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(54) **BIOMARKERS FOR EARLY DIAGNOSIS AND DIFFERENTIATION OF MYCOBACTERIAL INFECTION**

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CPC *G01N 33/6854* (2013.01); *C12Q 1/6851* (2013.01); *G01N 33/5695* (2013.01); *G01N 33/68* (2013.01); *G01N 2800/26* (2013.01); *G01N 2800/50* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

Mycobacterial-specific biomarkers and methods of using such biomarkers for diagnosis of mycobacterial infection in a mammal are disclosed.

10 Claims, 10 Drawing Sheets
(8 of 10 Drawing Sheet(s) Filed in Color)

Specification includes a Sequence Listing.

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FIG. 1

FIGS. 2A-2B

A.

B.

FIGS. 3A–3C

FIGS. 4A-4C

A

Infected vs. naïve   LAV-vaccinated vs. naïve

158   68   950

Total analyzed genes: 17,380

B

Infected vs. naïve   Infected vs. LAV-vaccinated

150   76   1057

Total analyzed genes: 17,380

C

LAV-vaccinated vs. naïve   Mycopar-vaccinated vs. naïve

351   667   1047

Total analyzed genes: 17,380

FIG. 5

Infected vs Naive

Percent of genes

■ Input list   ▩ Background/Reference

GO annotation
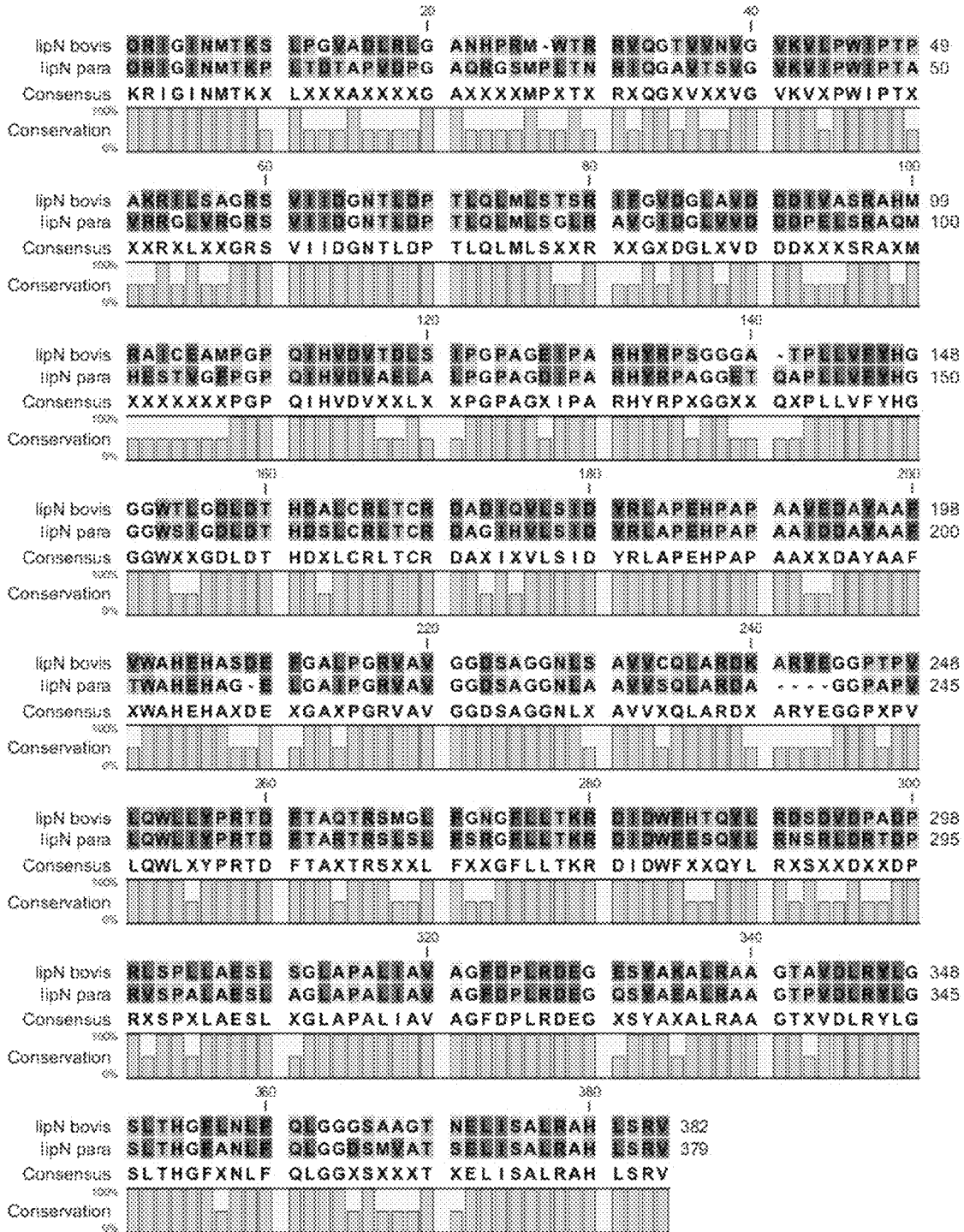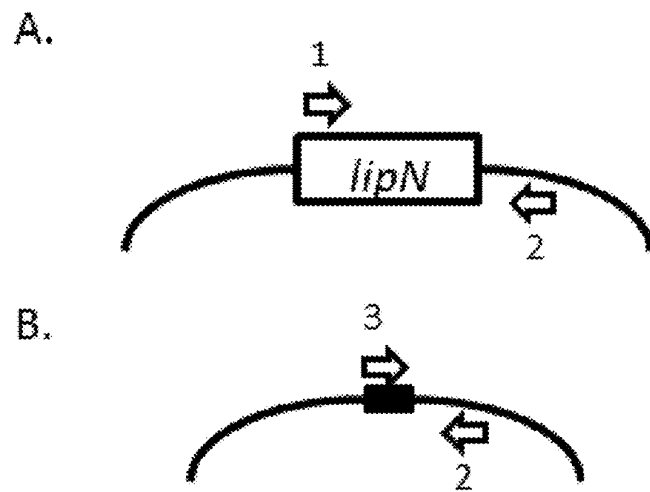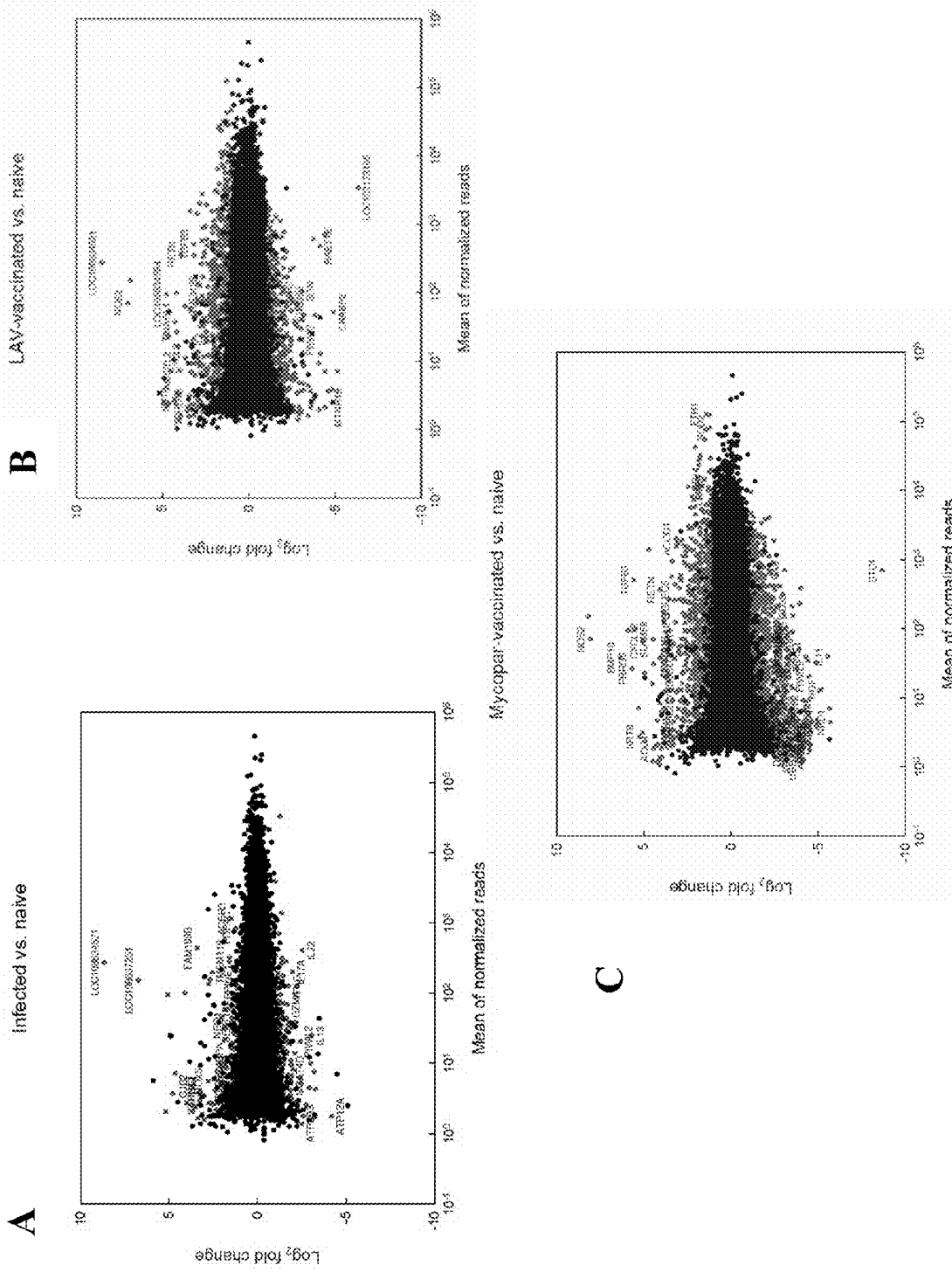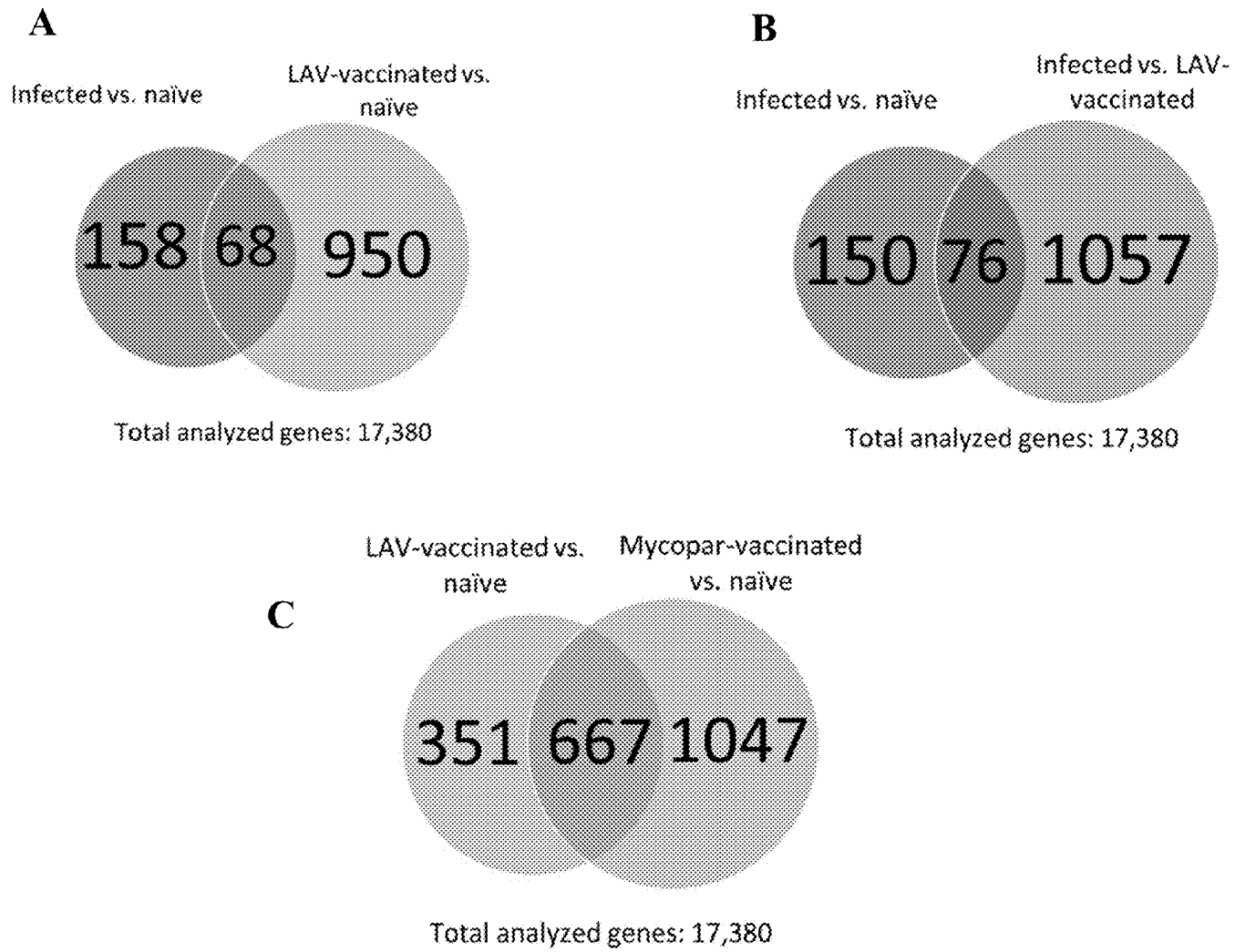
FIGS. 6A-6B

A

B

FIGS. 7A-7D

FIGS. 8A-8C

A.
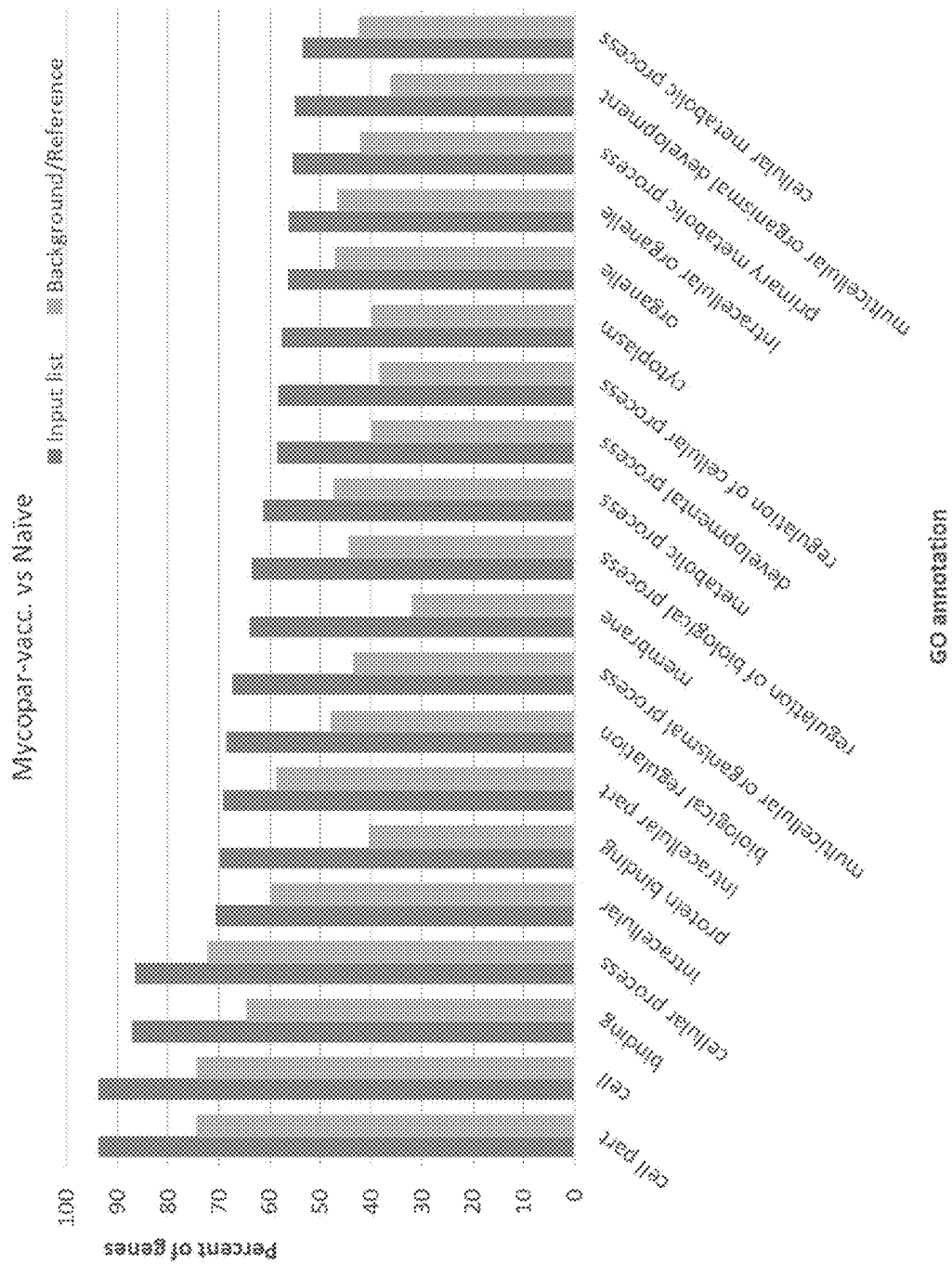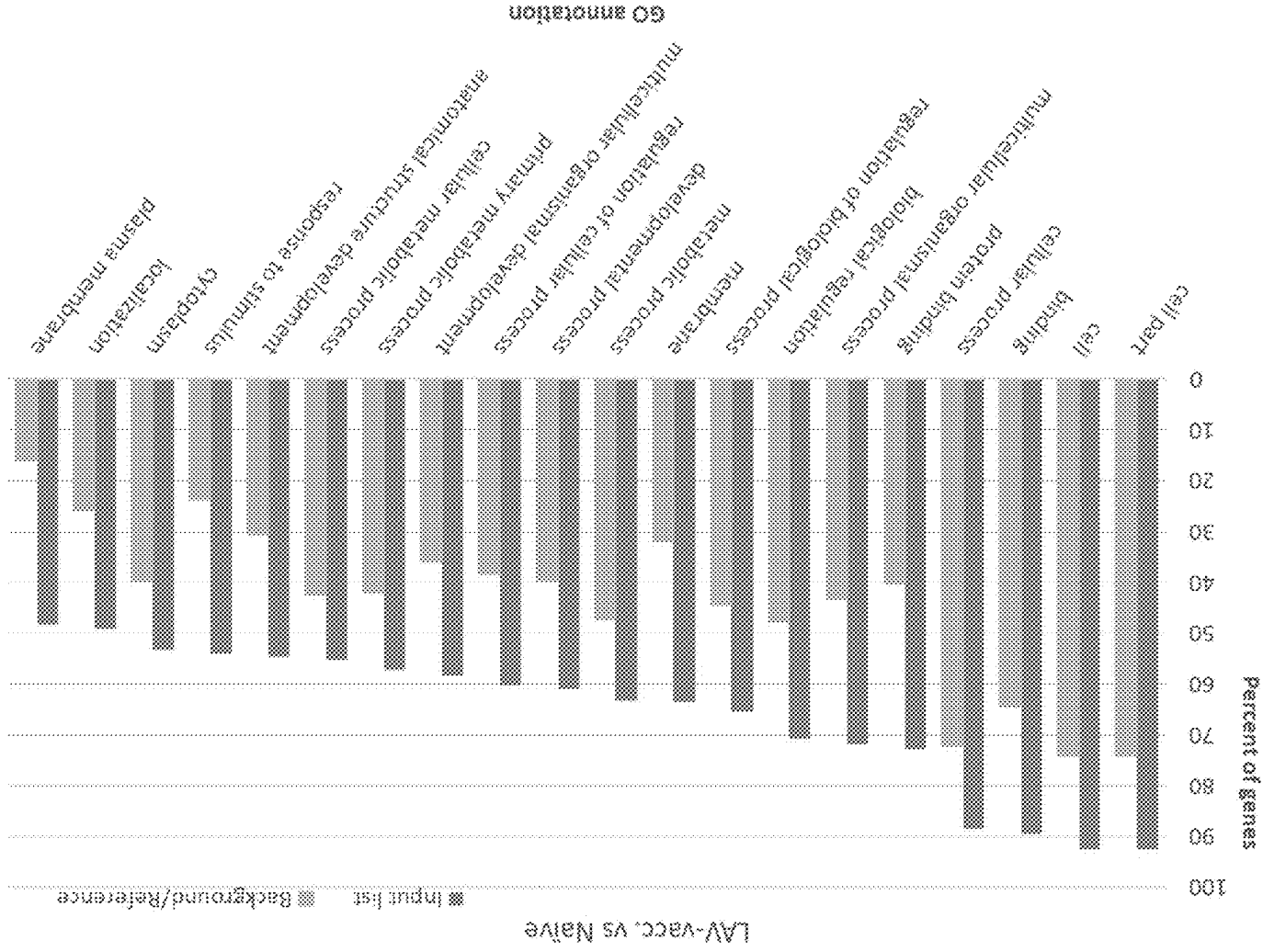
FIGS. 8A-8C (CONTINUED)

FIGS. 8A-8C (CONTINUED)

C.

