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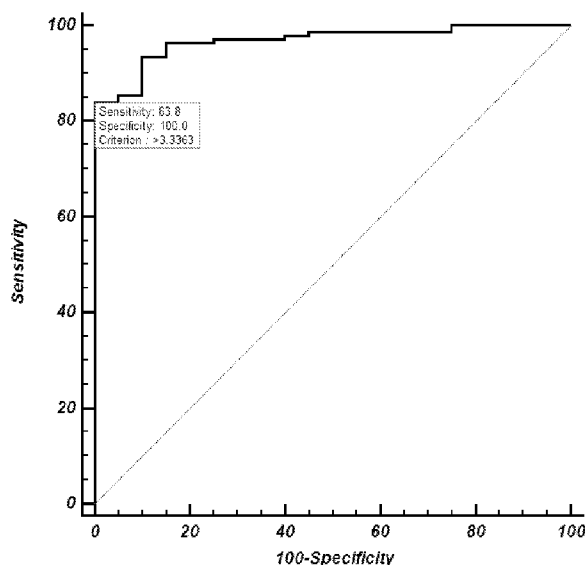
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(54) Title: COMPOSITIONS AND METHODS FOR DIAGNOSIS AND PROPHYLAXIS OF MASTITIS IN RUMINANTS

Figure 3.



(57) **Abstract:** Provided is a method for determining whether or not a ruminant has mastitis. The method includes testing a milk sample from the ruminant to determine *Fusobacterium necrophorum* as a percentage of bacteria in the milk sample. The ruminant is diagnosed as having mastitis if the percentage of *Fusobacterium necrophorum* is >3.3% of the bacteria in the milk sample, or the ruminant is diagnosed as not having mastitis if the percentage of *Fusobacterium necrophorum* is <3.3% of the total bacteria in the milk sample. In another aspect the disclosure provides a method for determining that a sample of milk was obtained from a ruminant which does not have clinical mastitis. The method involves testing a milk sample from the ruminant to determine *Fusobacterium necrophorum* as a percentage of bacteria in the milk sample, and identifying the sample of milk as having been obtained from a ruminant that does not have mastitis if the *Fusobacterium necrophorum* is less than 3.3% of the bacteria in the milk sample. The ruminants tested include bovine animals. Compositions and methods for prophylaxis against mastitis in ruminants are also provided and involve veterinary compositions which contain whole cells of *Fusobacterium necrophorum*, *Fusobacterium necrophorum* leukotoxin (LKT), or a combination thereof.

ruminant, *Fusobacterium necrophorum* leukotoxin (LKT), or a combination thereof.

COMPOSITIONS AND METHODS FOR DIAGNOSIS AND PROPHYLAXIS OF MASTITIS IN RUMINANTS

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CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional application no. 61/710,946, filed on October 8, 2012, the disclosure of which is incorporated herein by reference.

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FIELD OF THE INVENTION

[0002] The present invention relates generally to bovine mastitis and more specifically to compositions and methods for diagnosing and protecting against mastitis and/or its symptoms.

BACKGROUND OF THE INVENTION

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[0003] Dairy cow mastitis is arguably the most important disease for the dairy industry worldwide, causing economic losses due to reduced milk production, discarded milk, premature culling, and antibiotic usage. Clinical mastitis is also a serious animal welfare issue as it is associated with pain and reduced well-being of the affected animals. Currently, bacterial culture is the gold standard method for identification of mastitis-causing microorganisms. However, classical bacterial culture has undesirable limitations, such as a delay of 24-48 hours to obtain results. Further, bacteria are not detected in conventional culture in approximately 25% of milk samples from clinical mastitis cases. Further still, there are no satisfactory approaches for prophylaxis of mastitis. Thus, there is an ongoing and unmet need for improved methods of diagnosis and prophylaxis of bovine mastitis and for mastitis in ruminants generally. The present disclosure addresses these needs.

