala Phe  
           35          40  
 Ile Asn Pro Leu Val Met Leu Val Ala Leu Ala Val Ser Met Ile  
           50          55  
 Ala Gly Gly Val Pro Phe Met Leu Phe Leu Thr Arg Val Arg His  
           65          70  
 Ala Gly Met Val Thr Val Phe Ala Ile Ile Thr Ala Gly Leu Leu  
           80          85  
 Ala Leu Thr Gly His Pro Pro Ile Cys Phe Val Ile Thr Val Ala  
           95          100  
 Cys Ala Leu Val Ala Glu Val Val Leu Trp Leu Gly Arg Tyr Arg  
          110         115  
 Ser Arg Thr Met Gly Val Leu Ala Tyr Ala Ile Tyr Ala Ala Trp  
          125         130  
 Tyr Ile Gly Pro Leu Leu Pro Ile Phe Tyr Ala Arg Asp Glu Tyr  
          140         145  
 Phe Ser Ser Pro Gly Met Ala Gln Met Gly Pro Arg Tyr Leu Glu  
          155         160  
 Glu Met Glu Arg Leu Leu Ser Pro Ala Val Leu Ile Ala Phe Asp  
          170         175  
 Leu Ser Thr Val Val Phe Gly Leu Ile Gly Gly Leu Leu Gly Val  
          185         190  
 Arg Leu Leu Arg Lys His Phe Gln Arg Ala Gly Leu Ala  
          200         205         208

<210> 9  
 <211> 27  
 <212> DNA  
 <213> Mycobacterium avium subspecies paratuberculosis

<220>  
 <223> 85A forward primer

<400> 9  
 cgggatccatgatgacgcttgtcgaca 27

<210> 10  
 <211> 21  
 <212> DNA  
 <213> Mycobacterium avium subspecies paratuberculosis

<220>  
 <223> 85A reverse primer

<400> 10  
 cgggatccttaggtgccctgg 21

<210> 11  
 <211> 20  
 <212> DNA  
 <213> Mycobacterium avium subspecies paratuberculosis

<220>  
 <223> 85B forward primer

## Chang Sequence Listing

<400> 11  
cgggatccatgacagatctg 20

<210> 12  
<211> 20  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> 85B reverse primer

<400> 12  
cgggatccttatccgccg 20

<210> 13  
<211> 23  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> 85C forward primer

<400> 13  
cgggatccatgtcgttcacgaa 23

<210> 14  
<211> 21  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> 85C reverse primer

<400> 14  
cgggatcctcaggtggcgggc 21

<210> 15  
<211> 20  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> SOD forward primer

<400> 15  
ggatcctgggactatgcagc 20

<210> 16  
<211> 23  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> SOD reverse primer

<400> 16  
agatcttcagccgaagatcaggc 23

<210> 17  
<211> 20  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> 35 kDa forward primer

## Chang Sequence Listing

<400> 17  
ggatccccacttggtgatct 20

<210> 18  
<211> 26  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> SOD reverse primer

<400> 18  
agatcttcacttggtcatggaact 26

<210> 19  
<211> 18  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> MptC forward primer

<400> 19  
ggatcccgcggtcggcgt 18

<210> 20  
<211> 23  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> MptC reverse primer

<400> 20  
agatcttcattggtcgaggtgcct 23

<210> 21  
<211> 18  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> MptD forward primer

<400> 21  
ggatcccgccgcatcgac 18

<210> 22  
<211> 21  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> MptD reverse primer

<400> 22  
agatcttcaagctaggccggc 21

<210> 23  
<211> 18  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis

<220>  
<223> ESAT6-like forward primer

<400> 23  
ggatccccgggcgcggtg 18

## Chang Sequence Listing

<210> 24  
<211> 19  
<212> DNA  
<213> Mycobacterium avium subspecies paratuberculosis  
  
<220>  
<223> ESAT6-like reverse primer  
  
<400> 24  
agatcttcagaacaggccg 19