LAV-vacc. vs Infected

Percent of genes

GO annotation

BIOMARKERS FOR EARLY DIAGNOSIS AND DIFFERENTIATION OF MYCOBACTERIAL INFECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 62/728,387 filed Sep. 7, 2018, which is incorporated herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under 2013-67015-21347, 2018-67015-28243 and 2016-33610-25438 awarded by the USDA/NIFA. The government has certain rights in the invention.

REFERENCE TO A SEQUENCE LISTING SUBMITTED VIA EFS-WEB

The content of the ASCII text file of the sequence listing named "960296_02450_ST25.txt" which is 21.4 kb in size was created on Aug. 28, 2019 and electronically submitted via EFS-Web herewith the application is incorporated herein by reference in its entirety.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, and patent application was specifically and individually indicated to be incorporated by reference.

BACKGROUND OF THE INVENTION

The field of the invention is vaccine and diagnostic biomarkers. More particularly, the invention relates to a set of biomarkers for diagnosing mycobacterial infection and distinguishing between vaccinated and infected animals.

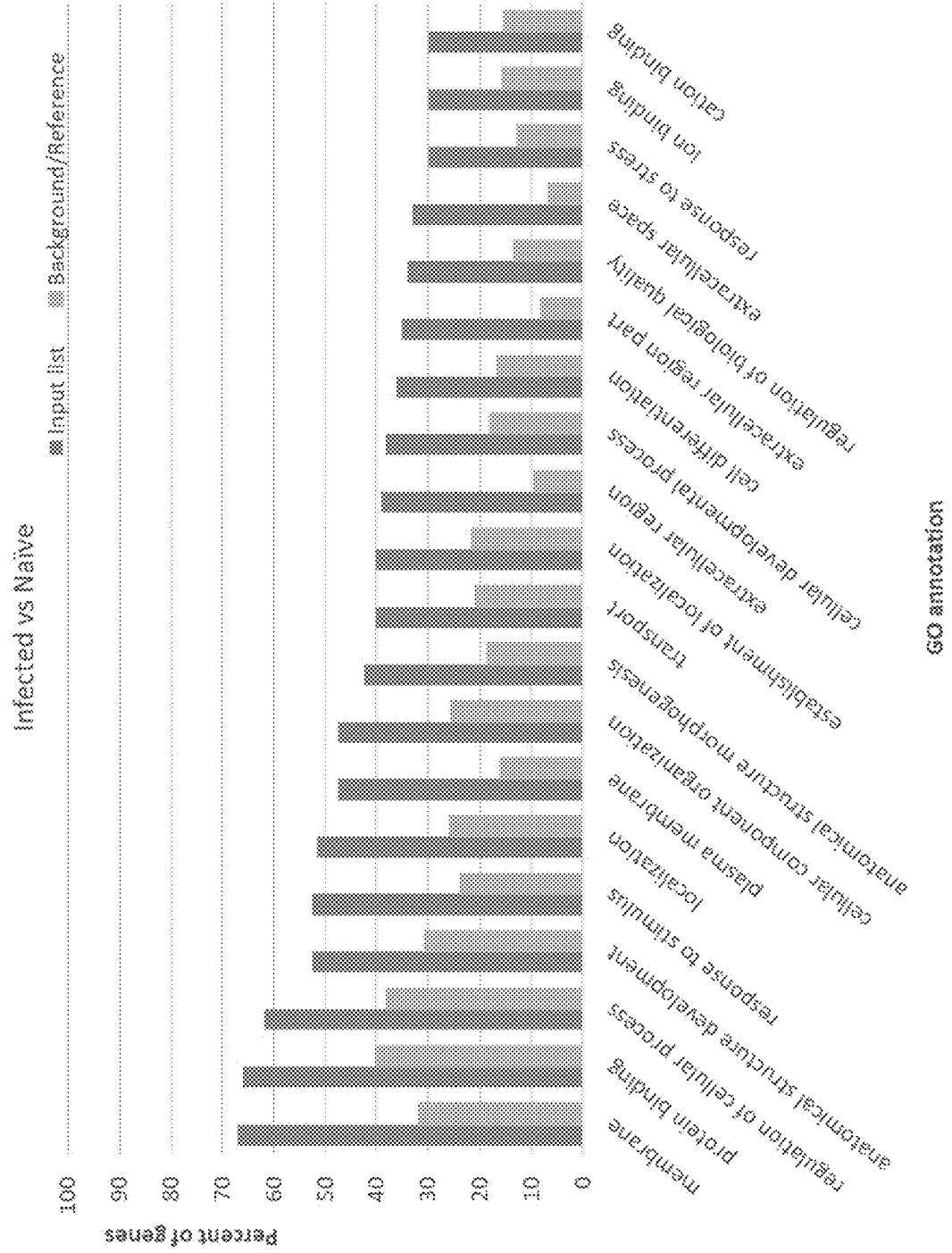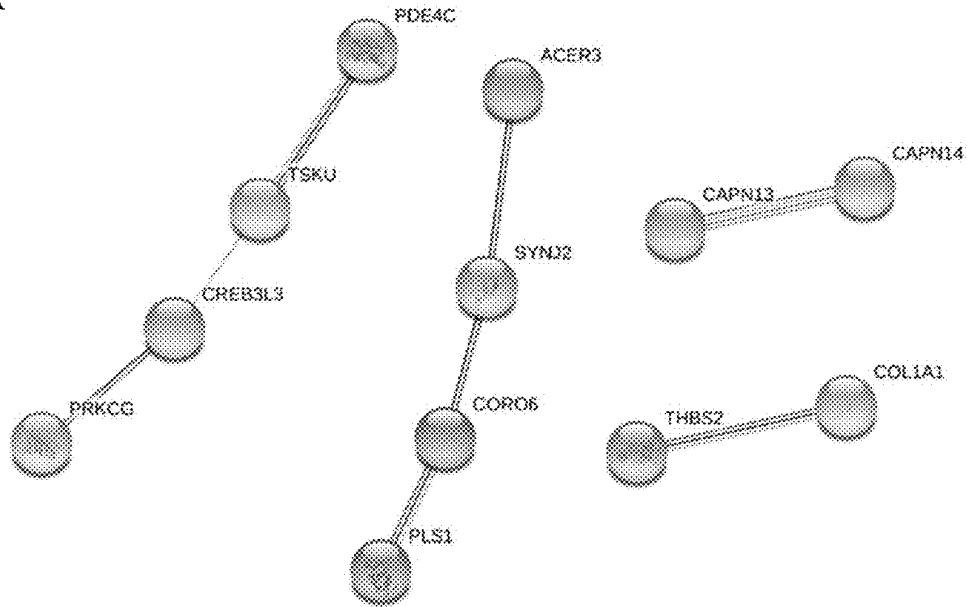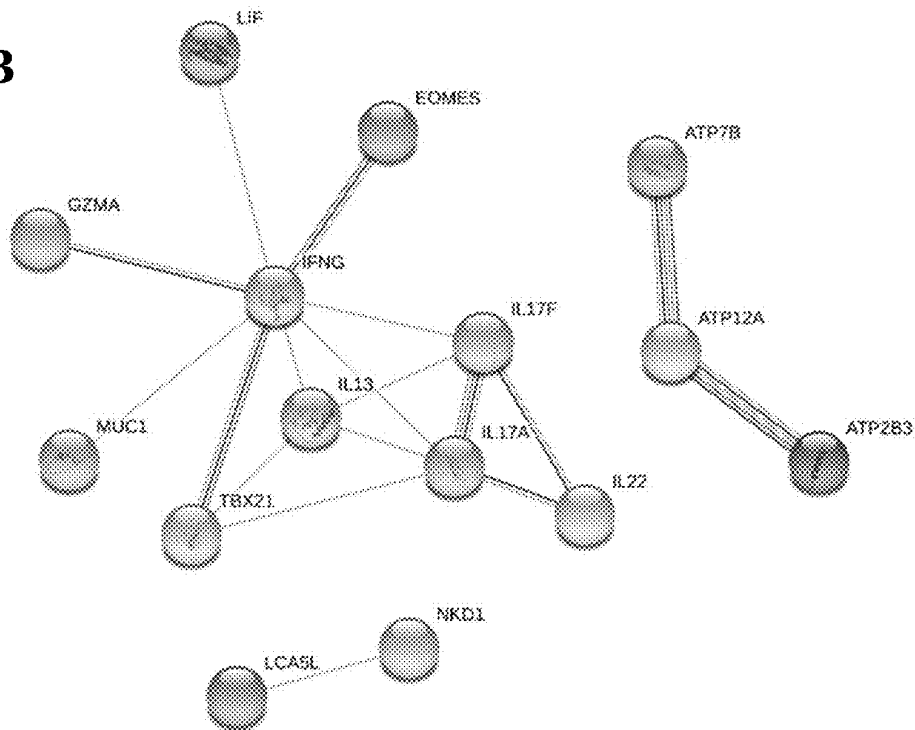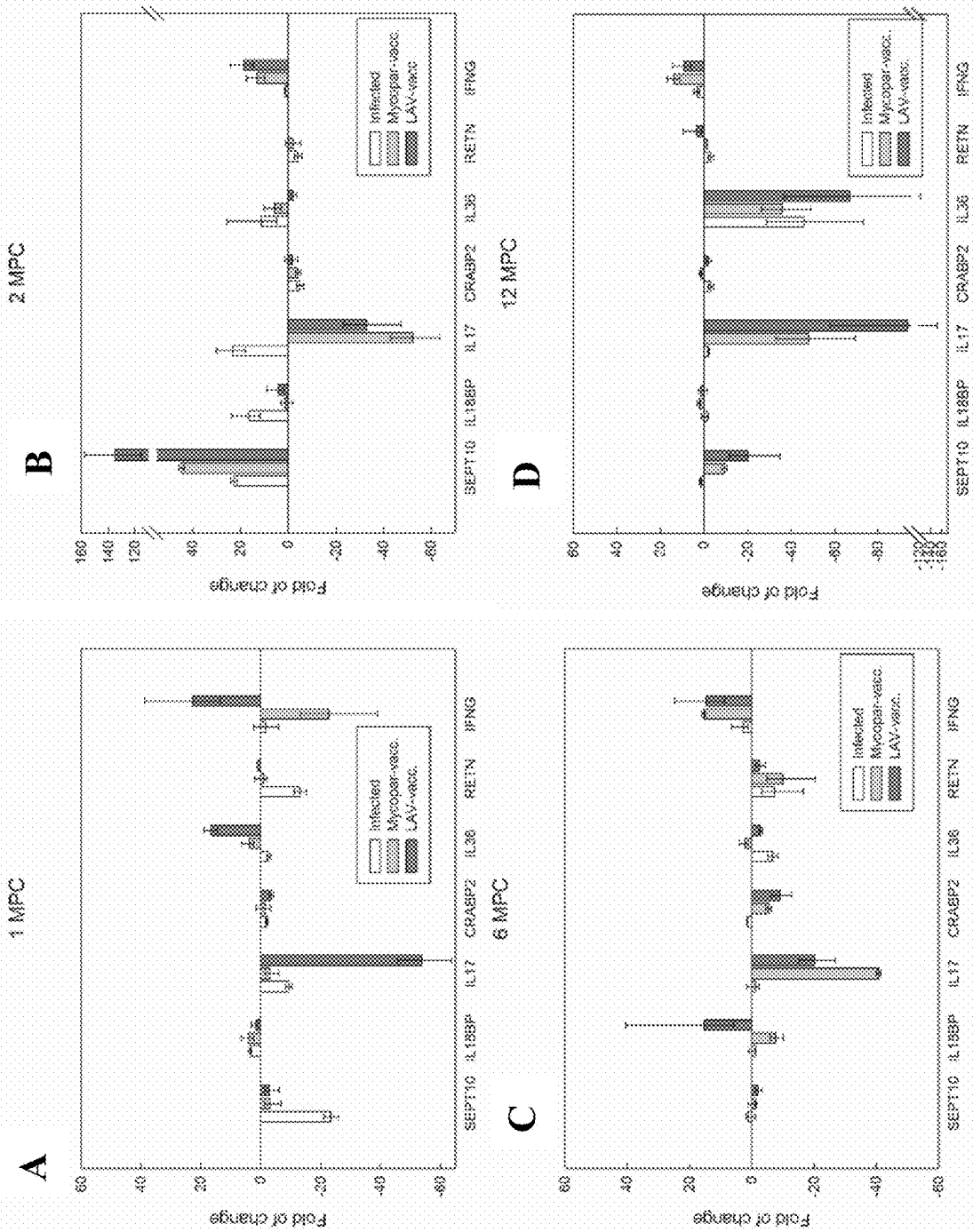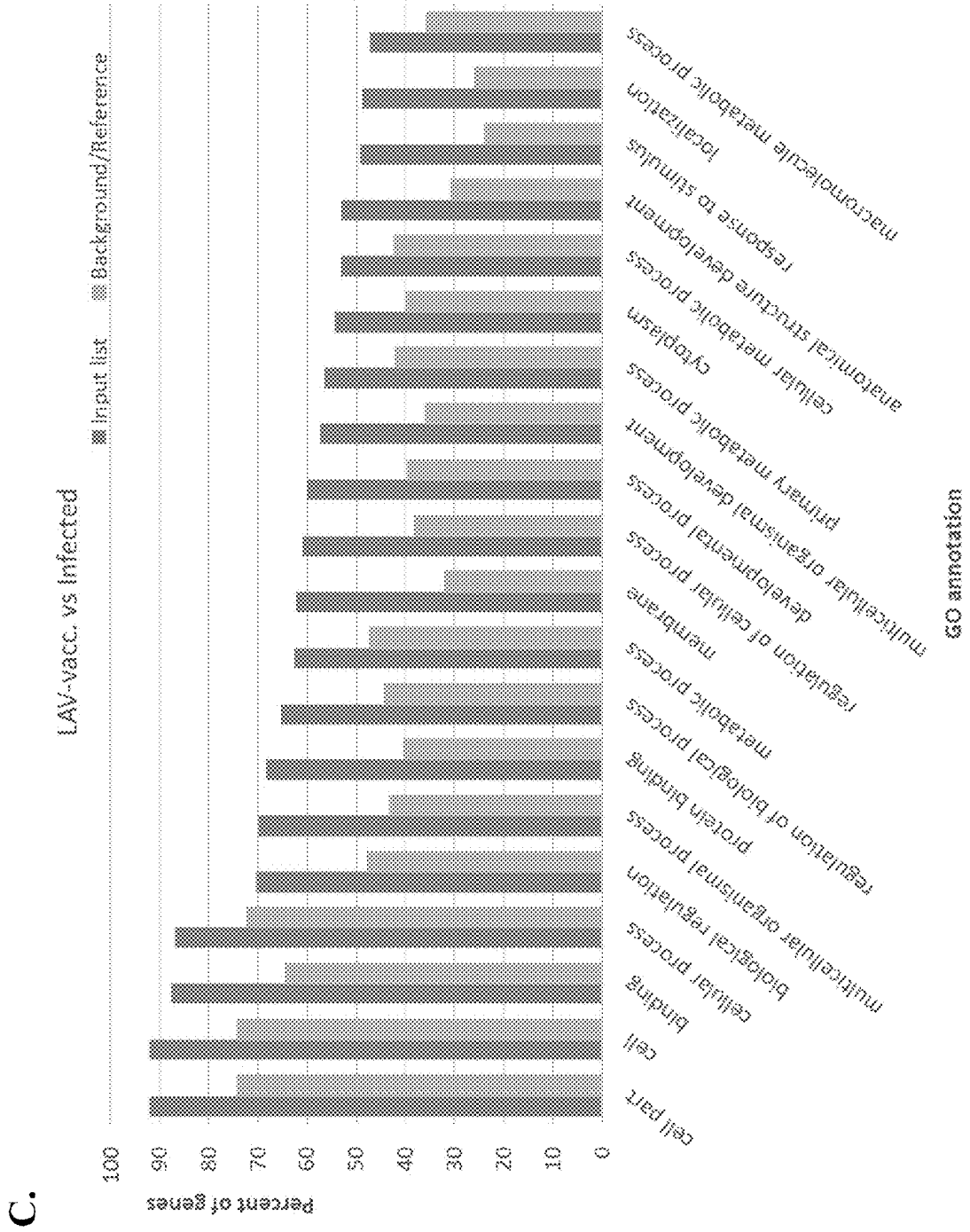Mycobacterial infections cause significant health problems to humans and animals including human tuberculosis, bovine tuberculosis, and Johne's disease. Johne's disease (aka *paratuberculosis*) is caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* (*M. ap*); this disease causes severe economic losses estimated at $500 million per year for the US dairy industry alone, and these infections constitute a problem for 91% of dairy herds. Bovine tuberculosis, which is caused by infection with *M. bovis*, is endemic in dairy herds in several parts of the developing world and a significant problem for the wildlife animals in several developed countries (e.g., UK, USA, and Australia).

Current diagnostics can detect mycobacterial infections in cattle that have started to shed the bacteria or developed an antibody response. The available diagnostic tools are unreliable to detect early stages of infection or to differentiate infected from vaccinated animals (aka the DIVA principle). Early detection of mycobacterial infections is imperative to control the infection in herds. Further, the availability of a DIVA-based assay will facilitate adoption of new vaccines that can prevent *M. ap* infection.