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BRIEF DESCRIPTION OF FIGURES

[0004] Figure 1 provides a representative depiction of data summarizing mean prevalence of bacterial genera that were found to be significant for the discriminant analysis of mastitic milk samples. *Fusobacteria* is represented by the darkest bar.

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[0005] Figure 2 provides a representation of a phylogenetic tree of the ten most predominant sequences (OTU1-OTU10, also presented in Table 7) of samples characterized as *Staphylococcus* spp. mastitis. *Escherichia coli* served as outgroup. GenBank accession numbers are indicated in parentheses.

[0006] Figure 3 is a graphical depiction of a receiver operating characteristic curve

(ROC) illustrating the sensitivity (Y axis) and 100-specificity (X axis) of the % *Fusobacterium* spp. (percent of the overall bacterial population that was classified as *Fusobacterium* spp.) as a predictor of clinical mastitis.

[0007] Figure 4 provides a graphical representation of the effect of vaccination by ELISA-detected serum IgG against *F. necrophorum*. The X-axis represents days relative to calving, while Y-axis represents OD650 of ELISA-detected serum IgG against several antigens. Standard errors of the means are represented by the error bars. As described in the Examples, Vaccine 1 was composed of inactivated bacterial whole cells and proteins; Vaccine 2 was composed of proteins only; and Vaccine 3 was composed of inactivated bacterial whole cells only. Two intravaginal vaccines were formulated: Vaccine 4 was composed of inactivated bacterial whole cells and proteins; and Vaccine 5 was composed of PLO and LKT.

SUMMARY

[0008] The present disclosure provides in one aspect a method for diagnosis of mastitis in a female ruminant. The method comprises testing a milk sample from the ruminant to determine *Fusobacterium necrophorum* as a percentage of bacteria in the milk sample. The ruminant is diagnosed as having mastitis if the percentage of *Fusobacterium necrophorum* is $\geq 3.3\%$ of the bacteria in the milk sample, or the ruminant is diagnosed as not having mastitis if the percentage of *Fusobacterium necrophorum* is $< 3.3\%$ of the total bacteria in the milk sample. In embodiments testing the milk sample comprises determining prevalence of a plurality of bacteria types in the milk sample. In embodiments, the prevalence of the bacteria types is performed by determining polynucleotide sequences which are unique to each of the bacteria types. In embodiments, the polynucleotide sequences comprise 16S rRNA that is distinct for each of the bacteria types. In embodiments the method is used for diagnosis of mastitis in a bovine animal, such as a dairy cow.

[0009] In another aspect the disclosure provides a method for determining that a sample of milk was obtained from a ruminant which does not have clinical mastitis comprising (a) testing a milk sample from the ruminant to determine *Fusobacterium necrophorum* as a percentage of bacteria in the milk sample, and b) identifying the sample of milk as having been obtained from a ruminant that does not have mastitis if the *Fusobacterium necrophorum* is less than 3.3% of the bacteria in the milk sample.

[0010] In other aspects the present disclosure also provides a veterinary composition comprising whole cells of *Fusobacterium necrophorum*, recombinant or isolated

Fusobacterium necrophorum leukotoxin (LKT), or a combination thereof, and a veterinarily acceptable carrier, excipient or diluent. Also provided is an article of manufacture comprising packaging and at least one sealed container. The container comprises a veterinary composition comprising whole cells of *Fusobacterium necrophorum*, recombinant or isolated

5 *Fusobacterium necrophorum* leukotoxin (LKT), or a combination thereof, the packaging further comprising printed material providing an indication that the veterinary composition is for vaccination of ruminant. In embodiments, the indication includes description of administering the vaccination subcutaneously. The disclosure also includes a method for prophylaxis of mastitis in a ruminant comprising subcutaneously administering to the

10 ruminant a veterinary composition of the invention.