Needed in the art are methods or diagnostic tools for detecting early stages of mycobacterial infection. Addition-

ally, needed in the art are methods or diagnostic tools for distinguishing vaccinated from infected animals.

SUMMARY OF THE INVENTION

The present invention overcomes the aforementioned drawbacks by providing a set of biomarkers to diagnose infected animals and distinguishing between vaccinated and infected animals.

In a first aspect, provided herein is a method for diagnosis of mycobacterial infection in a mammal, the method comprising the steps of a) obtaining a first type of sample from the mammal; b) measuring the expression level in the sample of at least one biomarker selected from the group consisting of LOC108634521, LOC108637251, LOC108637252, LOC108634594, FAM198B, LOC108637671, CDCP1, TMTC1, BAIAP2L1, MEI1, SEPT10, IFNG, IL17F, FCER2, ADGRG1, APBB1, PIWIL2, AOAH, and homologs thereof and comparing the level of the biomarker against the level detected in a first type of sample from an uninfected mammal of the same species; and c) determining the infection status of the mammal, wherein differential expression of the biomarker is indicative of a mycobacterial infection in the mammal. In some embodiments, the method is used for early diagnosis and detection of mycobacterial infection in a mammal. In some embodiments, the measuring is via ELISA assay for antibodies formed against the biomarker. In some embodiments, measuring is via quantitative PCR. In some embodiments, the sample is a blood sample. In some embodiments, the mammal is selected from the group consisting of cow, sheep and goat.

In a second aspect, provided herein is a method for differentiating between mammals who have been vaccinated with a live-attenuated mycobacterial vaccine and non-vaccinated mammals, the method comprising the steps of a) obtaining a first type of sample from the mammal; b) measuring the expression level in the sample of at least one biomarker selected from the group consisting of LOC108634521, NOS2, LOC108637251, TINAGL1, RETN, C1QL2, TDRD10, TGFB3, ADGRE2, LIPG, KCNJ2, AQP9, BPI, IL9, IL1R2, IL36B, IGF1, BGN, PIWIL2, RAET1E, CRABP2, AOAH, and homologs thereof and comparing the level of the biomarker against the level detected in a first type of sample from a non-vaccinated mammal of the same species; and c) determining the vaccination status of the mammal, wherein differential expression of the biomarker is indicative of a mammal who has been vaccinated with a live-attenuated mycobacterial vaccine. In some embodiments, the measuring is via ELISA assay for antibodies formed against the biomarker. In some embodiments, the measuring is via quantitative PCR. In some embodiment, the sample is a blood sample. In some embodiments, the mammal is selected from the group consisting of cow, sheep and goat.

In some embodiments, the live-attenuated mycobacterial vaccine is a *mycobacterium* mutant vaccine. In some embodiments, the *mycobacterium* mutant vaccine comprises at least one mutation in at least one gene sequence encoding global gene regulators (GGRs) selected from the group consisting of sigH, sigL and LipN.

In a third aspect, provided herein is a method for differentiating between mammals who have been vaccinated with a live-attenuated mycobacterial vaccine and infected mammals, the method comprising the steps of a) obtaining a first type of sample from the mammal; b) measuring the expression level in the sample of at least one biomarker selected

from the group consisting of LOC106503226, PMP22, ART5, LOC102169116, GNLY, ASAP3, LOC108633178, TBKBP1, SLC17A7, LOC108638192, IFNG and homologs thereof and comparing the level of the biomarker against the level detected in a first type of sample from an infected mammal of the same species; and c) determining the vaccination status of the mammal, wherein differential expression of the biomarker is indicative of a mammal who has been vaccinated with a live-attenuated mycobacterial vaccine. In some embodiments, the measuring is via ELISA assay for antibodies formed against the biomarker. In some embodiments, the measuring is via quantitative PCR. In some embodiments, the sample is a blood sample. In some embodiments, the mammal is selected from the group consisting of cow, sheep and goat.

In some embodiments, the live-attenuated mycobacterial vaccine is a *mycobacterium* mutant vaccine. In some embodiments, the *mycobacterium* mutant vaccine comprises at least one mutation in at least one gene sequence encoding global gene regulators (GGRs) selected from the group consisting of sigH, sigL and LipN.

In a fourth aspect, provided herein is a method for differentiating between mammals who have been vaccinated with a live-attenuated mycobacterial vaccine, non-vaccinated naïve mammals, and infected mammals, the method comprising the steps of obtaining a first type of sample from the mammal; measuring the expression level of FAM198B and AOAH in the sample; determining the vaccination or infection status of the mammal, wherein when relative expression of FAM198B is higher than AOAH the subject is infected, when relative expression of AOAH is higher than FAM198B the subject is vaccinated, and when the relative expression of AOAH and FAM198B are equal the subject is naïve.

In some embodiments, the measuring is via ELISA assay for antibodies formed against the biomarker. In some embodiments, the measuring is via quantitative PCR. In some embodiments, the sample is a blood sample. In some embodiments, the mammal is selected from the group consisting of cow, sheep, and goat. In some embodiments, the live-attenuated mycobacterial vaccine is a *mycobacterium* mutant vaccine. In some embodiments, the *mycobacterium* mutant vaccine comprises at least one mutation in at least one gene sequence encoding global gene regulators (GGRs) selected from the group consisting of sigH, sigL and LipN.

The foregoing and other aspects and advantages of the invention will appear from the following description. In the description, reference is made to the accompanying drawings which form a part hereof, and in which there is shown by way of illustration a preferred embodiment of the invention. Such embodiment does not necessarily represent the full scope of the invention, however, reference is made therefore to the claims and herein for interpreting the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

FIG. **1** is a graph showing the alignment plot of amino acids deduced from the protein sequence in LipN of both *M. paratuberculosis* (SEQ ID NO:1) and *M. bovis* (SEQ ID NO:2). The consensus sequence is SEQ ID NO:3. Peptides

conserved in *M. paratuberculosis* sequence but absent from*M. bovis* were targets for previous DIVA testing methods.

FIGS. **2A-2B** is a diagram showing multiplex PCR strategy using 3 primers. (A) Wild-type (virulent) strain with intact lipN gene. (B) LAV strain with scar sequence from hygromycin cassette removal represented by the black rectangle.

FIGS. **3A-3C** show RNA sequencing analysis of different goat groups following infection or vaccination with LAV or Mycopar vaccines. MAplots of (A) the infected group compared to the naïve group, (B) the LAV-vaccinated group compared to the naïve group and (C) the Mycopar-vaccinated group compared to the naïve group are shown. Red dots represent differentially expressed transcripts (fold change>2.0 or <−2.0, p<0.05).

FIGS. **4A-4C** show comparative transcriptome analysis of the infected and vaccinated goat groups. Venn diagrams show numbers of common DE transcripts between (A) the infected and vaccinated groups, each compared to the naïve group. (B) infected vs. naïve and infected vs. LAV-vaccinated groups and (C) LAV-vaccinated vs. naïve and Mycopar-vaccinated vs. naïve groups.

FIG. **5** shows significant terms in gene ontology analysis for the differentially expressed genes in infected goats compared to naïve control goats. The agriGO for automated identification of GO terms were used on the list of genes with significant differential expression when the transcriptomes of infected and naïve goats were compared.

FIGS. **6A-6B** show gene network analysis of DE genes in the infected group. (A) Genes that were significantly up-regulated. (B) Genes that were significantly down-regulated. IL-17A and IL-17F are two homologs in the IL-17 family. Light green lines represent connection between genes co-mentioned in an abstract in published studies, cyan lines represent putative pathway connections found in homologs in other species, black lines represent co-expression in *Bos taurus* or homologs in other species and pink lines represent experimentally determined association.

FIGS. **7A-7D** show the transcriptional profile of key caprine genes over 12 months post challenge with *M. paratuberculosis*. Panels display quantitative real-time PCR analysis of total RNA extracted from blood samples collected from goat groups at 1 (A), 2 (B), 6 (C) and 12 (D) months post challenge (MPC). Expression levels were calculated with $\Delta\Delta$Ct relative quantitation method relative to the GAPDH gene expression in the naïve group. Target gene names are listed below each panel and fold change for the infected, Mycopar® or LAV-vaccinated relative to naïve goat groups are listed on the Y-axis. At each time point, samples from three animals in each group except the infected group at 1 MPC (N=2) were included and standard errors of the mean (SEM) of the three measurements were presented as error bars.

FIGS. **8A-8C** show significant terms in gene ontology analysis, using agriGO, for the differentially expressed genes. The significant terms for the GO analysis for the Mycopar-vaccinated vs naïve group is shown in (A). Chart in (B) shows the significant terms for LAV-vaccinated vs naïve group and in (C) shows the significant terms for LAV-vaccinated vs. infected group. The significant GO terms for both comparisons are 1-binding, 2-intracellular, 3-intracellular part and 4-metabolic process.

DETAILED DESCRIPTION OF THE INVENTION

The term "*mycobacterium*," as used herein, refers to a genus of actinobacteria given its own family, the mycobac-

teriaceae. The genus includes pathogens known to cause serious diseases in mammals, including tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*).

Mycobacterium tuberculosis complex (MTBC) members are causative agents of human and animal tuberculosis. Species in this complex may include *M. tuberculosis*, the major cause of human tuberculosis, *M. bovis, M. bovis* BCG, *M. africanum, M. canetti, M. caprae, M. microti*, and *M. pinnipedii*.

Mycobacterium avium complex (MAC) is a group of species that, in a disseminated infection but not lung infection, used to be a significant cause of death in AIDS patients. Species in this complex include *M. avium, M. avium paratuberculosis*, which has been implicated in Crohn's disease in humans and is the causative agent of Johne's disease in cattle and sheep, *M. avium silvaticum, M. avium "hominissuis,"* *M. colombiense*, and *M. indicus pranii*.

Mycobacterial infections are notoriously difficult to treat. The organisms are hardy due to their cell wall, which is neither truly Gram negative nor Gram positive. In addition, they are naturally resistant to a number of antibiotics that disrupt cell-wall biosynthesis, such as penicillin. Due to their unique cell wall, they can survive long exposure to acids, alkalis, detergents, oxidative bursts, lysis by complement, and many antibiotics. Most mycobacteria are susceptible to the antibiotics clarithromycin and rifamycin, but antibiotic-resistant strains have emerged.

The term "biomolecule," as used herein, refers to any organic molecule that is part of or from a living organism. Biomolecules may include nucleic acids, a nucleotide, a polynucleotide, an oligonucleotide, a peptide, a protein, a carbohydrate, a ligand, a receptor, among others. In one embodiment of the present invention, biomolecules may include genes and their expression products.

The term "expression product," as used herein, refers to any product produced during the process of gene expression. These products are often proteins, but in non-protein coding genes such as ribosomal RNA (rRNA), transfer RNA (tRNA) or small nuclear RNA (snRNA) genes, the product is a functional RNA.

The terms "polypeptide," "peptide," and "protein," as used herein, refer to a polymer comprising amino acid residues predominantly bound together by covalent amide bonds. The terms apply to amino acid polymers in which one or more amino acid residue may be an artificial chemical mimetic of a naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms may encompass amino acid chains of any length, including full length proteins, wherein the amino acids are linked by covalent peptide bonds. The protein or peptide may be isolated from a native organism, produced by recombinant techniques, or produced by synthetic production techniques known to one skilled in the art.

The term "recombinant protein," as used herein, refers to a polypeptide of the present disclosure which is produced by recombinant DNA techniques, wherein generally, DNA encoding a polypeptide is inserted into a suitable expression vector which is in turn used to transform a heterologous host cell (e.g., a microorganism or yeast cell) to produce the heterologous protein.

The term "recombinant nucleic acid" or "recombinant DNA," as used herein, refers to a nucleic acid or DNA of the present disclosure which is produced by recombinant DNA techniques, wherein generally, DNA encoding a polypeptide

is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein.

The term "mammal," as used herein, refers to any living species which can be identified by the presence of sweat glands, including those that are specialized to produce milk to nourish their young. In one embodiment, the mammal suitable for the present invention may include bubaline, elephantine, musteline, pardine, phocine, rhinocerine, caprine, hircine, leonine, leporine, lupine, lyncine, murine, rusine, tigrine, ursine, vulpine, zebrine, vespertilionine, porcine, bovine, equine, swine, elaphine, ovine, caprine, camelidae, feline, cervine, primate, human and canine mammals. In one preferred embodiment of the present invention, the mammal may be one of the ruminants such as cattle, goats, sheep, giraffes, yaks, deer, camels, llamas, antelope, and some macropods. In one specific embodiment of the present invention, the mammal may include any of the milk cattle species, such as cow, sheep and goat.

The term "antibody," as used herein, refers to a class of proteins that are generally known as immunoglobulins. The term "antibody" herein is used in the broadest sense and specifically includes full-length monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity. Various techniques relevant to the production of antibodies are provided in, e.g., Harlow, et al., ANTIBODIES: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1988).

The term "marker" or "biomarker," as used herein, refers to a biomolecule (e.g., protein, nucleic acid, carbohydrate, or lipid) that is differentially expressed in the cell, differentially expressed on the surface of an infected cell, differentially phosphorylated, or differentially secreted by a infected cell in comparison to a normal cell or in a paracrine fashion by neighboring uninfected cells, and which is useful for the diagnosis of mycobacterial infection, differentiating between infected and vaccinated animals, and for preferential targeting of a pharmacological agent to an infected mammal. In some embodiments, the biomarker is differentially expressed in an infected subject in comparison to a normal subject. In some embodiments, the biomarker is differentially expressed in a vaccinated subject in comparison to a non-vaccinated subject. In some embodiments, the biomarker is differentially expressed in a vaccinated subject in comparison to an infected subject.

The term "differentially expressed," as used herein, refers to a change in expression of at least 2-fold. In some embodiments, differential expression indicates that a given biomarker is over-expressed in a first subject or cell in comparison to a second subject or cell, for instance, at least 2-fold over-expression, at least 3-fold over-expression, at least 4-fold over-expression or more in comparison to the second subject or cell. In some embodiments, differential expression indicates that a given biomarker has decreased expression in a first subject or cell in comparison to a second subject or cell, for instance, at least a 2-fold decease in expression, at least a 3-fold decrease in expression, at least a 4-fold decrease in expression or more.

The term "mycobacterial-specific biomarkers," as used herein, refers to biomarkers which are specifically related to mycobacterial infection. Some of these biomarkers are listed in FIG. 1 and Tables 4 and 5.

The term "lyophilization," as used herein, refers to freezing of a material at low temperature followed by dehydration by sublimation, usually under a high vacuum. Lyophiliza-

tion is also known as freeze drying. Many techniques of freezing are known in the art of lyophilization such as tray freezing, shelf freezing, spray-freezing, shell-freezing and liquid nitrogen immersion. Each technique will result in a different rate of freezing. Shell freezing may be automated or manual. For example, flasks can be automatically rotated by motor driven rollers in a refrigerated bath containing alcohol, acetone, liquid nitrogen, or any other appropriate fluid. A thin coating of product is evenly frozen around the inside "shell" of a flask, permitting a greater volume of material to be safely processed during each freeze drying run. Tray freezing may be performed by, for example, placing the samples in lyophilizer, equilibrating 1 hr at a shelf temperature of 0° C., then cooling the shelves at 0.5° C./min to −40° C. Spray-freezing, for example, may be performed by spray freezing into liquid, dropping by ~20 μl droplets into liquid N_2, spray freezing into vapor over liquid, or by other techniques known in the art.

The term "antigen," as used herein, refers to any molecule that is capable of eliciting an immune response, whether a cell-mediated or humoral immune response, whether in the presence or absence of an adjuvant. An antigen can be any type of molecule, e.g., a peptide or protein, a nucleic acid, a carbohydrate, a lipid, and combinations thereof. A "vaccine antigen" is an antigen that can be used in a vaccine preparation. A "therapeutic antigen" is an antigen that can be used for therapeutic purposes.

The term "vaccine," as used herein, refers to an antigenic preparation used to produce active immunity to a disease, in order to prevent or ameliorate the effects of infection. The antigenic moiety making up the vaccine may be either a live or killed microorganism, or a natural product purified from a microorganism or other cell including, but not limited to tumor cells, a synthetic product, a genetically engineered protein, peptide, polysaccharide or similar product or an allergen.

The term "immunologically active," as used herein, refers to the ability to raise one or more of a humoral response or a cell mediated response specific to an antigen.

The term "adjuvant," as used herein, refer to compounds that, when used in combination with specific vaccine antigens in formulations, augment or otherwise alter or modify the resultant immune responses. An adjuvant combined with a vaccine antigen increases the immune response to the vaccine antigen over that induced by the vaccine antigen alone. An adjuvant may augment humoral immune responses or cell-mediated immune responses or both humoral and cell-mediated immune responses against vaccine antigens.

The term "detecting," as used herein, refers to confirming the presence of the biomarker or marker present in the sample. Quantifying the amount of the biomarker or marker present in a sample may include determining the concentration of the biomarker present in the sample. Detecting and/or quantifying may be performed directly on the sample, or indirectly on an extract therefrom, or on a dilution thereof.

The term "homology," as used herein, refers to the resemblance or similarity between two nucleotide or amino acid sequences. As applied to a gene, "homolog" may refer to a gene similar in structure and/or evolutionary origin to a gene in another organism or another species. As applied to nucleic acid molecules, the term "homolog" means that two nucleic acid sequences, when optimally aligned (see below), share at least 80 percent sequence homology, preferably at least 90 percent sequence homology, more preferably at least 95, 96, 97, 98 or 99 percent sequence homology. "Percentage nucleotide (or nucleic acid) homology" or "percentage

nucleotide (or nucleic acid) sequence homology" refers to a comparison of the nucleotides of two nucleic acid molecules which, when optimally aligned, have approximately the designated percentage of the same nucleotides or nucleotides that are not identical but differ by redundant nucleotide substitutions (the nucleotide substitution does not change the amino acid encoded by the particular codon). For example, "95% nucleotide homology" refers to a comparison of the nucleotides of two nucleic acid molecules which, when optimally aligned, have 95% nucleotide homology.

In one aspect, the present invention relates to a method for diagnosis of mycobacterial infection in a mammal. In one embodiment, the present invention discloses a method for early detection of mycobacterial infection. The term "early detection," as used herein, refers to detection of mycobacterial infection during the early stage of infection, e.g., a stage before the development of chronic diarrhea.

In another embodiment, the present invention discloses a method for differentiating a vaccinated mammal from a non-infected mammal or a mycobacterial infected mammal.

The detection of mycobacterial infection and related diseases such as Johne's disease is very difficult because the disease generally takes many years to develop, and the organism is shed by the mammal periodically, so every mammal must be repeatedly tested over long time periods.

Applicants have identified mycobacterial-specific biomarkers and host-specific biomarkers, such as genes and/or expression products derived thereof, useful for detection of mycobacterial infection. Mycobacterial-specific biomarkers, host-specific biomarkers or a combination of such biomarkers may also be used to differentiate a vaccinated mammal (e.g., from genetically engineered vaccines) from a non-infected mammal or a mycobacterial-infected mammal.

Differentiating Vaccinated Mammals from Mycobacterial-Infected Mammals

In one embodiment, the present invention discloses a method for differentiating a vaccinated mammal from a mycobacterial infected mammal.

In one embodiment, the method for differentiating a vaccinated mammal from a mycobacterial-infected mammal comprises the steps of (a) obtaining a sample from the mammal; (b) testing the sample for the concentration level of at least one biomarker and comparing the level of the biomarker against the level detected in an infected mammalian sample; and (c) determining the infection or vaccination status of the mammal.

A sample suitable for the present invention may include any biological sample from the mammal. The biological sample may include, without limitation, saliva, sputum, blood, plasma, serum, urine, feces, cerebrospinal fluid, amniotic fluid, wound exudate, or tissue of the subject of mammal. In one specific embodiment, the biological sample is a blood sample. In some embodiments, when comparing the expression level of a biomarker between two subjects, it is desirable to compare expression levels in the same type of sample.

A major problem in employing mass vaccination program for the control of Johne's disease in dairy herds is the inability to differentiate between infected and vaccinated animals with the current vaccine (DIVA principal). Applicants have previously proposed using genetically engineered vaccines (PCT patent application publication WO2014164055, U.S. Pat. Nos. 9,663,758, and 9,446,110, each of which is incorporated herein by reference). One would wish to consider the DIVA principal and wish to distinguish between *M. paratuberculosis* infected and

Johne's disease vaccinated animals that have been vaccinated with genetically engineered vaccines.

In one embodiment, Applicants identify biomolecules as mycobacterial-specific biomarkers. For example, the biomolecules of mycobacterial-specific biomarkers may include genes and their expression products which are present in a *M. ap* wild-type strain but not present or have a low expression level in genetically engineered vaccines and vaccinated animals. In one embodiment involving sigL and sigH mutants, the mycobacterial-specific biomarker may comprise at least one member selected from the group consisting of gene sequences Q73SF4, Q73Y73, Q73ZE6, Q73SL7, Q73VK6, Q73XZ0, Q740D1 and Q73UE0 and expression products derived thereof. In another embodiment, the mycobacterial-specific biomarker may comprise at least two, three, four, five, six, seven or eight members selected from the group consisting of gene sequences Q73SF4, Q73Y73, Q73ZE6, Q73SL7, Q73VK6, Q73XZ0, Q740D1 and Q73UE0 and expression products derived thereof. Preferably, the mycobacterial-specific biomarker may comprise at least two members selected from the group as discussed above.

In one embodiment involving sigL and sigH mutants, the mycobacterial-specific biomarker comprises at least one member selected from the group consisting of gene sequences Q73VL6, Q73YW9, Q741L4, Q744E5, Q73YP5, Q73WE5, Q73U21, Q73UH9, Q741M5, Q742F4, and Q73SU6 and expression products derived thereof. In one embodiment, the mycobacterial-specific biomarker comprises at least two, three, four, five, six, seven, eight, nine or ten members selected from the group consisting of gene sequences Q73VL6, Q73YW9, Q741L4, Q744E5, Q73YP5, Q73WE5, Q73U21, Q73UH9, Q741M5, Q742F4, and Q73SU6 and expression products derived thereof.

Applicants envision that the present invention may be applicable to any genetically engineered vaccines. In some embodiments, the vaccine may be an inactived vaccine (e.g., Mycopar™). In one specific embodiment, the present invention is applicable to live attenuated vaccines. The example of the live attenuated vaccines may include sigL, sigH, or LipN mutants. PCT patent application publication WO20141640055A1 discloses live attenuated vaccines, such as sigL and sigH mutants. PCT patent application publication WO20141640055A1 is incorporated herein by reference in its entirety.

In one embodiment, the presence or absence of the biomarkers in a mammal may demonstrate the infection status of the mammal. In one specific embodiment, the biomarkers that are significantly over-expressed in the wild type strain and not in the mutant vaccine and could be used for the mutant vaccine-DIVA testing.

For example, when the biomarkers are those significantly over-expressed in the wild type strain and not in the mutant vaccine, the presence of at least one biomarker in a mammal shows that the mammal may be infected and not merely vaccinated. On the other hand, the absence of at least one biomarker in a mammal shows that the mammal may be vaccinated.

In one embodiment, Applicants envision that the present invention is also applicable when antigens are inoculated to a mammal and the infection status of the mammal needs to be identified. Specifically, the infection status may include whether a mammal is vaccinated or whether a mammal is infected with *M. paratuberculosis*.

Table 7, which tabulates the result of one of the Examples drawn to host transcriptome analysis of goats, lists additional markers that will be useful for embodiments of the

invention. The first part of Table 7 lists DNA markers that are useful for early diagnosis of John's disease in ruminants, as described above, because the markers differentiate infected from naïve animals. Table 7 lists the locus in goats and provides homologous locus in cows, if it is known. Table 7 also lists host markers that can differentiate live attenuated vaccine (LAV) vaccinated animals from naïve animals and markers that can differentiate inactivated-vaccine immunized from naïve animals. Table 7 also lists host markers to distinguish between infected and vaccinated animals.

As used herein, "naïve" refers to animals that are not vaccinated nor infected.

In addition to the biomarkers recited herein, additional biomarkers useful in the disclosed methods include those described in U.S. Pat. No. 10,054,586, which is incorporated herein in its entirety.

In some embodiments, the present invention provides methods of distinguishing between infected and naïve animals by measuring expression of one or more biomarkers selected from the group of LOC108634521, LOC108637251, LOC108637252, LOC108634594, FAM198B, LOC108637671, CDCP1, TMTC1, BAIAP2L1, MEI1, SEPT10, IFNG, IL17F, FCER2, ADGRG1, APBB1, PIWIL2, AOAH, and homologs thereof (Table 1) In some embodiments, two, three, four, five, six or more of the recited biomarkers may be measured.

In some embodiments, the present invention provides methods of distinguishing between live attenuated vaccine (LAV) vaccinated animals from naïve animals by measuring expression of one or more biomarker selected from the group of LOC108634521, NOS2, LOC108637251, TINAGL1, RETN, C1QL2, TDRD10, TGFB3, ADGRE2, LIPG, KCNJ2, AQP9, BPI, IL9, IL1R2, IL36B, IGF1, BGN, PIWIL2, RAET1E, CRABP2, AOAH, and homologs thereof (Table 2) In some embodiments, two, three, four, five, six or more of the recited biomarkers may be measured.

In some embodiments, the present invention provides methods of distinguishing between LAV-vaccinated animals from infected animals by measuring expression of one or more biomarker selected from the group of LOC106503226, PMP22, ART5, LOC102169116, GNLY, ASAP3, LOC108633178, TBKBP1, SLC17A7 and homologs thereof (Table 3) In some embodiments, two, three, four, five, six or more of the recited biomarkers may be measured.

In some embodiments, biomarkers may be used to distinguish between naïve, infected, and vaccinated animals. By measuring expression of two or more biomarkers recited herein, an animal may be identified as naïve, infected, or vaccinated. A selection of suitable biomarkers and their relative expression is outlined in Table 4A and Table 4B. For example, as demonstrated in Table 4A, when relative expression of FAM198B is higher than relative expression of AOAH, the subject is infected with a mycobacterial infection. When relative expression of AOAH is higher than relative expression of FAM198B, the subject has been vaccinated with an LAV vaccine. When relative expression of AOAH and FAM198B are equal, or within error of the method used to quantify relative expression, the subject is naïve. Similar comparisons and conclusions may be drawn using other biomarkers described herein, such as those outlined in Table 4B or any of Tables 1-3.

TABLE 1

List of host (goat and cow) genes that can differentiation infected from naïve animals.

Symbol	Entrez Gene ID	Description	Expression in a Naïve Animal	Expression in an Infected Animal	Log₂ Fold Change in Expression Infected vs Naïve
LOC108634521	108634521	non-coding RNA	0.66	258.7	8.66
LOC108637251	108637251	multidrug resistance-associated protein 4-like	1.28	137.44	6.74
LOC108637252	108637252	multidrug resistance-associated protein 4-like	5.31	90.39	4.11
LOC108634594	108634594	multidrug resistance-associated protein 4-like	3.35	110.84	5.06
FAM198B	102191727	family with sequence similarity 198 member B	71.81	768.85	3.42
LOC108637671	108637671	tripartite motif-containing protein 5-like	46.57	316.63	2.77
CDCP1	102187276	CUB domain containing protein	8.9	36.66	2.02
TMTC1	102185637	transmembrane and tetratricopeptide repeat	13.2	47.85	1.86
BAIAP2L1	102173150	BAI1 associated protein 2 like	8.17	29.54	1.85
MEI1	102169168	meiotic double-stranded break formation protein	203.8	609.18	1.58
SEPT10	102171885	septin 10	16.83	40.16	1.27
IFNG	100860815	interferon gamma	63.77	19.26	−1.75
IL17F	102171111	interleukin 17F	625.76	267.69	−1.22
FCER2	102171507	Fc fragment of IgE receptor II	626.49	243.68	−1.36
ADGRG1	102171366	G protein-coupled receptor G	72.43	21.2	−1.78
APBB1	102179305	amyloid beta precursor protein binding family B member	97.39	21.75	−2.16
PIWIL2	102173845	piwi like RNA-mediated gene silencing	57.32	10.67	−2.41
AOAH	102189546	acyloxyacyl hydrolase	258.05	268.21	0.06

TABLE 2

List of host (goat and cow) genes that can differentiate LAV vaccinated from naïve animals.

Symbol	Entrez Gene ID	Description	Expression in a Naïve Animal	Expression in an LAV vaccinated Animal	Log₂ Fold Change in Expression Vaccinated vs Naïve
LOC108634521	108634521	non-coding RNA	0.66	238.29	8.54
NOS2	100860742	nitric oxide synthase 2	0.78	103.4	7.03
LOC108637251	108637251	multidrug resistance-associated protein 4-like	1.28	150.28	6.87
TINAGL1	102169636	tubulointerstitial nephritis antigen like	4.62	90.01	4.27
RETN	102170965	resistin	44.98	708.35	3.96
C1QL2	102176742	complement C1q like 2	2.29	30.2	3.69
TDRD10	102174259	tudor domain containing 10	0	2.49	3.53
TGFB3	102189962	transforming growth factor beta 3	40.44	367.3	3.19
ADGRE2	102171592	adhesion G protein-coupled receptor E2	101.97	601.2	2.56
LIPG	102191574	lipase G endothelial type	33.18	175.34	2.40
KCNJ2	102168940	potassium voltage-gated channel subfamily J member 2	78.28	378.07	2.27
AQP9	102181396	aquaporin 9	72.94	342.39	2.24
BPI	102185756	bactericidal/permeability-increasing protein	13.67	49.85	1.85
IL9	102179848	interleukin 9	13.72	4.82	−1.54
IL1R2	102186601	interleukin 1 receptor type 2	170.86	47.04	−1.86
IL36B	102182235	interleukin 36 beta	80.82	14.69	−2.45
IGF1	100860838	insulin like growth factor	109.36	19.9	−2.45
BGN	102183219	biglycan	227.52	26.44	−3.10
PIWIL2	102173845	piwi like RNA-mediated gene silencing	57.32	7	−3.10
RAET1E	108636743	retinoic acid early transcript	832.23	48.24	−4.11
CRABP2	102174348	cellular retinoic acid binding protein 2	103.46	3.31	−4.91
AOAH	102189546	acyloxyacyl hydrolase	258.05	1014.3	1.98

TABLE 3

List of host (goat and cow) genes that can differentiate LAV vaccinated from infected animals.

Symbol	Entrez Gene ID	Description	Expression in an Infected Animal	Expression in an LAV vaccinated Animal	Log₂ Fold Change in Expression Infected vs Vaccinated
LOC106503226	106503226	non-coding RNA	37.38	5.83	2.74
PMP22	102184371	peripheral myelin protein 22	268.96	36.55	2.87
ART5	102169686	ADP-ribosyltransferase 5	16.98	103.7	−2.62
LOC102169116	102169116	ecto-ADP-ribosyltransferase 5	43.11	198.79	−2.20

TABLE 3-continued

List of host (goat and cow) genes that can differentiate LAV vaccinated from infected animals.

Symbol	Entrez Gene ID	Description	Expression in an Infected Animal	Expression in an LAV vaccinated Animal	Log_2 Fold Change in Expression Infected vs Vaccinated
GNLY	102191341	granulysin	32.21	149.78	−2.21
ASAP3	102182646	ArfGAP with SH3 domain ankyrin repeat and PH domain 3	15.62	70.26	−2.18
LOC108633178	108633178	granzyme B-like	6.6	50.4	−2.98
TBKBP1	102172659	TBK1 binding protein	74.03	499.27	−2.76
SLC17A7	102169042	solute carrier family 17 member 7	3.1	53.98	−4.10
FAM198B	102191727	family with sequence similarity 198 member B	768.85	311.61	1.30
AOAH	102189546	acyloxyacyl hydrolase	268.21	1014.3	−1.92

TABLE 4A

Use of host genes, for example, as measured by quantitative PCR, to differentiate between naïve, infected, and vaccinated animals

Status of the Animal	Relative expression of FAM198B	Relative expression of AOAH
Naïve	1	1
Infected	6.89	1.10
Vaccinated with LAV vaccine	3.49	10.47

TABLE 4B

Summary of biomarkers for use in differentiation of infected and vaccinated animals.

	FAM198B	AOAH	MEI1	IL-22	CDCP1
Infected with MAP vs. Naïve animas	6.89	1.1	−1.58	−3.84	−1.05
Vaccinated with LAV vaccine vs. Naïve Animals	3.49	10.47	−3.17	−1.29	1.64

Applicants envision that the biomarker may include genes or the polynucleotides containing less than an entire gene sequence of the above genes. The biomarker of genes or the polynucleotides may be either single- or double-stranded nucleic acids. A polynucleotide may be RNA, DNA, cDNA, genomic DNA, chemically synthesized RNA or DNA or combinations thereof. The polynucleotides can be purified free of other components, such as proteins, lipids and other polynucleotides. For example, the polynucleotide may be 50%, 75%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% purified. The purified polynucleotides may comprise additional heterologous nucleotides. The purified polynucleotides of the invention can also comprise other nucleotide sequences, such as sequences coding for linkers, primer, signal sequences, TMR stop transfer sequences, transmembrane domains, or ligands.

The gene or the polynucleotides of the invention may also comprise fragments that encode immunogenic polypeptides. Polynucleotides of the invention may encode full-length polypeptides, polypeptide fragments, and variant or fusion polypeptides. Polynucleotides of the invention may comprise coding sequences for naturally occurring polypeptides or may encode altered sequences that do not occur in nature. If desired, polynucleotides may be cloned into an expression vector comprising expression control elements, including for example, origins of replication, promoters, enhancers, or other regulatory elements that drive expression of the polynucleotides of the invention in host cells.

Detection of Biomarkers or Markers

The present biomarkers or markers may be detected by any suitable method. In one embodiment, the testing is via ELISA assay for antibodies formed against the biomarkers or markers.

The biomarker or marker in the present invention may be directly detected, e.g., by SELDI or MALDI-TOF. Alternatively, the biomarker may be detected directly or indirectly via interaction with a ligand or ligands such as an antibody or a biomarker-binding fragment thereof, or other peptide, or ligand, e.g. aptamer, or oligonucleotide, capable of specifically binding the biomarker. The ligand may possess a detectable label, such as a luminescent, fluorescent or radioactive label, and/or an affinity tag.

For example, detecting and/or quantifying may be performed by one or more method(s) selected from the group consisting of: SELDI (-TOF), MALDI (-TOF), a 1-D gel-based analysis, a 2-D gel-based analysis, Mass spectrometry (MS), reverse phase (RP) LC, size permeation (gel filtration), ion exchange, affinity, HPLC, UPLC and other LC or LC MS-based techniques. Appropriate LC MS techniques may include ICAT® (Applied Biosystems, CA, USA), or iTRAQ® (Applied Biosystems, CA, USA). Liquid chromatography (e.g., high pressure liquid chromatography (HPLC) or low pressure liquid chromatography (LPLC)), thin-layer chromatography, NMR (nuclear magnetic resonance) spectroscopy may also be used. Methods of diagnosing and/or monitoring according to the invention may comprise analyzing a plasma, serum or whole blood sample by a sandwich immunoassay to detect the presence or level of the biomarker. These methods are also suitable for clinical screening, prognosis, monitoring the results of therapy, identifying patients most likely to respond to a particular therapeutic treatment, for drug screening and development, and identification of new targets for drug treatment.

Detecting and/or quantifying the biomarkers or markers may be performed using an immunological method, involving an antibody, or a fragment thereof capable of specific binding to the biomarker. Suitable immunological methods include sandwich immunoassays, such as sandwich ELISA, in which the detection of the analyte biomarkers is performed using two antibodies which recognize different epitopes on a analyte biomarker; radioimmunoassays (RIA), direct, indirect or competitive enzyme linked immunosorbent assays (ELISA), enzyme immunoassays (EIA), Fluorescence immunoassays (FIA), western blotting, immunoprecipitation and any particle-based immunoassay (e.g., using gold, silver, or latex particles, magnetic particles, or

Q-dots). Immunological methods may be performed, for example, in microtiter plate or strip format.

The gene or the polynucleotides of the invention may be detected by, for example, a probe or primer or a PCR primer. The gene or the polynucleotides of the invention may be the basis for designing a complimentary probe or primer, to detect the presence and/or quantity of biomarker in a subject, such as a biological sample. Probes are molecules capable of interacting with a target nucleic acid, typically in a sequence specific manner, for example, through hybridization. Primers are a subset of probes that can support specific enzymatic manipulation and that can hybridize with a target nucleic acid such that the enzymatic manipulation occurs. A primer may be made from any combination of nucleotides or nucleotide derivatives or analogs available in the art that do not interfere with the enzymatic manipulation. "Specific" means that a gene sequence recognizes or matches another gene of the invention with greater affinity than to other non-specific molecules. Preferably, "specifically binds" or "specific to" also means a gene sequence recognizes and matches a gene sequence comprised in a biomarker described herein, with greater affinity than to other non-specific molecules.

The hybridization of nucleic acids is well understood in the art. Typically a primer may be made from any combination of nucleotides or nucleotide derivatives or analogs available in the art. The ability of such primers to specifically hybridize to polynucleotide sequences of the recited biomarkers will enable them to be of use in detecting the presence of complementary sequences in a given subject. The primers of the invention may hybridize to complementary sequences in a subject such as a biological sample, including, without limitation, saliva, sputum, blood, plasma, serum, urine, feces, cerebrospinal fluid, amniotic fluid, wound exudate, or tissue of the subject. Polynucleotides from the sample can be, for example, subjected to gel electrophoresis or other size separation techniques or can be immobilized without size separation.

The probes or the primers may also be labeled for the detection. Suitable labels, and methods for labeling primers are known in the art. For example, the label may include, without limitation, radioactive labels, biotin labels, fluorescent labels, chemiluminescent labels, bioluminescent labels, metal chelator labels and enzyme labels. The polynucleotides from the sample are contacted with the probes or primers under hybridization conditions of suitable stringencies. Preferably, the primer is fluorescently labeled. Also, the detection of the presence or quality of the gene sequence of interest can be accomplished by any method known in the art. For instance, the detection can be made by a DNA amplification reaction. In some embodiments, "amplification" of DNA denotes the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixtures of DNA sequences.