DETAILED DESCRIPTION

[0011] The present disclosure provides in various embodiments compositions and methods for diagnosis and/or aiding in the diagnosis of mastitis in ruminants, as well as compositions and methods for prophylaxis of mastitis in ruminants. The method is expected

15 to be suitable for use with any ruminant. In embodiments, the ruminant is a bovine animal. In the context of this disclosure, the terms “bovine animal” and “bovine” mean mammals of the genus *Bos*, such as an ox, cow, or buffalo. In embodiments, the bovine animal is a dairy cow.

[0012] Bovine mastitis refers to inflammation of bovine mammary glands. Two

20 general types of mastitis include clinical (acute) mastitis and subclinical mastitis. The clinical form is characterized by the classical symptom of inflammation of the mammary glands, which can be evidenced on site by indicators which include but are not limited to swelling, pain, redness and elevated temperature in the gland. Visually detectable changes in the milk may also be observed at the time of milking, such as the presence of flakes, clots or serous

25 milk.

[0013] Subclinical mastitis is generally difficult to detect using classical methods because the symptoms are typically less noticeable than in the clinical cases, but subclinical mastitis is believed to be a source of infection for initially non-infected bovines who are housed with subclinically infected animals.

30 [0014] Clinical and subclinical mastitis have been evaluated using traditional bacterial culturing techniques, and by determining leukocytes (somatic cells) that enter the milk during inflammation. The concentration of somatic cells in milk is referred to in the art as the somatic cell count (SCC). It is considered that the greater the SCC, the higher the level

of inflammation in the tissue. In general, somatic cell counts of 50,000 cells/ml or less are taken as indicating the absence of infection. However, cell SCC numbers much higher than that can be present in samples from healthy and mastitic cells. Further, while techniques such as differential cell counts have been proposed for use in evaluating mastitis, these involve significant investments of time, equipment and expertise. Further still, traditional bacterial culturing techniques for determining distinct types of bacteria in milk samples involve significant time, cost and expertise, with the results not necessarily being reliably predictive of mastitis or a distinct cause of it. Moreover, as demonstrated in the present disclosure, the mastitis causative agent diagnosed on a culture by culture basis using traditional techniques is not necessarily clinically informative and does not suggest any compositions or methods for vaccinating against mastitis in a manner that would provide broad protection against most forms of bovine mastitis, regardless of traditional culture-based causative-agent analysis.

[0015] In contrast to previously available approaches, the present disclosure provides improved methods for determining mastitis in ruminants, as well as compositions and methods for protecting against development of the mastitis. These aspects are related at least in part to the present discovery that the presence and amount of the strict anaerobe *Fusobacterium necrophorum* in bovine milk samples is highly and unexpectedly predictive of the presence or absence of mastitis. In particular, using one illustrative approach comprising determining 16S rRNA gene sequences, we classified bacterial species in milk samples from classically diagnosed mastitic cows and determined that the presence and amount of *Fusobacterium necrophorum* in the milk is highly specific and sensitive for diagnosing the bovine mammal as having mastitis.

[0016] In particular, based on bacterial culture of milk samples from 136 mastitic dairy cows, 16 cases were diagnosed as infected with *Streptococcus uberis*, 17 as *Escherichia coli*, 11 as *Klebsiella spp.*, 17 as *Staphylococcus aureus*, 20 as *Staphylococcus spp.*, 17 as *Streptococcus dysgalactiae*, 2 as *Streptococcus spp.*, 3 as *Trueperella pyogenes*, and 33 samples were characterized as culture negative. Twenty milk samples obtained from healthy cows were also found to be culture negative. We analyzed the same 156 milk samples using metagenomic pyrosequencing of the bacterial 16S rRNA genes. The results of this analysis reveal a threshold level of *F. necrophorum* to be highly predictive of clinical mastitis. In particular, our analysis shows that a % *Fusobacterium spp.* of $\geq 3.3\%$ is highly predictive of clinical mastitis with a Sensitivity of 83.8 and a Specificity of 100%. Therefore, 83.8% of the cows which were affected with mastitis had *Fusobacterium spp.* $\geq 3.3\%$, with an average of

8.96% (Standard deviation = 6.67). Further, 100% of the cows not affected with mastitis had % *Fusobacterium* spp. <3.3. The threshold level determined to the fourth decimal place is 3.3363 %. Accordingly, a threshold of 3.3%, or 3.33%, or 3.336%, or 3.3363% can be used, and any of these values can be rounded if desired.