In some embodiments, quantitative polymerase chain reaction (qPCR) or real-time quantitative polymerase chain reaction (RT-qPCR) is used to measure expression levels of nucleotide biomarkers. These methods detect and quantify the products generated during each cycle of the PCR process which are directly proportionate to the amount of the messenger RNA, DNA, or cDNA prior to the start of the PCR process. Some qPCR and RT-qPCR methods may use non-specific fluorescent dyes that intercalate with any double stranded DNA or sequence specific DNA probes with fluorescently labeled oligonucleotides to permit detection only after hybridization of the probe with the complementary

sequence. Suitable RT-qPCR and qPCR methods, probes and dyes are known in the art.

In another embodiment, the amplification of DNA may be done by the loop-mediated isothermal amplification (LAMP). Similar to PCR, LAMP utilizes a polymerization-based reaction to amplify DNA from examined samples, but the enzyme for LAMP, Bst DNA polymerase large fragment, possesses a DNA strand displacement activity. This makes the DNA extension step possible without having to fully denature DNA templates. Moreover, the primers are designed in a way that a hairpin loop structure is formed in the first cycle of amplification, and the following products are further amplified in an auto-cycling manner. Therefore, in about an hour, the repeated reactions can amplify by ~10^9 copies of DNA molecules and can be done at a constant temperature in a single heat block, instead of at various cycles of temperature in a relatively expensive thermal cycler. The detection of LAMP has been described in PCT patent application publication WO20141640055A1, which is incorporated herein in its entirety.

In one embodiment, the detection of the presence of the gene or the specific binding between the gene in *mycobacterium* mutant and a gene that is not a component of a subject's immune response to a particular vaccine may indicate a natural or experimental *mycobacterium* infection. For example, the absence of such binding or presence may indicate the absence of *mycobacterium* infection. Or, a second, separate gene, such as a mutated *mycobacterium* gene that is specific to a component of a mammal's immune response to a particular *mycobacterium* vaccine, may be used to detect corresponding antibodies produced in response to vaccination. Thus, if an antibody specific to a gene in *mycobacterium* vaccine is detected, then the mammal has been vaccinated and/or infected. The detection of neither genes indicates no infection and no vaccination. As such, various combinations can lead to a determination of the vaccination and/or infection status of the mammal.

Kits of the Present Invention

In another aspect, the present invention discloses a diagnostic kit suitable for carrying out the diagnostic method of the first aspect of the invention. In one embodiment, the kit may be a "one-day" kit, meaning that it is capable of providing the diagnostic result within one day of sample collection. In another embodiment, the kit may be able to provide a diagnostic result within 12, 10, 8, 6, 4, 2, 1 or 0.5 hours of sample collection.

In one embodiment, the diagnostic kit may be used for early detection of mycobacterial infection in a mammal. The diagnostic kit may also be used to differentiate a vaccinated mammal from an infected mammal.

In one embodiment, the diagnostic kit may be portable. The portable diagnostic kit may specifically suitable for field testing. Applicants envision that the present diagnostic kit may be used in a farm field such as a milk farm, where farmers/veterinarians may collect samples and run the assay on the field (point of care assay) to identify early stages of Johne's disease infection and to differentiate infected from vaccinated mammals.

The kit may include a substrate. In one embodiment, the substrate may be coated with biomolecules such as antibodies, which are specifically binding to the specific biomarkers as discussed above. The biomolecules may further possess a detectable label, such as a luminescent, fluorescent or radioactive label, and/or an affinity tag.

In one embodiment of the present invention, the substrate may be used as a sample holder. Exemplary substrates may include microtiter strips or plates. In one specific embodi-

ment, a sample such as a diluted serum may be pipetted into the wells of the microtiter plate or strip. A binding between the biomarkers in the serum and the biomolecules takes place. The presence or absence of the specific biomarkers or a combination of biomarkers as discussed above may indicate the infection status of the mammal.

The kit may further include a means of detection. The means of detection may include any detection method as discussed above. In one embodiment, the means of detection may be a spectroscopic technique, such as UV-Vis or MS. In one specific embodiment, the means of detection may be ELISA.

In one embodiment, the kit may include standard data for specific biomarker or a combination of biomarkers as discussed. One may compare the test result of a mammalian sample with the standard data for specific biomarker or a combination of biomarkers to determine the infection status of the mammal. For example, specific biomarkers or a combination of biomarkers may be visualized by a simple means of detection such as different colors. The detection result (e.g., showing one specific color) of a mammalian sample may be compared with the standard data (e.g., different colors for different biomarkers) to determine the infection status of the mammals.

In one embodiment, the kit may also be in the form of reagents (e.g. protein extract) that can be inoculated into animals to estimate the level of cell-mediate immunity (e.g. single intradermal comparative skin test, SICST). The reagents may include any of the biomarkers as discussed above. In one embodiment, the reagents may also include any genetically engineered vaccines. Suitable genetically engineered vaccines may include those Applicants previously proposed in PCT patent application publication WO20141640055A1, which is incorporated herein in its entirety.

The diagnostic kit may also include one or more of the following: instructions for use (detailing the method of the first aspect of the invention); sample collection apparatus (such as a needle and syringe); a chart for interpretation of the results; an electronic readout system; software providing a database for accurate data management.

The present invention has been described in terms of one or more preferred embodiments, and it should be appreciated that many equivalents, alternatives, variations, and modifications, aside from those expressly stated, are possible and within the scope of the invention.

EXAMPLES

Example 1—Proteomic Analysis of *M. avium* Subsp. *Paratuberculosis* Vaccine Candidates

Johne's disease (JD) is a worldwide health problem for dairy herds that carries a heavy economic burden for producing safe food. Infected cattle suffer from chronic diarrhea, weight loss, low milk yield and low, but persistent mortality (1). For the dairy industry alone, the economic losses caused by JD are estimated to range between $200-$500 million annually, in the USA alone (2, 3). Identifying protective vaccine candidates against JD could be the cornerstone of controlling this widespread infection. In our group, we deciphered genomic information available for *M. ap* to identify key gene regulators that could control the expression of large number of genes. Throughout the genome of *M. ap* there are 19 sigma factors that act as global gene regulators that could contribute to the ability of *M. ap* to grow in many environments (4). Through previous fund-

ing from USDA, we examined several *M. ap* sigma (σ) factors that were important for growth in murine macrophages. Using transcriptional profiling, we compared mid-log phase *M. ap* to *M. ap* that had infected IFN-γ activated macrophages for 2 and 24 hours. Of the 19 sigma factors monitored, 6 sigma factor transcripts were up-regulated and one sigma factor transcript was down-regulated during the 24 hour time frame. Of the up-regulated transcripts, the sigL transcript was the only transcript up-regulated 2 hours after infection while sigH was up-regulated at 24 hrs (5). SigL is implicated in cell membrane protein biosynthesis as well as virulence in *M. tuberculosis* (6) while SigH was shown to be involved in combating the host intracellular responses such as oxidative stress (7).

To assess the role of sigL and sigH in *M. ap* virulence, we replaced the target sigma factors gene coding regions with a hygromycin-resistant gene cassette in *M. ap* K-10 using a specialized transduction protocol that was adapted for *M. ap*. Both genes were shown to be necessary for *M. ap* virulence in different stages of murine infection as detailed before (5, 8). Interestingly, the same mutants were shown to provide protective immunity against challenge with the virulent strain of *M. ap* when they were used as vaccine candidates in mice. To better analyze proteins expressed in each mutant, we grow cultures of *M. ap* ΔIsigL, *M. ap* ΔsigH mutants and the wild type parent strain, *M. ap* K10 to mid-log phase. All cultures were washed twice in PBS, resuspended in buffer cocktail with endonuclease before proteomic analysis using nano-Liquid Chromatography-Mass Spectroscopy-MS (nano-LC MS/MS) at the University of Wisconsin Biotechnology Center. From 3 biological replicates, a total of ~900 proteins were identified in this analysis comparing sigL and sigH mutant to *M. ap* K10 proteome.

Diagnostic Markers for JD-Vaccinated Animals.

A major problem in employing mass vaccination program for the control of JD in dairy herds is the inability to differentiate between infected and vaccinated animals with the current vaccine (DIVA principal). In addition, vaccinated animals could not be differentiated from positive reaction to the infection with *M. bovis*, a significant health problem for domesticated and wildlife animals. However, the DIVA principal and ability to distinguish between *M. bovis* and JD vaccinated animals could be achieved in genetically engineered vaccines (such as live attenuated vaccines based on sigL and sigH mutant) using a novel approach designed by the Applicant. In this approach, a simple blood test targeting proteins or sequences present in *M. ap* wild type strain and with lower expression level in the vaccine strain or even not encoded in the *M. bovis* genome would be developed. The target proteins include the following list of genes that could be used for the sigL-based vaccines.

TABLE 5

	M. ap proteins that are significantly over-expressed in the wild type strain and not in the sigL-vaccine and could be used for sigL-DIVA testing.		
Number	Accession Number	Fold Change (K10/sigH)	Name/Function
1	Q73SF4	1.75	hypothetical protein
2	Q73Y73	2.66	aldehyde dehydrogenase (NAD+)
3	Q73ZE6	2.13	nucleotide-sugar epimerase EpiA
4	Q73SL7	2.69	hypothetical protein Mb0574c
5	Q73VK6	1.14	oxidoreductase
6	Q73XZ0	1.88	antigen CFP2
7	Q740D1	4.71	peptide synthetase Nrp
8	Q73UE0	1.99	cutinase

TABLE 6

Table 6 title:
M. ap proteins that are significantly over-expressed in the wild type strain and not in the sigH-vaccine and could be used for sigH-DIVA testing.

Number	Accession Number	Fold Change (WT/sigH)	Name/Function
1	Q73VL6	3.05	diguanylate cyclase (GGDEF) domain-containing protein
2	Q73YW9	1.64	PE family protein, partial
3	Q741L4	1.88	hypothetical protein
4	Q744E5	2.67	ABC transporter ATPase
5	Q73YP5	2.47	Pup--protein ligase
6	Q73WE5	1.78	arginine decarboxylase
7	Q73U21	1.88	PE family protein PE17
8	Q73UH9	2.16	XRE family transcriptional regulator
9	Q741M5	2.11	nitroreductase
10	Q742F4	2.72	metallo-beta-lactamase
11	Q73SU6	2.47	3-ketoacyl-ACP reductase

In addition, another vaccine candidate is based on lipN mutant. In this case, epitopes that are different in the *M. ap* protein compared to those in *M. bovis* will be the target for DIVA diagnostic test.

FIG. 1 shows the alignment plot of amino acids deduced from the protein sequence in LipN of both *M. paratuberculosis* and *M. bovis*. Peptides conserved in *M. paratuberculosis* sequence but absent from *M. bovis* would be the target for developing the DIVA test.

Example 2—Biomarkers for Early Diagnosis and Differentiation of Mycobacterial Infections

Johne's disease, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a chronic gastroenteritis of ruminants. Although infection often occurs within the first

few months of life, clinical signs do not appear until 2-5 years of age. Current diagnostic tests, such as fecal culture and ELISA, have poor sensitivity for detection of the sub-clinical phase of disease. Therefore, biomarkers have been increasingly investigated as a method for sub-clinical detection.

In this project, we set out to develop rapid assays (e.g. PCR or field skin test) for early detection of presence of Johne's disease and for the differentiation of Johne's disease vaccinated vs. infected animals (with MAP or *M. bovis*). To speed up the project outcome, we capitalized on ongoing vaccine study in goats (*Capra hircus*) and collected Peripheral blood mononuclear cells (PBMC's) for transcriptional profiling followed by gene prediction for disease initiation and progression.

The PBMC's have been shown to be a predictor of infection and inflammatory disease. The PBMC transcriptomes of the goats were profiled using RNA-sequencing (RNA-Seq) to evaluate differential gene expression between a subset of samples from either 30 days post-vaccination, 30 days post-infection, or a naive, non-infected control group (3-4 biological replicates per group). Preliminary results on differential gene expression indicated the presence of 88 significantly differentially expressed genes out of 11,009 genes between goats at 30 days post-infection and the naïve, non-infected controls. The 30 days post-vaccination group had 720 out of 10,985 and 746 out of 11,099 significantly differentially expressed genes compared to the naïve, non-infected control group and the 30 days post-infection group, respectively. However, preliminary evaluation of the expressed genes indicated a large number of genes with immunological and inflammatory functions, including IL-18 binding protein, IFN-γ, IL-17A, and IL-22. Because of this inquiry, Table 7 summarizes selected genes/targets suitable to use in the present invention.

TABLE 7

List of DNA markers that are derived from the host transcriptome analysis and can be used for early diagnosis of Johne's disease in ruminants (cattle, goats, sheep and camels).

Locus in goats	Symbol	Protein	Description	Homolog in Bos taurus (cows)	Homolog description
Selected list of host (goat and cow) markers that can differentiate infected from naïve animals.					
NW_005125111.1: 0-184	unplaced genomic scaffold	N/A	N/A		
NW_005101181.1: 1703-1858	unplaced genomic scaffold	N/A	N/A		
NW_005101181.1: 168292-168418	LOC102180841	XP_005701370.1	PREDICTED: multidrug resistance-associated protein 4-like	XP_005199610	multidrug resistance-associated protein 4-like isoform X1
NC_022320.1: 39973839-39974080	Non-coding region	N/A	N/A		
NC_022297.1: 44037534-44043184	IL-22	XP_005680263.1	interleukin 22	NP_001091849.1	interleukin 22
NC_022296.1: 81262820-81263390	Non-coding region	N/A	N/A		
NW_005101844.1: 141791-142987	ABCC4	XP_005701761.1	PREDICTED: multidrug resistance-associated protein 4-like, partial	XP_010820300.1	PREDICTED: multidrug resistance-associated protein 4 isoform X1
NW_005101711.1: 48628-48757	LOC102185556	XP_005701708.1	PREDICTED: multidrug resistance-associated protein 4-like	XP_003585348.3	PREDICTED: multidrug resistance-associated protein 4 isoform X1
NW_005132660.1: 0-240	unplaced genomic scaffold	N/A	N/A		
NW_005109943.1: 2-224	unplaced genomic scaffold	N/A	N/A		
NW_005149706.1: 0-366	unplaced genomic scaffold	N/A	N/A		

TABLE 7-continued

List of DNA markers that are derived from the host transcriptome analysis and can be used
for early diagnosis of Johne's disease in ruminants (cattle, goats, sheep and camels).

Locus in goats	Symbol	Protein	Description	Homolog in Bos taurus (cows)	Homolog description
NW_005153011.1: 2-407	unplaced genomic scaffold	N/A	N/A		
NW_017189548.1: 2899 . . . 17746	LOC108634521	N/A	ncRNA	N/A	N/A
NC_030819.1: complement (13836329 . . . 13914672)	LOC108637251	N/A	N/A	N/A	multidrug resistance-associated protein 4-like
NC_030819.1: 13926013 . . . 14000960	LOC108637252	N/A	N/A	N/A	multidrug resistance-associated protein 4-like
NW_017189646.1: complement (5337 . . . 40350)	LOC108634594	N/A	N/A	N/A	multidrug resistance-associated protein 4-like
NC_030824.1: complement (30222726 . . . 30294233)	FAM198B	N/A	N/A	N/A	family with sequence similarity 198 member B
NC_030822.1: 34764774 . . . 34772382	LOC108637671	N/A	N/A	N/A	tripartite motif-containing protein 5-like
NC_030829.1: 54084869 . . . 54144689	CDCP1	102187276	CUB domain containing protein	XP_002697164; XP_612363	
NC_030812.1: 78337529 . . . 78655742	TMTC1	102185637	transmembrane and tetratricopeptide repeat	N/A	
NC_030832.1: 38530654 . . . 38607511	BAIAP2L1	102173150	BAI1 associated protein 2 like	XP_003584109; XM_003584061; XP_003587892; XM_003587844	
NC_030812.1: 111693222 . . . 111748937	MEI1	102169168	meiotic double-stranded break formation protein	NP_001295589	
NC_030818.1: 43871666 . . . 43923217	SEPT10	102171885	septin 10	NP_001039641	
NC_030812.1: 44984285 . . . 44988400	IFNG	100860815	interferon gamma	NP_776511	
NC_030830.1: 24511444 . . . 24519042	IL17F	102171111	interleukin 17F	NP_001179011	
NC_030814.1: 93891943 . . . 93902156	FCER2	102171507	Fc fragment of IgE receptor II	N/A	
NC_030825.1: 26945415 . . . 26990225	ADGRG1	102171366	G protein-coupled receptor G	NP_001077125	
NC_030822.1: complement (35694810 . . . 35718058)	APBB1	102179305	amyloid beta precursor protein binding family B member	NP_001068654	
NC_030815.1: 69261357 . . . 69324811	PIWIL2	102173845	piwi like RNA-mediated gene silencing	XP_015320079; XM_015464593; XP_015328077; XM_015472591	

Selected list of host (goat and cow) markers that can differentiate
live attenuated vaccinated (LAV) from naïve animals.

Locus in goats	Symbol	Protein	Description	Homolog in Bos taurus (cows)	Homolog description
NC_022296.1: 32351255-32351413	Non-coding region	N/A	N/A		
NC_022307.1: 44045143-44403012	Non-coding region	N/A	N/A		
NC_022295.1: 13176472-13182094	Non-coding region	N/A	N/A		
NC_022321.1: 6000551-6000875	Non-coding region	N/A	N/A		
NW_005126018.1: 16-203	unplaced genomic scaffold	N/A	N/A		
NW_005101711.1: 48628-48757	LOC102185556	XP_005701708.1	PREDICTED: multidrug resistance-associated protein 4-like	XP_003585348.3	PREDICTED: multidrug resistance-associated protein 4 isoform X1
NW_005101844.1: 141790-142987	ABCC4	XP_005701761.1	PREDICTED: multidrug resistance-associated protein 4-like, partial	XP_010820300.1	PREDICTED: multidrug resistance-associated protein 4 isoform X1
NW_005101645.1: 16151-23647	unplaced genomic scaffold	N/A	N/A		
NW_017189548.1: 2899 . . . 17746	LOC108634521	108634521	ncRNA		
NC_030826.1: complement (19203362 . . . 19245850)	NOS2	100860742	nitric oxide synthase 2	NP_001070267	
NC_030819.1: complement (13836329 . . . 13914672)	LOC108637251	108637251	multidrug resistance-associated protein 4-like		

TABLE 7-continued

List of DNA markers that are derived from the host transcriptome analysis and can be used
for early diagnosis of Johne's disease in ruminants (cattle, goats, sheep and camels).

Locus in goats	Symbol	Protein	Description	Homolog in Bos taurus (cows)	Homolog description
NC_030809.1: 13806906 . . . 13817913	TINAGL1	102169636	tubulointerstitial nephritis antigen like	XP_015315454; XP_015317919; XP_015315453; XM_015459967; XP_015317918; XM_015462432	
NW_017189666.1: complement (10281 . . . 11678)	RETN	102170965	resistin	NP_899206	
NC_030809.1: 65236017 . . . 65240282	C1QL2	102176742	complement C1q like 2	NP_001192765	
NC_030810.1: 104057279 . . . 104111513	TDRD10	102174259	tudor domain containing 10	XP_005197751; XM_005197694; XP_005203865; XM_005203808	
NC_030817.1: 15809952 . . . 15835252	TGFB3	102189962	transforming growth factor beta 3	NP_001094653; XP_005212263; XP_005212264	
NC_030814.1: 96350745 . . . 96400437	ADGRE2	102171592	adhesion G protein-coupled receptor E2		
NC_030831.1: 49397175 . . . 49422345	LIPG	102191574	lipase G endothelial type		
NC_030826.1: complement (59803404 . . . 59814242)	KCNJ2	102168940	potassium voltage-gated channel subfamily J member 2	NP_776798	
NC_030817.1: 51022717 . . . 51074079	AQP9	102181396	aquaporin 9	XP_015328629; XM_015473143; XP_015328630; XM_015473144	
NC_030820.1: 66719462 . . . 66768979	BPI	102185756	bactericidal/permeability-increasing protein	NP_776320	
NC_030814.1: 63194434 . . . 63197324	IL9	102179848	interleukin 9	XP_015319783; XM_015464297; XP_015327708; XM_015472222	
NC_030818.1: 6611732 . . . 6648492	IL1R2	102186601	interleukin 1 receptor type 2	NP_001039675; XP_010808117; XP_010808118	
NC_030818.1: 46359268 . . . 46368303	IL36B	102182235	interleukin 36 beta	XP_002691396; XM_002691350; XP_002700827; XM_002700781	
NC_030812.1: complement (64862983 . . . 64943172)	IGF1	100860838	insulin like growth factor	XP_005206547; XP_015326547; XP_015326549	
NW_017190169.1: complement (56783 . . . 70690)	BGN	102183219	biglycan	NP_847888; XP_005227715	
NC_030815.1: 69261357 . . . 69324811	PIWIL2	102173845	piwi like RNA-mediated gene silencing	XP_015320079; XM_015464593; XP_015328077; XM_015472591	
NC_030816.1: 74364855 . . . 74372073	RAET1E	108636743	retinoic acid early transcript		
NC_030810.1: complement (105983749 . . . 105989432)	CRABP2	102174348	cellular retinoic acid binding protein 2	NP_001008670	

Selected list of host (goat and cow) markers that can differentiate
inactivated-vaccine immunized from naïve animals.

NW_005125111.1: 0-184	unplaced genomic scaffold	N/A	N/A		
NC_022320.1: 39973839-39974080	Non-coding region	N/A	N/A		
NC_022303.1: 46207878-46237242	Non-coding region	N/A	N/A		
NC_022296.1: 81262820-81263390	Non-coding region	N/A	N/A		
NW_005101711.1: 48628-48757	LOC102185556	XP_005701708.1	PREDICTED: multidrug resistance-associated protein 4-like	XP_003585348.3	PREDICTED: multidrug resistance-associated protein 4 isoform X1

TABLE 7-continued

List of DNA markers that are derived from the host transcriptome analysis and can be used
for early diagnosis of Johne's disease in ruminants (cattle, goats, sheep and camels).

Locus in goats	Symbol	Protein	Description	Homolog in Bos taurus (cows)	Homolog description
NW_005102056.1: 2049-9786	LOC102190036	XP_005701827.1	PREDICTED: tyrosine-protein phosphatase non-receptor type substrate 1-like, partial	NP_786982.1	tyrosine-protein phosphatase non-receptor type substrate 1 precursor
NC_022309.1: 40520580-40588889	—	XP_005691363.1	PREDICTED: protein FAM198B	NP_001077247.1	protein FAM198B
NW_005101931.1: 48185-54192	LOC102180487	XP_005701808.1	PREDICTED: interferon alpha-inducible protein 27-like protein 2-like	NP_001069925.2	uncharacterized protein LOC617420
NW_005164924.1: 1-636	unplaced genomic scaffold	N/A	N/A		

Selected list of host (goat and cow) markers that can differentiate
LAV-vaccine immunized from infected animals.

NC_030826.1: 39149778 . . . 39151507	LOC106503226	106503226			
NC_030826.1: complement (32435859 . . . 32463764)	PMP22	102184371	peripheral myelin protein 22	NP_001094626; XP_005220437; XP_010814341	
NC_030822. 1: complement (31912716 . . . 31916296)	ART5	102169686	ADP-ribosyl-transferase 5		
NC_030822.1: complement (31947901 . . . 31951735)	LOC102169116	102169116	ecto-ADP-ribosyl-transferase 5		
NC_030818.1: complement (48786756 . . . 48789216)	GNLY	102191341	granulysin	NP_001068611	
NC_030809.1: complement (6533315 . . . 6583406)	ASAP3	102182646	ArfGAP with SH3 domain ankyrin repeat and PH domain 3	NP_001076915	
NC_030828.1: complement (69374237 . . . 69377734)	LOC108633178	108633178	granzyme B-like		
NC_030826.1: complement (38379103 . . . 38396803)	TBKBP1	102172659	TBK1 binding protein	XP_001253301; XP_005195770; XP_005220704; XP_010814497; XP_010822429; XP_015314281	
NC_030825.1: complement (56899764 . . . 56911150)	SLC17A7	102169042	solute carrier family 17 member 7	NP_001091515	
NC_030826.1: 53112179 . . . 53116348	LOC108638192	108638192			
NC_030812.1: 44984285 . . . 44988400	IFNG	100860815	interferon gamma	NP_776511	

Materials and Methods

Animals—Approximately one week-old kids were purchased from a farm with no previous history of Johne's disease. All study kids, and their dams, tested negative for *M. paratuberculosis* by ELISA for serum antibody (Paracheck®, Biocor Animal Health, Omaha, NE). Additionally, fecal samples collected from the originating farm environment were negative for *M. paratuberculosis* by culture. All kids were housed in a restricted biosafety animal facility (BSL-2). All animal care was handled in accordance to the standards of the University of Wisconsin-Madison Animal Care and Use Committee. The kids were randomly assigned to one of four groups as shown in Table 8. One group of kids (n=6 but only 4 used for transcriptome analysis) were vaccinated with a live-attenuated vaccine (LAV) construct (*M. paratuberculosis* ΔlipN mutant (Wu et al., 2007)) at a dose of 1×10⁹ CFU/animal. The second groups of kids (n=4) were vaccinated with the USDA-licensed inactivated vaccine (Mycopar®). A third group inoculated with PBS served as the vaccine control. Both vaccines and PBS were given subcutaneously. At 60 days post-vaccination, kids in these three groups were inoculated with *M. paratuberculosis* strain JTC1285 at a dose of 1×10⁸ CFU administered orally in the milk replacer for three consecutive days. A fourth group (n=4), inoculated with PBS and not challenged with *M. paratuberculosis* served as a naïve control. Goat kids were monitored daily for signs of clinical disease and evaluated monthly for potential weight loss. A detailed report on the outcome of this vaccine/challenge study was previously published (Shippy et al., 2017).

TABLE 8

Experimental Groups

Group	No.	Vaccine*	Vaccine Dose	Challenge Strain/Dose**
Infected	4	PBS	0.5 ml	*M. paratuberculosis* JTC1285/ 1 × 10⁸ CFU
LAV-vaccinated	4	*M. ap* ΔlipN	1 × 10⁹ CFU	*M. paratuberculosis* JTC1285/ 1× 10⁸ CFU

TABLE 8-continued

Experimental Groups

Group	No.	Vaccine*	Vaccine Dose	Challenge Strain/Dose**
Mycopar-vaccinated	3	Mycopar ®	0.5 ml	*M. paratuberculosis* JTC1285/ 1 × 10^8 CFU
Naïve Control	4	PBS	0.5 ml	None

*All vaccines were given subcutaneously.
**Challenge dose was given orally in milk replacer for three consecutive days and was performed at 60 days post-vaccination in LAV- and Mycopar- vaccinated groups.

Isolation of blood cells—Blood samples (10 ml) were collected from the jugular vein of goats into EDTA vacutainer tubes pre-vaccination, 1 week, 30 days, 60 days post-vaccination and 1 week post-challenge (for 3 groups), and then monthly for 12 months. Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque®-1077 (Sigma-Aldrich®) with the following modifications. Anti-coagulated blood was diluted with an equal volume of RPMI-1640 medium (Sigma Aldrich®), layered over 10 ml of Histopaque®-1077, and centrifuged at 400×g for 30 minutes at room temperature. Following centrifugation, PBMC's were aspirated from the interface and washed twice with RPMI-1640 medium. Residual red blood cells were lysed with 0.83% NH_4Cl_2. The PBMC's were then resuspended in complete culture medium (RPMI-1640 containing 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin (final concentration 100 IU/ml), and 1% non-essential amino acids). Cell density was determined by use of 0.4% Trypan blue stain and a hemocytometer.

PBMC stimulation and RNA extraction—PBMC's were plated at a density of 1×10^6/well in 96 well plates with either medium alone (non-stimulated) or *M. paratuberculosis* whole cell lysate (WCL). The WCL was prepared by resuspending the centrifuged cell pellet of actively grown *M. paratuberculosis* (O.D.~1.0) in protein lysis buffer (100 mM Tris-Cl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, complete ultra-protease inhibitor cocktail (Roche, Indianapolis, IN; pH 7.5) and bead-beating to homogenize (maximum pulse for 45 sec for a total of 4 pulses; with cooling on on ice for 30 sec between pulses). The supernatant was then transferred to a new 1.5 ml tube and non-soluble material was removed by centrifugation at 10,000×g for 5 min at 4° C. The protein content of the supernatant was measured via the Pierce™ BCA protein assay (Thermo Fisher Scientific), aliquoted and stored at −80° C. until used. Final concentrations of WCL was 10 μg/ml. IL-2 was added to all wells at a concentration of 100 U/ml. Plates were incubated at 37° C. with 5% CO_2 for 24 hours. Supernatants were then removed and cell pellets were stored in 100 μl TRIzol® and frozen at −80° C. until used for RNA extraction. RNA was extracted from stimulated PBMC's using TRIzol® and RNeasy® Mini Kit (Qiagen®) according to manufacturer's directions for the remainder of the extraction. TURBO DNA-Free™ DNase Treatment (Ambion®) was used to eliminate residual genomic DNA. RNA quantity and quality was assessed using the RNA Pico Series Chip on the Bioanalyzer 2100 (Agilent). RNA integrity numbers (RINs)>8 were obtained for all total RNA samples purified.

RNA Sequence Analysis—RNA-Sequencing (RNA-Seq) was performed by the University of Wisconsin-Madison Biotechnology Center on RNA extracted from WCL-stimulated PBMC's from goats at 30 days post-vaccination, 30 days post-challenge (PBS vaccinated), or at the same time for the naïve control group (4 goats/biological replicates per group). A total of 1 μg of RNA was used as input for TruSeq® RNA Sample Prep Rev.F (March 2014; Illumina®). Paired-end RNA Sequencing was performed on the Illumina HiSeq 2000 sequencer according to manufacturer's instructions.

Raw RNA-Seq reads were uploaded to CLC Genomics Workbench 8.5 (Qiagen, Redwood City, CA) for processing. Two read files from one RNA sample were paired and trimmed. The ambiguous trim limit was set at 1 and quality trim limit was at 0.05. Reads shorter than 25 nucleotides were excluded. The trimmed sequences were then mapped to the reference genome sequence of *Capra hircus* assembly ARS1 (Bickhart et al., 2017) and read counts against the reference genome annotation tracks, generated with files, available at ncbi.nlm.nih.gov/genomes/Capra_hircus, were compiled and tabulated using the CLC Genomics Workbench NGS tools. The mapping parameters were set as follows: mismatch cost, 2; insertion and deletion cost, 3; length and similarity fraction, 0.8. Unique gene reads from each sample were exported from CLC Genomics Workbench and used for normalization and differential gene expression analysis with an R package, DESeq2 version 1.16.1 (Love et al., 2014). Transcripts that had an average of normalized read count<3 in all three tested groups were excluded from the analysis (N=11,541). Differentially expressed transcripts are defined as transcripts with fold changes≥2.0 or ≤−2.0 (or Loge-transformed fold changes≥1.0 or ≤−1.0), and p-value<0.05 when compared to the naïve control group.

Gene ontology (GO) analysis was performed for the differentially expressed genes with agriGO, an automated tool to identify enriched GO terms, which is specially focused on agricultural species (Du et al., 2010). The gene products are categorized with respect to biological processes, cellular components, and molecular functions. Because the gene ontology in the goat genome is poorly annotated, we chose the *Bos taurus* ENSEMBL genome B2G list (2010 version) as the reference genome. Goat genes (assembly ARS1) with an Entrez gene name were mapped to the counterparts in the bovine genome, resulting in a total gene list of 9,115 GO-annotated genes. Goat DE genes identified in the RNA-Seq analysis were also mapped to the bovine genome and used as query lists against the 9,115-gene reference. FDR was calculated using the Fisher test.

Network analysis was performed using the STRING database (Szklarczyk et al., 2015) with DE transcripts identified in this study. The input DE transcripts were treated as homologues of *Bos taurus* because of availability in the database.

Quantitative RT-PCR—cDNA was synthesized from each RNA sample using SuperScript III Reverse Transcriptase (Invitrogen, Waltham, MA) and oligo(dT)$_{12-18}$ Primer according to manufacturer's instructions. Quantitative PCR (qPCR) assays were performed in triplicates for each cDNA sample. Primers were designed across adjacent exons in order to differentiate products from genomic DNA and cDNA. The GAPDH gene served as an internal control to normalize the data for the ΔΔCt relative quantitation method. The assays were performed on an Applied Biosystems StepOne Plus Real-Time PCR System (Foster City, CA), and the cDNA amplifications were monitored by the measurement of SYBR Green fluorescence at a specific cycle threshold. Each reaction was carried out in a 20 μl volume that contained 10 μl of 2× GoTaq qPCR Master Mix (Promega, Madison, WI), 5.0 μl of ddH2O, 0.5 μl of each primer (10 μm) and 4.0 μl of the template (100-150 ng/ul). The qPCR amplification process began with the temperature

at 95° C. for 2 min, followed by 40 cycles of the amplification process (95° C. for 3 s, 60° C. for 30 s). Subsequent to the cycling process, melting curves were generated by inclining the temperature from 60° C. to 95° C. at 0.3° C./s increments. With the exception of the infected group at 1 month post-challenge where two samples were used, cDNA samples from three animals in each group were included in the qPCR analysis. Average $\Delta\Delta$Ct values and standard errors of the mean (SEM) of the three measurements were calculated and transformed to linear fold change.

qRT-PCR primers

Primer ID	Primer name	Primer sequence	SEQ ID NO:
AMT2341	SEPT10_F	ggtgagcgccagaggaa	4
AMT2342	SEPT10_R	cagcttctcctcttggtggac	5
AMT2343	IL18BP_F	aactggatcccagacccc	6
AMT2344	IL18BP_R	gtagctgctgggagcgc	7
AMT2351	IL17A_F	ggaacacgaactccagaaggc	8
AMT2352	IL17A_R	acagagttcatgtgatggtccac	9
AMT2353	CRABP2_F	accaccgtgcgtaccac	10
AMT2354	CRABP2_R	ggaggtcttgggaccctctc	11
AMT2355	IL36_F	cgttaatagcagttccttctagcaac	12
AMT2356	IL36_R	ggatagccctggatttctgtgc	13
AMT2361	RETN_F	tgaggcagtaaggaacattggc	14
AMT2362	RETN_R	agtccatgcctgcgcac	15
AMT2363	IFNG_F	gcagctctgagaaactggagg	16
AMT2364	IFNG_R	tccggcctcgaaagagattct	17
AMT2365	GAPDH_F	ggcgtgaaccacgagaagtataa	18
AMT2366	GAPDH_R	ggcagtgatggcgtggac	19
AMT 2899	ABCC4_F	cttggatcgccataccctc	20
AMT 2900	ABCC4_R	gggctccgggttgtagattc	21

qRT-PCR primers

Primer ID	Primer name	Primer sequence	SEQ ID NO:
AMT 2914	IL 17F_F	gaggaccacattgtgagggt	22
AMT 2915	IL 17F_R	cgggtgatgttgtaatcccag	23
AMT 2918	TINAGL1_F	cgacgaggggttgtgtctg	24
AMT 2919	TINAGL1_R	acatagctattgggggcagcg	25
AMT 2971	FAM198B_F	tcatccaagatggccgcc	26
AMT 2972	FAM198B_R	gccagcacttctgtttcagc	27
AMT 2973	AOAH_F	gaaatcacggaggagtggca	28
AMT 2974	AOAH_R	aacagctgtgaaaccacctca	29
AMT 2988	IL 22_F	cagggaatcaatcaggtgacga	30
AMT 2989	IL 22_R	atgggggtggaattcatcgg	31
AMT 2992	MEI1_F	cagtgaagtgctcgtctggt	32
AMT 2993	MEI1_R	cgactcaatcccatacaccgt	33
AMT 2994	CDCP1_F	aagccaagcttccgctatca	34
AMT 2995	CDCP1_R	cgatgacagtcaggtccgtg	35

Results

Transcriptome analysis of goat groups. The transcriptome analysis of goats infected with *M. paratuberculosis* and/or vaccinated LAV vaccine strain *M. ap* ΔlipN is a proportion of a larger study that examined the performance of this vaccine published earlier (Shippy et al., 2017). The transcriptome analysis is the focus of this report. The summary statistics of the RNA-Seq data for each replicate are shown in Table 9. Mean values of 58.88 million raw reads were generated per library (each RNA sample). Following trimming of reads based on read length, quality score and adapter sequences, an average of 20.04 million paired reads remained. Alignment of the trimmed RNA-Seq reads to the *Capra hircus* reference genome yielded mean values per library of 18.71 million paired reads (93.32%) mapped to unique locations.

TABLE 9

			Total	% Total		
		Number of	Paired	Paired		%
	Total	Read Pairs	Reads	Reads	Uniquely	Uniquely
Group/Replicate	Number of	Being	After	After	mapped	mapped
Number*	Reads	Trimmed	mapping	Trimming	reads	reads
Infected 1	73,105,634	22,810,292	25,147,671	68.8	23,533,313	93.58
Infected 2	72,155,108	23,491,132	24,331,988	67.44	22,701,378	93.30
Infected 3	68,005,382	21,295,316	23,355,033	68.69	21,941,420	93.95
Infected 4	48,108,726	15,062,194	16,523,266	68.69	15,433,817	93.41
LAV-vaccinated 1	73,973,058	24,360,882	24,806,088	67.07	23,253,486	93.74
LAV-vaccinated 2	63,076,126	20,765,850	21,155,138	67.08	19,668,167	92.97
LAV-vaccinated 3	34,967,370	11,169,558	11,898,906	68.06	11,062,093	92.97
LAV-vaccinated 4	66,996,260	21,387,972	22,804,144	68.08	21,282,727	93.33
Mycopar-vaccinated 1	60,074,726	20,141,682	19,966,522	66.47	18,707,059	93.69

Summary statistics for Illumina RNA sequencing data

TABLE 9-continued

			Total	% Total		%
		Number of	Paired	Paired		Uniquely
	Total	Read Pairs	Reads	Reads	Uniquely	mapped
Group/Replicate	Number of	Being	After	After	mapped	reads
Number*	Reads	Trimmed	mapping	Trimming	reads	
Mycopar-	70,284,036	23,645,792	23,319,122	66.36	21,978,596	94.25
vaccinated 2						
Mycopar-	64,746,832	21,633,920	21,556,456	66.59	20,141,993	93.44
vaccinated 3						
Naïve 1	73,575,076	22,859,182	25,357,947	68.93	23,681,558	93.39
Naïve 2	50,851,250	16,107,620	17,371,815	68.32	16,180,977	93.14
Naïve 3	46,913,012	15,083,746	15,914,633	67.85	14,815,443	93.09
Naïve 4	34,829,124	11,209,870	11,809,627	67.81	10,972,712	92.91

Summary statistics for Illumina RNA sequencing data

*Time when blood samples were taken: Infected: 30 days post-infection; LAV-vaccinated: 30 days post-LAV vaccination; Mycopar-vaccinated: 30 days post-Mycopar ® vaccination; Naïve:: 30 days post-PBS vaccination.