5 [0017] Figure 3 provides a representation of data that includes the threshold value and is discussed in additional detail below. Thus, the present disclosure provides for the first time a highly accurate and reliable method for diagnosing a bovine as having bovine mastitis based on testing a milk sample for the presence / amount of *F. necrophorum*. Further, the present disclosure provides for establishing that a bovine is free from mastitis based on
10 determining a threshold amount of *F. necrophorum* in a milk sample. These approaches do away with previous requirements for classical bacterial culture, cell counting and the like. Thus it is expected that embodiments of the present disclosure will contribute to a significant increase in production of commercially usable milk by at least eliminating previous false positive results generated from traditional bacteria culturing techniques.

15 [0018] It will be apparent from the foregoing that the present disclosure provides, in various embodiments, methods for determining whether or not a ruminant, such as a bovine animal has mastitis. The method in general involves testing a milk sample from the bovine. The sample can be tested directly, or it can be subjected to a processing step, such as by subjecting it to incubation, dilution, mixing with any of a variety of reagents, centrifugation,
20 filtering, or otherwise separating milk components and bacteria for testing if desired. The samples can be processed to concentrate or otherwise separate and/or purify bacterial cells, and/or to isolate polynucleotides from the bacteria using any of a wide variety of conventional techniques.

[0019] Testing the samples for *F. necrophorum* can be performed using any suitable
25 technique. Several approaches comprise forming a complex between a component of *F. necrophorum* and a specific binding partner to detect and/or quantify *F. necrophorum* in a sample.

[0020] In embodiments, the specific binding partner can be an antibody or fragment thereof which specifically binds to a component of *F. necrophorum*, which can include but is
30 not necessarily limited to epitopes present on surface exposed proteins, or proteins that are characteristic of *F. necrophorum* which can be immunologically detected after, for example, cell lysis. Thus, the present disclosure includes immunological detection of *F. necrophorum*. Immunological approaches can be used for quantifying the amount of *F. necrophorum* in a

sample and can include techniques such as ELISA assays, fluorescence activated cell-sorting assays, and may be adapted for use with immunological detection devices, such as a lateral flow strip, dot blots, and the like.

[0021] In embodiments the specific binding partners can be synthetic oligonucleotide primers that are targeted to *F. necrophorum* genetic material. In embodiments, the primers can be designed to amplify any portion of the *F. necrophorum* genome. In embodiments, the primers are designed for amplification of 16S rRNA gene sequences. Thus, in one aspect the present disclosure includes forming complexes of synthetic oligonucleotide primers and bacterial genetic material and amplifying a portion of a bacterial genome via reactions that can include a polymerase chain reaction (PCR).

[0022] In embodiments, the disclosure includes amplifying a plurality of distinct regions of a plurality of distinct bacterial genomes in order to determine the bacterial composition in a milk sample. In embodiments, the presence, and/or type(s) and/or amount(s) of bacteria in a milk sample can be determined by amplifying and quantifying portions of the respective bacterial chromosomes. In embodiments, the disclosure includes amplifying and sequencing of bacterial 16S rRNA from a plurality of bacteria types. Any suitable technique for determining polynucleotide sequences can be used, which include but are not necessarily limited to deep sequencing and pyrosequencing. Those skilled in the art will recognize how to quantify amplified nucleic acids and correlate that measurement with other parameters, such as amount of bacterial cells in a sample and/or the prevalence of any type or multiple types of bacteria in a sample, such as the percentage of *F. necrophorum* in a sample with a mixed population of bacteria types.

[0023] In addition to *F. necrophorum*, in embodiments, the bacteria types that are analysed can include but are not necessarily limited to any one or any combination of the bacteria types that are listed in the Tables of this disclosure. In embodiments, the presence, and/or type(s) and/or amount(s) of bacteria determined in performing a method of this disclosure include testing for at least 2, and up to and including 32 distinct bacteria types. In embodiments, at least two different strains of at least one bacteria type are analyzed. In embodiments, the presence and/or amounts of any combination of bacteria shown in Figure 2 can be tested. It will be recognized that Figure 2 and other portions of this disclosure provide GenBank accession numbers which are readily accessible by the public. Each nucleotide sequence and amino acid sequence associated with each GenBank accession number of the present disclosure is incorporated herein as they exist as of the priority date of this

application or patent.

[0024] The amount of *F. necrophorum* in the sample can be quantified using any suitable technique and can be compared to any suitable reference. The reference can be established in parallel with the test sample, can be pre-established or established at a later time. The reference can be a single value or a range of values. For example, a reference can be a standardized curve or an area on a graph. In a particular embodiment, a reference can be obtained using a known amount and/or a titration curve generated from amplification of *F. necrophorum* polynucleotide sequences such that a test measurement can be compared against the reference value to establish that the amount of *F. necrophorum* in any given sample is less than, more than, or equal to a threshold value, such as 3.3% of the total bacteria in the sample.

[0025] In embodiments, a threshold value of $\geq 3.3363\%$ *F. necrophorum* as a percentage of total bacteria in a milk sample is used to aid in a diagnosis or is itself a diagnosis of mastitis. Likewise, a threshold value of $< 3.3363\%$ *F. necrophorum* as a percentage of total bacteria in a milk sample tested as described herein is used to aid in a diagnosis or is itself a diagnosis that the bovine animal from which the sample was obtained does not have mastitis, and/or is used to identify a sample of milk as having been obtained from a bovine animal that either has, or does not have mastitis, as the case may be. In embodiments, the diagnosis of the bovine animal can be expressed and fixed in a tangible medium of expression, such as an electronic file, compact disk, or as a paper-based report. In embodiments, the disclosure includes communicating the result of the diagnosis to an animal health care provider, or an animal caretaker, or to a public health authority. In embodiments, the disclosure includes diagnosing one or more bovine animals as having mastitis and causing distribution of milk obtained from the one or more bovine animals to be prevented or stopped, and/or the milk to be destroyed. In embodiments, the disclosure includes diagnosing a bovine animal as having mastitis and administering to the bovine a veterinary composition as described herein, and/or administering one or more antibiotics to the bovine animal. In an embodiment, diagnosing a member of a group of bovine animals as having mastitis is followed by vaccination of additional members of the herd with a veterinary composition as disclosed herein.

[0026] It will be apparent from the foregoing that a threshold amount of *F. necrophorum* as a percentage of total bacteria in a milk sample is positively correlated with the presence or absence of mastitis. Given this novel finding, it is reasonable to expect that

reducing the presence of *F. necrophorum* in a bovine animal will result in a prophylactic effect towards mastitis. In this regard, the present disclosure includes compositions and methods for use as vaccines against bovine mastitis. The compositions will have the effect of reducing or eliminating *F. necrophorum* in the milk of a vaccinated bovine mammal, and/or
5 will result in a lessening or prevention of the classical symptoms of bovine mastitis that will be readily apparent to those skilled in the art, and/or will stimulate a cell-mediated and/or humoral immune response against the *F. necrophorum*.

[0027] In embodiments, the present disclosure includes compositions comprising whole cells of *Fusobacterium necrophorum* and/or one or more other immunogens. In
10 embodiments, the composition comprises a recombinant *Fusobacterium necrophorum* surface protein, and/or one or more *Fusobacterium necrophorum* proteins that would be expected and/or are known to be capable of stimulating an immune response in a bovine animal. In embodiments, the compositions comprise *Fusobacterium necrophorum* leukotoxin (LKT), or a combination thereof with whole *Fusobacterium necrophorum* cells.