Changes in the goat transcriptomes related to infection or vaccination—Transcriptomes of different animal groups were analyzed to identify differentially expressed (DE) genes with significant change using a p-value threshold of >0.05 and >2-fold change. A summary of comparative numbers of differentially expressed genes is presented in Table 10. MA-plots in FIGS. 3A-3C depict the distributions of the DE transcripts PI and post-vaccination groups compared to naïve control group. Generally, the infected goat group had 226 significantly DE transcripts out of 17,380 (total goat transcripts identified by RNA-Seq) at 30 days PI in comparison to the naïve, non-infected controls. Of the 226 significantly DE transcripts, 113 were up-regulated in the PI group, while the other 113 were down-regulated. A total of 106 out of the 226 DE transcripts had more than a 2.8 fold change (or 1.5 loge fold change) with a selected group of known function listed in Table 11. On the other hand, the LAV-vaccinated goat group had 1018 significantly DE transcripts out of 17,380 compared to the naïve, non-infected control group. A total of 628 and 390 transcripts were up- and down-regulated, respectively. A total of 517 out of the 1018 had >2.8 fold change with a selected group of known

function listed in Table 11. Additionally, when the transcripts of both LAV-vaccinated and infected groups were compared, at total of 1133 transcripts were significantly DE out of 17,380 (Table 10). Of these transcripts, 629 and 504 transcripts were up- and down-regulated, respectively. A total of 575 out of the 1133 DE transcripts were greater than a 2.8 fold change. Interestingly, the immunization with the inactivated, oil-based vaccine (Mycopar) triggered significant changes in a large number of goat genes (N=1714) including key genes involved in immune responses (Table 10).

TABLE 10

Differentially Expressed (DE) Genes for each comparison group

Comparison	Total analyzed Genes	DE Genes*
Infected vs Naïve	17,380	226
LAV-vaccinated vs Naïve		1018
Mycopar-vaccinated vs Naïve		1714
LAV-vaccinated vs Infected		1133

*DE genes were identified as those with a p value threshold of ≤ 0.05

TABLE 11

Selected differentially up- or down-regulated genes by fold change, between 30 days post-infection and naïve groups

Gene symbol	Gene ID	Fold change	P value	Description
FAM198B	102191727	10.70	0.0016	family with sequence similarity 198 member B
CDCP1	102187276	4.06	0.0143	CUB domain containing protein 1
TMTC1	102185637	3.63	0.0217	transmembrane and tetratricopeptide repeat containing 1
BAIAP2L1	102173150	3.61	0.0196	BAI1 associated protein 2 like 1
MEI1	102169168	2.99	0.0155	meiotic double-stranded break formation protein 1
SEPT10	102171885	2.41	0.0239	septin 10
IFNG	100860815	−3.36	0.0047	interferon, gamma
IL17F	102171111	−2.33	0.0098	interleukin 17F
FCER2	102171507	−2.57	0.0001	Fc fragment of IgE receptor II
ADGRG1	102171366	−3.43	0.0037	adhesion G protein-coupled receptor G1
APBB1	102179305	−4.47	0.0002	amyloid beta precursor protein binding family B member 1
PIWIL2	102173845	−5.31	0.0400	piwi like RNA-mediated gene silencing 2

TABLE 12

Selected differentially up- or down-regulated genes by fold change,
between 30 days post-LAV-vaccination and naïve groups

Gene symbol	Gene ID	Fold change	p-value	Description
NOS2	100860742	130.42	2.3E−09	nitric oxide synthase 2
TINAGL1	102169636	19.31	1.2E−05	tubulointerstitial nephritis antigen like
RETN	102176742	12.91	4.4E−13	resistin
C1QL2	102176742	12.89	0.002	complement C1q like 2
TDRD10	102174259	11.54	0.019	tudor domain containing 10
TGFB3	102189962	9.13	0.0020	transforming growth factor beta 3
ADGRE2	102171592	5.90	0.0135	adhesion G protein-coupled receptor E2
LIPG	102191574	5.28	0.0001	lipase G, endothelial type
KCNJ2	102168940	4.82	0.0003	potassium voltage-gated channel subfamily J member 2
AQP9	102181396	4.72	0.0007	aquaporin 9
BPI	102185756	3.61	0.0140	bactericidal/permeability-increasing, protein
IL9	102179848	−2.91	0.0083	interleukin 9
IL1R2	102186601	−3.63	0.0055	interleukin 1 receptor type 2
IL36B	102182235	−5.46	0.0013	interleukin 36 beta
IGF1	100860838	−5.46	0.0463	insulin, like, growth, factor, 1
BGN	102183219	−8.57	0.0045	biglycan
PIWIL2	102173845	−8.57	0.009	piwi like RNA-mediated gene silencing 2
RAET1E	108636743	−17.27	0.0008	retinoic acid early transcript 1E
CRABP2	102174348	−30.12	2.0E−20	cellular retinoic acid binding protein 2

TABLE 13

Selected differentially up- or down-regulated genes by fold change, between 30
days post-Mycopar ®-vaccination and naïve groups

Gene symbol	Gene ID	Fold change	p-value	Description
NOS2	100860742	269.200	3.7E−11	nitric oxide synthase 2
BMP10	102185577	82.746	0.0003	bone morphogenetic protein 10
TDRD10	102174259	18.438	0.0061	tudor domain containing 10
RETN	102170965	16.901	4.2E−12	resistin
AMOTL2	102169708	14.389	0.0065	angiomotin like 2
KLRG2	102177407	12.733	2.9E−10	killer cell lectin like receptor G2
IL21	100861248	8.124	4.6E−05	interleukin 21
C2	102176085	7.95	2.4E−7	complement C2
C3	100860826	6.495	0.0002	complement C3
MCEMP1	102172348	6.436	7.4E−08	mast cell expressed membrane protein 1
IL34	102173115	5.434	0.0084	interleukin 34
IL12A	100861293	3.907	0.0035	interleukin 12A
TLR4	100860955	3.423	3.8E−07	toll like receptor 4
TNF	100861232	3.399	0.0003	tumor necrosis factor
IL18	100861190	−4.441	3.6E−06	interleukin 18
IL9	102179848	−4.802	0.0012	interleukin 9
IL9R	102191479	−4.961	9.4E−08	interleukin 9 receptor
IL5	102188034	−4.964	0.0396	interleukin 5
IL36B	102182235	−9.557	0.0001	interleukin 36 beta
IL13	102187477	−9.675	3.4E−07	interleukin 13
PIWIL2	102173845	−22.152	0.0009	piwi like RNA-mediated gene silencing 2
IL11	102184367	−46.823	1.6E−07	interleukin 11

Several genes involved in immune responses were significantly regulated in all goat groups. For example, leukemia inhibitory factor (LIF), interferon-gamma (IFN-γ), and interleukin 22 (IL-22), were found to be DE genes in the infected group when compared to both the control and the LAV-vaccinated groups. More gene lists are provided in the Tables included in Appendices A-G. In the infected group, LIF was down-regulated by −2.51 fold change when compared to the control group and by 3.84 fold when compared to the vaccinated group. IL-22, a Th17-related cytokine, was also down-regulated by a −5.78 fold in the infected group vs the control group and by −33.82 fold when compared to the LAV-vaccinated group. Interestingly, NOS2 gene involved in controlling infection of a closely related mycobacteria, *M. tuberculosis* (Kutsch et al., 1999; Velez et al., 2009), was significantly induced (>100 fold) in both vaccine groups, suggesting an important role of this gene in adaptive immune responses following immunization with LAV (Table 12) or inactivated (Table 13) vaccine. A group of genes with unique diphasic regulatory responses in both LAV and infected goats included immune response genes (e.g. IFN-γ, Granulysin) as well as basic cell metabolic process (e.g. ART5). This list of genes (Table 14) could expand gene categories utilized as targets for developing a sensitive assay to differentiate infected from vaccinated animals (DIVA).

TABLE 14

Common differentially expressed genes regulated in opposite direction
between 30 days post-infection and 30 days post-LAV-vaccinated
groups, each compared to the naïve group

Gene symbol	Gene ID	Fold change in Infected group	Fold change in Vaccinated group	Description
LOC106503226	106503226	2.62	−2.53	non-coding RNA
PMP22	102184371	2.11	−3.46	peripheral myelin protein 22
ART5	102169686	−2.01	3.05	ADP-ribosyltransferase 5
LOC102169116	102169116	−2.03	2.27	ecto-ADP-ribosyltransferase 5
GNLY	102191341	−2.13	2.19	granulysin
ASAP3	102182646	−2.16	2.10	ArfGAP with SH3 domain ankyrin repeat and PH domain 3
LOC108633178	108633178	−2.68	2.95	granzyme B-like
TBKBP1	102172659	−3.03	2.23	TBK1 binding protein transcript
SLC17A7	102169042	−3.12	5.50	solute carrier family 17 member 7
LOC108638192	108638192	−3.27	5.28	non-coding RNA
IFNG	100860815	−3.36	3.89	interferon gamma

Among those identified DE transcripts in the infected and LAV-vaccinated groups (each referenced against the naïve group), there were 68 transcripts in common (FIG. 4A). The majority of those transcripts were regulated in the same direction in both groups, but 11 transcripts were regulated in the opposite direction. A non-coding RNA transcript, LOC106503226 and a gene, PMP22, were the only two that were up-regulated 30 days PI and down-regulated 30 days post-vaccination. The remaining 9 transcripts (e.g. ART5 and IFNG) were down-regulated 30 days PI and up-regulated 30 days post-vaccination (Table 14). More comparative analysis of transcript profiles identified 76 transcripts commonly up- or down-regulated shared between the lists of genes from comparing infected vs. naïve control and *M. paratuberculosis*-infected vs. LAV-vaccinated transcripts (FIG. 4B). Those common genes could be considered the core responsive genes for *M. paratuberculosis* infection or vaccination with an LAV vaccine. For the inactivated vaccine, a total of 667 core genes were also regulated when compared to the LAV-vaccine group (Appendix F). Such core genes included those with potential rules in immunity (e.g. NOS2, RETN and IL21), another indication of core genes responsive to any *M. paratuberculosis*-specific vaccines whether live-attenuated or inactivated were used.

Pathways and networks of differentially expressed genes—To better define gene pathways involved in *M. paratuberculosis* infection, genes with significant differential expression were evaluated through gene ontology (GO) analysis using agriGO. This analysis provides categories of genes involved in different biological or molecular functions and those integral for different cellular components. Interestingly, the most abundant significant terms for the GO analysis for the infected vs naïve control group included genes involved in protein binding, regulation of cellular process and response to stimulus, which includes significant subcategories immune responses (GO:0006955) and inflammatory response (GO:0006954) (FIG. 3), suggesting the importance of controlling immune genes by *M. paratuberculosis* following infection. On the other hand, the largest gene groups with significant GO terms for the Mycopar®- or LAV-vaccinated vs infected groups included genes involved in binding, cellular process and metabolic process while those for the LAV-vaccinated vs infected group included genes involved in cellular process and biological regulation (FIGS. 8A-8C).

To better characterize gene networks activated during infection and vaccination, gene transcripts were further analyzed to identify co-regulated genes. FIGS. 6A-6B display gene network analysis in the post-infection group. Several in the up-regulated group of genes (FIG. 6A), such as ACER3, SYNJ2, CORO6 and PLS1, showed physical associations and co-expression among transcripts of the *M. paratuberculosis*-infected group. In addition, homologs of PDE4C and TSKU were also found associated (Halls and Cooper, 2010; Schlecht et al., 2012; Costanzo et al., 2016) and suggested to be involved in signaling and relaxin regulation (Halls and Cooper, 2010). In FIG. 6B, a co-down-regulation of ATP7B, ATP12A and ATP2B3 suggests a possible reduced activity of calcium transport in infected cells. This analysis also highlighted the negative regulation by *M. paratuberculosis* of host cytokines such as IFN-γ, IL-13, IL-17A, IL-17F and IL-22.

Prolonged Changes of Key Host Genes.

To further analyze the utility of transcriptome analysis for prediction of unique transcripts associated with infection or vaccination, we used real-time, quantitative PCR to compare transcript levels among animal groups over 12 months post-challenge (MPC) (FIGS. 7A-7D). Interestingly, IL-17 cytokine was repressed in the challenged and Mycopar® and LAV-vaccinated goats compared to the naïve control group for all examined times, except for the infected group at 2 MPC. Similarly, the Sept10 gene was induced, only at 2 MPC. On the other hand, IL-36 was activated soon after vaccination (1 and 2 MPC) but then repressed for the rest of the examined time points, i.e. 6 and 12 MPC. More interestingly, the IFN-γ expression profile was refractive to elicited immune responses. IFN-γ was induced soon in the LAV-vaccine group (1 MPC) but then continued to be expressed in the Mycopar®-vaccinated and *M. paratuberculosis*-challenged groups starting from 2 MPC until the end of the experiment. At all of these sampling times, the IFN-γ was consistently higher in the LAV-vaccine group compared to the challenged group.

Differential Expression in LAV vaccinated animals, Mycopar™ vaccinated animals, and infected animals.

TABLE 15

List of host genes (goat and cow) differentially expressed in both LAV
and Mycopar vaccinated animals compared to infected animals

| Gene symbol | Gene ID | LAV vs. Infected | | Mycopar vs Infected | | Fold change difference | Description |
		Fold change	p-value	Fold change	p-value		
STC1	102179386	−4.668	1.4E−07	−8.937	5.6E−17	4.268	stanniocalcin 1
NGF	100862660	−1.778	2.8E−02	−5.001	1.2E−04	3.223	nerve growth factor
FAM150B	102183516	−1.406	4.8E−02	−4.355	2.0E−05	2.949	family with sequence similarity 150 member B
FOXE1	106502413	−1.679	1.4E−02	−4.290	4.5E−07	2.611	forkhead box E1
C28H10orf71	102181364	−2.870	5.2E−03	−5.463	1.3E−04	2.593	chromosome 28 C10orf71 homolog
HEBP2	102174123	−1.584	4.2E−02	−4.133	1.9E−03	2.549	heme binding protein 2
IL11	102184367	−2.218	8.9E−03	−4.659	1.1E−05	2.440	interleukin 11
KRT82	102183763	−1.630	5.6E−03	−3.951	4.0E−04	2.320	keratin 82
NTNG1	102190191	−1.704	8.1E−06	−3.726	7.9E−16	2.022	netrin G
SORCS2	102176511	−1.357	4.9E−03	−3.365	5.8E−09	2.008	sortilin related VPS10 domain containing receptor 2
HS3ST2	102183286	−3.638	1.0E−07	−1.432	3.4E−02	−2.206	heparan sulfate-glucosamine 3-sulfotransferase 2
TGFB3	102189962	2.706	8.5E−03	5.103	4.2E−06	−2.397	transforming growth factor beta 3

TABLE 16

List of host genes (goat and cow) differentially expressed in LAV
vaccinated animals compared to infected animals but not differentially expressed in Mycopar ®
vaccinated animals compared to infected animals.

| Gene symbol | Gene ID | LAV vs. Infected | | Mycopar vs Infected | | Description |
		Fold change	p-value	Fold change	p-value	
LOC102176439	102176439	3.865	0.003	0.763	0.603	misc_RNA
LOC102187130	102187130	3.341	0.002	−0.300	0.800	protein ARMCX6-like
LOC108633178	108633178	2.976	0.000	0.895	0.129	granzyme B-like
CPNE6	102180500	2.755	0.000	0.841	0.289	copine 6
IL13	102187477	2.752	0.000	−0.220	0.752	interleukin 13
CCR10	102184001	2.581	0.007	0.700	0.502	C-C motif chemokine receptor 10
C1QL2	102176742	2.427	0.032	−0.520	0.684	complement C1q like 2
MGAT3	102185445	2.418	0.005	−0.570	0.553	mannosyl (beta-4-)-glycoprotein beta-4-N-acetylglucosaminyltransferase
GNLY	102191341	2.212	0.000	0.807	0.146	granulysin transcript
KY	102169426	−3.013	0.003	−0.144	0.884	kyphoscoliosis peptidase
RAET1E	108636743	−3.784	0.002	−0.895	0.497	retinoic acid early transcript 1E

TABLE 17

List of host genes (goats and cow) differentially expressed in Mycopar ®
vaccinated animals compared to infected animals but not differentially expressed in LAV
vaccinated animals compared to infected animals.

| Gene symbol | Gene ID | LAV vs. Infected | | Mycopar vs Infected | | Description |
		Fold change	p-value	Fold change	p-value	
LOC102174895	102174895	1.762	0.141	5.278	0.000	vascular cell adhesion protein
BMP10	102185577	0.557	0.721	4.192	0.012	bone morphogenetic protein 10
CXCL12	102169556	−0.755	0.302	3.613	0.000	C-X-C motif chemokine ligand 12
LOC108633303	108633303	−0.272	0.769	2.659	0.008	platelet glycoprotein 4-like
PPARG	100861309	0.904	0.053	2.339	0.000	peroxisome proliferator activated receptor gamma
F13A1	102169238	−0.132	0.883	2.239	0.021	coagulation factor XIII A chain
LOC108634012	108634012	−0.173	0.804	−2.736	0.000	homeobox protein MSX-3-like
LRRC3	102188902	0.357	0.786	−2.907	0.042	leucine rich repeat containing 3
MYO10	102175716	−0.967	0.023	−3.183	0.000	myosin X
LOC102179419	102179419	−0.471	0.759	−3.435	0.040	myeloid-associated differentiation marker

Discussion

Infection with *M. paratuberculosis* is costing the dairy industry significant economic losses (Cho et al., 2012) and is difficult to detect its presence, especially during early disease stages (Li et al., 2017). In this project, the goat PBMC transcriptome was profiled using RNA-Sequencing (RNA-Seq) to compare the early gene expression, 30 days post-infection and post-vaccination, compared to healthy, naïve controls. In addition to better understanding of disease progression, such analysis is expected to yield targets for further development into a diagnostic assay for early stages of Johne's disease. Many transcriptomic analyzing tools largely depend on information from an annotated genome. In this study, our quality of transcriptomic analyses improved as the goat genome assembly was significantly refined (Bickhart et al., 2017). According to NCBI *Capra hircus* Annotation Release 102, of 20,593 predicted coding genes, 20,256 had a protein aligned 50% or more of the query against the UniProtKB/Swiss-Prot curated proteins (NCBI, 2016). The updated annotation thus provides a much more reliable reference to our analysis. The generated RNA-Seq dataset could also benefit further improvement of goat genome annotation. As expected, a large number of differentially expressed (DE) transcripts were found between the vaccinated and infected groups (1133 genes) and between the vaccinated and naïve control group (1018 genes). In contrast, there was a relatively small number (226) of DE transcripts when comparing the infected and naïve control group. This large difference in the number of DE transcripts is most likely associated with the route of administration since both vaccines were administered subcutaneously (contrary to oral infection), allowing for increased contact with PBMCs in the bloodstream, while challenge dose of *M. paratuberculosis* could reach PBMC following intestinal invasion (Stabel et al., 2009). Our analysis, further illustrated the importance of route of infection and/or vaccination for the type and magnitude of the generated host responses.