15 With respect to LKT, it is known that it is highly toxic to bovine polymorphonuclear neutrophil (PMNs), and inducing apoptosis-mediated killing of them. However, we have determined LKT can be safely administered and stimulate an immune response in bovine animals that based on the present disclosure, can be expected to be prophylactic against mastitis. In embodiments, the composition comprises a veterinarily acceptable carrier,
20 excipient or diluent such that the composition is a veterinary composition. Suitable carriers, excipients and diluents for use with compositions intended for administration to animals are known in the art. Whole cells of other bacteria may be included in the veterinary composition. Further, other agents may optionally be included, such as Escherichia coli type 1 fimbrial adhesin (FimH), Trueperella pyogenes pyolysin (PLO), or combinations thereof.
25 The veterinary compositions may also comprise any other agents that would be expected to provide a therapeutic and/or prophylactic benefit to the recipient, such as antibiotics and/or adjuvants. Adjuvants that are suitable for use with veterinary compositions are well known in the art and can be included. In one embodiment, the adjuvant is aluminum hydroxide.

[0028] In an embodiment, the present disclosure includes an article of manufacture
30 comprising packaging and at least one sealed container. The sealed container comprises a veterinary composition comprising whole cells of *Fusobacterium necrophorum*, recombinant or isolated LKT, or a combination thereof, and may include additional components, such as other *F. necrophorum* immunogens, or immunogens obtained or derived from other types of

bacteria. The packaging comprises printed material providing an indication that the veterinary composition is for vaccination of a bovine mammal against bovine mastitis, and can include a description of subcutaneous administration.

[0029] The present disclosure also provides a method for stimulating an immune response against *F. necrophorum* for the purpose of prophylaxis of bovine mastitis. The method comprises administering to a female bovine mammal an effective amount of a composition comprising whole cells of *Fusobacterium necrophorum*, recombinant or isolated *Fusobacterium necrophorum* LKT, or a combination thereof. In embodiments, the administration stimulates an immunoglobulin response specific to an antigen expressed by the *F. necrophorum* cells. In connection with stimulating an immune response against *F. necrophorum* and/or a protein expressed by *F. necrophorum*, Figure 4 summarizes data which demonstrate stimulating an immune response in a bovine animal by vaccination against *F. necrophorum*. The data are provided as ELISA-detected serum IgG against *F. necrophorum*. Figure 4 is further described in the Examples.

[0030] The following specific examples are provided to illustrate the invention, but are not intended to be limiting in any way.

Example 1

[0031] This Example provides a description of the materials and methods used to obtain analyze bacteria in bovine milk samples.

[0032] **Sampling and microbiological culture** One hundred and thirty-six (136) milk samples were collected from cows with clinical or subclinical mastitis and sent to the Quality Milk Production Services (QMPS) laboratory at Cornell University for microbiological culture. For comparison purposes 20 milk samples obtained from healthy quarters from cows that had no history of mastitis and found to have a somatic cell count lower than 10,000 were also used. Samples were taken after teat ends had been disinfected with alcohol and the first streams of milk were discarded. Approximately 0.01 ml of each milk sample was inoculated using cotton swabs on trypticase soy agar plates containing 5% sheep blood and 0.1% esculin (bioMerieux, INC. Durham, NC 27704-0969 USA) and incubated aerobically at 37°C. Bacterial growth was identified after 24 and 48 h of incubation according to National Mastitis Council standards. Briefly, *Staphylococcus aureus* and *Staphylococcus* spp. were identified by haemolytic pattern and tube coagulate test. *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Streptococcus* spp. were differentiated

by presence or absence of esculin hydrolysis, Lancefield group C typing (PathoDx strep grouping latex agglutination test, Remel), and growth or growth inhibition on Bile Esculin Azide Agar (Enterococcosel™, Becton, Dickinson). *Escherichia coli* and *Klebsiella* spp. were identified using morphologic characteristics of colonies on MacConkey agar,

production of indole, motility, and utilization of citrate. *Trueperella pyogenes* was identified by colonial characteristic, presence of complete hemolysis and Gram stain. No mycoplasma culture or anaerobic culture was performed on the samples. Samples that showed a conclusive culture result with a single dominant pathogen or the absence of any growth in the aerobic culture process were selected for this study.

[0033] DNA extraction One millilitre of milk from the same sample that was used for bacterial culture was centrifuged for 10 min at room temperature at 13,200 rpm (16,100 rcf) in an Eppendorf 5415R centrifuge. The supernatant was discarded and the remaining pellet was resuspended in 400 µl of nuclease-free water. Isolation of genomic DNA was then performed by using a QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions, except that 400 µg of lysozyme was added to the bacterial suspension and incubated for 12 h at 56°C to maximize bacterial DNA extraction. DNA concentration and purity were evaluated by optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at wavelengths of 230, 260 and 280 nm.

[0034] PCR amplification of the V1-2 region of bacterial 16S rRNA genes The 16S rRNA genes were individually amplified from each sample using a composite pair of primers containing a unique 10-base barcode, which was used to tag the PCR products from the respective samples. The forward primer was 5'-
CGTATCGCCTCCCTCGCGCCATCAGNNNNNNNNNNTCAGAGTTTGATCCTGGCT
CAG-3' (SEQ ID NO:1); the bold sequence is the GS FLX Titanium Primer A, and the italicized sequence is the universal broadly conserved bacterial primer 27F. The reverse primer was 5'-**CTATGCGCCTTGCCAGCCCGCTCAGNNNNNNNNNN**
CATGCTGCCTCCCGTAGGAGT-3' (SEQ ID NO:2); the bold sequence is the GS FLX Titanium Primer B, and the italicized sequence is the broad-range bacterial primer 338R. The sequence NNNNNNNNNN, which is identical in the forward and reverse primer of each pair, designates the unique 10-base barcode used to tag each PCR product. A two-base linker sequence (underlined) was inserted between the barcode and the template-specific sequence to help diminish any effect the composite primer might have on the efficiency of the amplifications. The specific pair of primers used was checked against the bovine genome

with NCBI primer-BLAST and was not found to anneal with bovine DNA. PCRs were carried out in triplicate 20- μ l reactions containing 0.3 μ M forward and reverse primers, using approximately 50 ng of template DNA and 10 μ l HotStar Taq Plus Mix kit (Qiagen). A modified touchdown thermal cycling was used for amplification and consisted of initial
5 denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing (starting at 68°C and subsequently decreased by 2°C/2 cycles until it reached 58°C at which temperature the 20 remaining cycles were performed) for 30 sec, extension at 72°C for 60 sec, and a final extension at 72°C for 7 min. Replicate amplicons were pooled, purified with a QIAquick PCR Purification Kit (Qiagen), and visualized by electrophoresis using
10 1.2% (wt/vol) agarose gels stained with 0.5 μ g/ml ethidium bromide before sequencing. Blank controls, in which no DNA was added to the reaction, were performed. In all cases these blank controls failed to produce visible PCR products; these samples were not analysed further.

[0035] Barcoded pyrosequencing of bacterial 16S rRNA genes Amplicons were
15 quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) and combined in equimolar ratios into a single tube with a final concentration of 16 ng/ μ l. Pyrosequencing of the samples was carried at the Cornell University Life Sciences Core Laboratories Center using Roche 454 GS-FLX System Titanium Chemistry.