Although the comparison between the infected and naïve control group produced a relatively small number of DE transcripts, preliminary evaluation of these genes indicated a large number of genes with immunological and inflam-matory functions, including interferon gamma (IFN-γ), IL-18 binding protein, IL-17A, and IL-22. IFN-γ is an important player in the defense against intracellular pathogens including mycobacteria (Arsenault et al., 2012). A previous study in cattle showed that in the subclinical stages of infection, IFN-γ expression increased at the site of infection (Sweeney et al., 1998). Other studies indicate that *M. paratuberculosis*-infected animals produce IFN-γ but are unresponsive to it (Arsenault et al., 2012). In that study, IFN-γ was secreted significantly less (−3.36 fold change) in subclinically infected goats compared with the naïve, control goats. This IFN-γ profile was also evident in subclinically infected goats vs vaccinated goats (−13.0 fold change). Previously, IFN-γ was reported to be induced in PBMC's stimulated with *M. paratuberculosis* whole-cell sonicate from subclinically infected cows (Stabel, 2000). However, these cows ranged from 2-10 years of age and therefore were much further along in the infection pathogenesis than in the current study, which tested goats 30 days PI. The host response clearly changes over time and this data may demonstrate that. Potentially linked to the identified repression of IFN-γ, is the moderate up-regulation (+1.30 fold change) of interleukin 18 binding protein (IL-18 bp) in the infected vs naïve control group. IL-18 bp binds to IL-18 to block its biological activity (Novick et al., 1999). IL-18 is a pro-inflammatory cytokine that functions in the early Th1 cytokine response and induces IFN-γ production. A major source of IL-18 bp is from intestinal endothelial cells and macrophages (Corbaz et al., 2002). Therefore, IL-18 bp serves to modulate the early Th1 immune response in the intestine, the site of *M. paratuberculosis* infection. Interestingly, IL-18 bp has been found to be up-regulated during active Crohn's disease, an inflammatory bowel disease in humans with potential association to *M. paratuberculosis* infection (Corbaz et al., 2002).

As expected, genes involved in immune responses (e.g. LIF, IFN-γ and IL-22), were found to be DE among examined goat groups. LIF is a pleiotropic cytokine belonging to the IL-6 cytokine family with receptors primarily on monocytes/macrophages (Nicola and Babon, 2015). In the

infected group, both LIF and IL-22, a Th17-related cytokine, were down-regulated in the infected group vs the control or the vaccinated groups. These three genes, along with IL-13 and IL-17, were also found having associations in the protein network analysis. IL-17 was also down-regulated in the infected vs control group. Down-regulation of IFN-γ, IL-22 and IL-17 genes may suggest overall down-regulation of Th1 and Th17 cell activities and reduced cellular immunity against infections. Several studies in *Mycobacterium tuberculosis* and *Mycobacterium bovis* have shown significant IL-17 responses (Blanco et al., 2011; Jurado et al., 2012). A recent study on RNA-Seq analysis in cattle infected with *M. bovis* showed an up-regulation of IL-17, IL-22, and IFN-γ at one-month PI (Waters et al., 2015). This is in contrast to some of our findings in the present study (in case of IL-17) which was further confirmed by prolonged analysis of key genes up to 12 months post infection (FIGS. 7A-7D). Such difference could be attributed to the difference in host response to *M. bovis* vs. *M. paratuberculosis*. Further investigation into these key immune regulated genes as will aid in understanding how the host is dynamically responding to *M. paratuberculosis* infection or vaccination.

Our gene network analysis also shows associations among genes that were up-regulated in the infected group (FIG. 7A). Interestingly, homologs of ACER3, SYNJ2, CORO6 and PLS1 in animal species other than goats (mainly bovine, *Bos Taurus*) were also shown to have physical associations (Schlecht et al., 2012; Hein et al., 2015) and co-expression (Clancy et al., 2003; Janji et al., 2010) as well. Particularly, homologs of CORO6, an actin binding protein, was suggested to be involved in cytokinesis. In *M. tuberculosis*-infected macrophages, CORO6 homolog coronin-la was suggested to inhibit auto-phagosome formation and facilitate *M. tuberculosis* survival (Seto et al., 2012). In addition, homologs of PDE4C and TSKU were also found associated (Halls and Cooper, 2010; Schlecht et al., 2012; Costanzo et al., 2016) and suggested to be involved in signaling and

relaxin regulation (Halls and Cooper, 2010). It may thus imply a status of progression of an *M. paratuberculosis* infection in hosts as observed in *M. tuberculosis* infection (Seto et al., 2012). This observation, along with the likely reduced cellular immunity discussed above, is consistent with the infection status of the host. It is unclear, however, how bacterial or host factors regulate the expression of those genes. Understanding the host-pathogen interaction early in infection will allow for the identification of genes upregulated during initial infection. A useful biomarker for infection must be specific, detectable over the course of the disease with varying inoculation doses, and easily measurable. Moreover, it would improve interpretation of early disease detection if the biomarkers could differentiate infected and vaccinated animals. In our analyses, we identified 9 transcripts (out of 11 in Table 14) that were down-regulated 30 days PI and up-regulated 30 days post-vaccination. This biphasic regulation of those genes or transcripts might make them specific markers for differentiating vaccinated animals that are healthy or those infected with *M. paratuberculosis*.

The RNA-Seq analysis was performed only on samples taken one month post-infection or post-vaccination to identify early gene regulations in tested groups, notably, between one month after vaccinated only and infected only groups. This comparison differentiates host gene regulating responses after exposure to vaccine strains or virulent strains of *M. paratuberculosis*. The vaccinated animals were then challenged two months after the vaccination and several key gene expressions were profiled with quantitative PCR (FIGS. 7A-7D). The temporal expression patterns within the tested one year period could reflect unique characteristics of host responses after exposure to virulent *M. paratuberculosis* with or without prior vaccinations and could also benefit development of diagnostics. For example, IL-17 expressions in the vaccinated animals remained highly repressed at all time while peaking at 2 month post-challenge in the infected only group.

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Val Gly Val Lys Val Ile Pro Trp Ile Pro Thr Ala Val Arg Arg Gly
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Leu Val Arg Gly Arg Ser Val Ile Ile Asp Gly Asn Thr Leu Asp Pro
    50                  55                  60

Thr Leu Gln Leu Met Leu Ser Gly Leu Arg Ala Val Gly Ile Asp Gly
65                  70                  75                  80

Leu Val Val Asp Asp Asp Pro Glu Leu Ser Arg Ala Gln Met His Glu
                85                  90                  95

Ser Thr Val Gly Phe Pro Gly Pro Gln Ile His Val Asp Val Ala Glu
            100                 105                 110
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Pro Ala Gly Gly Glu Thr Gln Ala Pro Leu Leu Val Phe Tyr His Gly
    130             135             140

Gly Gly Trp Ser Ile Gly Asp Leu Asp Thr His Asp Ser Leu Cys Arg
145             150             155             160

Leu Thr Cys Arg Asp Ala Gly Ile His Val Leu Ser Ile Asp Tyr Arg
                165             170             175

Leu Ala Pro Glu His Pro Ala Pro Ala Ala Ile Asp Asp Ala Tyr Ala
            180             185             190

Ala Phe Thr Trp Ala His Glu His Ala Gly Glu Leu Gly Ala Ile Pro
        195             200             205

Gly Arg Val Ala Val Gly Gly Asp Ser Ala Gly Gly Asn Leu Ala Ala
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Val Val Ser Gln Leu Ala Arg Asp Ala Gly Gly Pro Ala Pro Val Leu
225             230             235             240

Gln Trp Leu Ile Tyr Pro Arg Thr Asp Phe Thr Ala Arg Thr Arg Ser
                245             250             255

Leu Ser Leu Phe Ser Arg Gly Phe Leu Leu Thr Lys Arg Asp Ile Asp
            260             265             270

Trp Phe Glu Ser Gln Tyr Leu Arg Asn Ser Arg Leu Asp Arg Thr Asp
        275             280             285

Pro Arg Val Ser Pro Ala Leu Ala Glu Ser Leu Ala Gly Leu Ala Pro
        290             295             300

Ala Leu Ile Ala Val Ala Gly Phe Asp Pro Leu Arg Asp Glu Gly Gln
305             310             315             320

Ser Tyr Ala Glu Ala Leu Arg Ala Ala Gly Thr Pro Val Asp Leu Arg
                325             330             335

Tyr Leu Gly Ser Leu Thr His Gly Phe Ala Asn Leu Phe Gln Leu Gly
            340             345             350

Gly Asp Ser Met Val Ala Thr Ser Glu Leu Ile Ser Ala Leu Arg Ala
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His Leu Ser Arg Val
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His Pro Arg Met Trp Thr Arg Arg Val Gln Gly Thr Val Val Asn Val
            20              25              30

Gly Val Lys Val Leu Pro Trp Ile Pro Thr Pro Ala Lys Arg Ile Leu
        35              40              45

Ser Ala Gly Arg Ser Val Ile Ile Asp Gly Asn Thr Leu Asp Pro Thr
    50              55              60

Leu Gln Leu Met Leu Ser Thr Ser Arg Ile Phe Gly Val Asp Gly Leu
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Ala Val Asp Asp Asp Ile Val Ala Ser Arg Ala His Met Arg Ala Ile
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Cys Glu Ala Met Pro Gly Pro Gln Ile His Val Asp Val Thr Asp Leu
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Val Trp Ala His Glu His Ala Ser Asp Glu Phe Gly Ala Leu Pro Gly
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Arg Val Ala Val Gly Gly Asp Ser Ala Gly Gly Asn Leu Ser Ala Val
        210                 215                 220

Val Cys Gln Leu Ala Arg Asp Lys Ala Arg Tyr Glu Gly Gly Pro Thr
225                 230                 235                 240

Pro Val Leu Gln Trp Leu Leu Tyr Pro Arg Thr Asp Phe Thr Ala Gln
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Thr Arg Ser Met Gly Leu Phe Gly Asn Gly Phe Leu Leu Thr Lys Arg
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Pro Ala Asp Pro Arg Leu Ser Pro Leu Leu Ala Glu Ser Leu Ser Gly
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Leu Ala Pro Ala Leu Ile Ala Val Ala Gly Phe Asp Pro Leu Arg Asp
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Glu Gly Glu Ser Tyr Ala Lys Ala Leu Arg Ala Ala Gly Thr Ala Val
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Gln Leu Gly Gly Gly Ser Ala Ala Gly Thr Asn Glu Leu Ile Ser Ala
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<222> LOCATION: (299)..(299)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
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<222> LOCATION: (305)..(305)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (325)..(325)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (329)..(329)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (337)..(337)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (351)..(351)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (359)..(359)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
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<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (365)..(365)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 3

Met Thr Lys Xaa Leu Xaa Xaa Xaa Ala Xaa Xaa Xaa Xaa Gly Ala Xaa
1               5                   10                  15

Xaa Xaa Xaa Met Pro Xaa Thr Xaa Arg Xaa Gln Gly Xaa Val Xaa Xaa
            20                  25                  30

Val Gly Val Lys Val Xaa Pro Trp Ile Pro Thr Xaa Xaa Xaa Arg Xaa
        35                  40                  45

Leu Xaa Xaa Gly Arg Ser Val Ile Ile Asp Gly Asn Thr Leu Asp Pro
    50                  55                  60

Thr Leu Gln Leu Met Leu Ser Xaa Xaa Arg Xaa Xaa Gly Xaa Asp Gly
65                  70                  75                  80

Leu Xaa Val Asp Asp Asp Xaa Xaa Xaa Ser Arg Ala Xaa Met Xaa Xaa
                85                  90                  95

Xaa Xaa Xaa Xaa Xaa Pro Gly Pro Gln Ile His Val Asp Val Xaa Xaa
            100                 105                 110

Leu Xaa Xaa Pro Gly Pro Ala Gly Xaa Ile Pro Ala Arg His Tyr Arg
        115                 120                 125

Pro Xaa Gly Gly Xaa Xaa Gln Xaa Pro Leu Leu Val Phe Tyr His Gly
    130                 135                 140
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Gly Gly Trp Xaa Xaa Gly Asp Leu Asp Thr His Asp Xaa Leu Cys Arg
145                 150                 155                 160

Leu Thr Cys Arg Asp Ala Xaa Ile Xaa Val Leu Ser Ile Asp Tyr Arg
                165                 170                 175

Leu Ala Pro Glu His Pro Ala Pro Ala Ala Xaa Xaa Asp Ala Tyr Ala
            180                 185                 190

Ala Phe Xaa Trp Ala His Glu His Ala Xaa Asp Glu Xaa Gly Ala Xaa
        195                 200                 205

Pro Gly Arg Val Ala Val Gly Gly Asp Ser Ala Gly Gly Asn Leu Xaa
    210                 215                 220

Ala Val Val Xaa Gln Leu Ala Arg Asp Xaa Ala Arg Tyr Glu Gly Gly
225                 230                 235                 240

Pro Xaa Pro Val Leu Gln Trp Leu Xaa Tyr Pro Arg Thr Asp Phe Thr
            245                 250                 255

Ala Xaa Thr Arg Ser Xaa Xaa Leu Phe Xaa Xaa Gly Phe Leu Leu Thr
            260                 265                 270

Lys Arg Asp Ile Asp Trp Phe Xaa Xaa Gln Tyr Leu Arg Xaa Ser Xaa
            275                 280                 285

Xaa Asp Xaa Xaa Asp Pro Arg Xaa Ser Pro Xaa Leu Ala Glu Ser Leu
    290                 295                 300

Xaa Gly Leu Ala Pro Ala Leu Ile Ala Val Ala Gly Phe Asp Pro Leu
305                 310                 315                 320

Arg Asp Glu Gly Xaa Ser Tyr Ala Xaa Ala Leu Arg Ala Ala Gly Thr
            325                 330                 335

Xaa Val Asp Leu Arg Tyr Leu Gly Ser Leu Thr His Gly Phe Xaa Asn
        340                 345                 350

Leu Phe Gln Leu Gly Gly Xaa Ser Xaa Xaa Xaa Thr Xaa Glu Leu Ile
        355                 360                 365

Ser Ala Leu Arg Ala His Leu Ser Arg Val
    370                 375


<210> SEQ ID NO 4
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 4

ggtgagcgcc agaggaa                                                  17


<210> SEQ ID NO 5
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 5

cagcttctcc tcttggtgga c                                             21


<210> SEQ ID NO 6
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 6
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aactggatcc cagacccc                                            18


<210> SEQ ID NO 7
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 7

gtagctgctg ggagcgc                                             17


<210> SEQ ID NO 8
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 8

ggaacacgaa ctccagaagg c                                        21


<210> SEQ ID NO 9
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 9

acagagttca tgtgatggtc cac                                      23


<210> SEQ ID NO 10
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 10

accaccgtgc gtaccac                                             17


<210> SEQ ID NO 11
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 11

ggaggtcttg ggaccctctc                                          20


<210> SEQ ID NO 12
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 12

cgttaatagc agttccttct agcaac                                   26


<210> SEQ ID NO 13
<211> LENGTH: 22
<212> TYPE: DNA
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 13

ggatagccct ggatttctgt gc   22

<210> SEQ ID NO 14
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 14

tgaggcagta aggaacattg gc   22

<210> SEQ ID NO 15
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 15

agtccatgcc tgcgcac   17

<210> SEQ ID NO 16
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 16

gcagctctga gaaactggag g   21

<210> SEQ ID NO 17
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 17

tccggcctcg aaagagattc t   21

<210> SEQ ID NO 18
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 18

ggcgtgaacc acgagaagta taa   23

<210> SEQ ID NO 19
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 19

ggcagtgatg gcgtggac   18

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<210> SEQ ID NO 20
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 20

cttggatcgc catacccctc                                               20


<210> SEQ ID NO 21
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 21

gggctccggg ttgtagattc                                               20


<210> SEQ ID NO 22
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 22

gaggaccaca ttgtgagggt                                               20


<210> SEQ ID NO 23
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 23

cgggtgatgt tgtaatccca g                                             21


<210> SEQ ID NO 24
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 24

cgacgagggg ttgtgtctg                                                19


<210> SEQ ID NO 25
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 25

acatagctat tggggcagcg                                               20


<210> SEQ ID NO 26
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 26

tcatccaaga tggccgcc   18

<210> SEQ ID NO 27
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 27

gccagcactt ctgtttcagc   20

<210> SEQ ID NO 28
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 28

gaaatcacgg aggagtggca   20

<210> SEQ ID NO 29
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 29

aacagctgtg aaaccacctc a   21

<210> SEQ ID NO 30
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 30

cagggaatca atcaggtgac ga   22

<210> SEQ ID NO 31
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 31

atggggtgg aattcatcgg   20

<210> SEQ ID NO 32
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 32

cagtgaagtg ctcgtctggt   20

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<210> SEQ ID NO 33
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 33

cgactcaatc ccatacaccg t                                                    21


<210> SEQ ID NO 34
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 34

aagccaagct tccgctatca                                                      20


<210> SEQ ID NO 35
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 35

cgatgacagt caggtccgtg                                                      20
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We claim:

1. A method comprising the steps of:
   a) obtaining a blood sample from a mammal, wherein the mammal is a ruminant;
   b) measuring the expression level of FAM198B and AOAH in the blood sample by quantitative polymerase chain reaction (qPCR);
   c) administering a live-attenuated or inactivated *Mycobacterium* vaccine to the mammal having equal relative expression of AOAH and FAM198B in the sample or having relative expression of AOAH and FAM198B within error of the qPCR used to measure the expression level of AOAH and FAM198b in the sample.

2. The method of claim 1, wherein the mammal is selected from the group consisting of cow and goat.

3. The method of claim 1, wherein the vaccine is a *mycobacterium* mutant vaccine.

4. The method of claim 3, wherein the *mycobacterium* mutant vaccine comprises at least one mutation in at least one gene sequence encoding global gene regulators (GGRs) selected from the group consisting of sigH, sigL and LipN.

5. The method of claim 1, wherein mammal is not actively shedding mycobacteria.

6. The method of claim 1, wherein the mammal has a negative mycobacteria antibody test.

7. A method comprising the steps of
   a) obtaining a blood sample from a mammal, wherein the mammal is a ruminant;
   b) measuring the expression level of at least two biomarkers consisting of FAM198B and AOAH in the blood sample by quantitative polymerase chain reaction (qPCR);
   c) administering antibiotics to the mammal having higher relative expression of FAM198B than AOAH to treat the mammal for a mycobacterial infection.

8. The method of claim 7, wherein the mammal is selected from the group consisting of cow and goat.

9. The method of claim 7, wherein the mammal is not actively shedding mycobacteria.

10. The method of claim 7, wherein the mammal has a negative mycobacteria antibody test.

*   *   *   *   *