[0036] Sequences library analysis Sorting by tag sequence, trimming and quality
20 control of sequences that derived from pyrosequencing, were done by Geneious. One mismatch was allowed in the barcode, while two mismatches were allowed in the primers. Primers were removed from the sequences, zero N's were allowed while sequences shorter than 250 bp were also removed. The Ribosomal Database Project (RDP) Classifier at the RDP's Pyrosequencing Pipeline was used to assign 16S rRNA gene sequences of each
25 sample to the new phylogenetically consistent higher-order bacterial taxonomy, using an 80% confidence threshold, providing information regarding different genera prevalence in each sample. Different genera prevalence in each sample derived from this analysis were used as covariates in a discriminant analysis that was performed in JMP Pro (SAS Institute Inc. North Carolina). The culture-based diagnosis was used as the categorical variable in this analysis.
30 To facilitate a detailed (species level) analysis of the sequences, the following steps were followed: 200 sequences from each sample with the same culture-based diagnosis were randomly selected, using the random number function of Excel, and used to create a new FASTA sequence file. This file was then processed through the RDP pyrosequencing

pipeline. Specifically, the file was first uploaded in the pipeline initial processor that trimmed the 16S primers and filtered out additional sequences of low-quality. The produced file was uploaded in the RDP's aligner, which aligns the sequences using the INFERNAL aligner, a Stochastic Context Free Grammar (SCFG)-based, secondary-structure aware aligner, and then processed by the complete linkage clustering tool (that clustered the aligned sequences in OTU). Finally, the dereplicate function was used to create one representative sequence for each OTU. Eventually, a new file of representative sequences was created. The Basic Local Alignment Search Tool (BLASTn algorithm) from the National Center for Biotechnology Information (NCBI) web pages (<www.ncbi.nlm.nih.gov/BLAST/>) was then used to examine the nucleotide collection (EMBL/GenBank/DDBJ/PDB) databases for sequences with high similarity to these representative sequences.

[0037] The cluster file that was obtained from the above described process was subsequently used for the evaluation of the samples richness and diversity through the estimation of Shannon and Chao1 indices, again using the RDP pyrosequencing pipeline. The Shannon index is a nonparametric diversity index that combines estimates of richness (the total number of OTUs) and evenness (the relative abundance of OTUs). For example, communities with one dominant species have a low index, whereas communities with a more even distribution have a higher index. Chao1 is a nonparametric estimator of the minimum richness (number of OTUs) and is based on the number of rare OTUs (singletons and doublets) within a sample.

Example 2

[0038] This Example provides a description of results obtained using the materials and methods described in Example 1.

[0039] To study the evolutionary relationships among the ten most predominant sequences from samples characterized as *Staphylococcus spp.* mastitis, the sequences obtained were imported into Geneious software and aligned to other 16S rRNA gene sequences using ClustalW. The alignment was further manually corrected, and calculation of the phylogenetic trees was based on these sequence alignments using the neighbour-joining algorithm. Evolutionary distances were computed using the Jukes-Cantor method.

[0040] **Diagnosis of mastitis etiology by bacterial culture** Based on bacterial culture of milk samples from 136 mastitic dairy cows, 16 cases were diagnosed as infected with *Streptococcus uberis*, 17 as *Escherichia coli*, 11 as *Klebsiella spp.*, 17 as *Staphylococcus aureus*, 20 as *Staphylococcus spp.*, 17 as *Streptococcus dysgalactiae*, 2 as *Streptococcus spp.*,

3 as *Trueperella pyogenes* , and 33 samples were characterized as culture negative. The 20 milk samples obtained from healthy cows were also found to be culture negative.

[0041] **Classification of bacterial species in milk from mastitic cows based on 16S rRNA gene sequences.** The same 156 milk samples were also subjected to metagenomic

5 pyrosequencing of bacterial 16S rRNA genes. Pyrosequencing produced 283,713 sequences; their size ranged from 36 to 1102 bp. The sequences that were finally analysed by the RDP classifier (after trimming and quality control) were 240,524; their size ranged from 250 to 504 bp. To facilitate comparison, the sequencing results have been grouped according to the culture-based mastitis diagnosis; sequences derived from the milk samples obtained from
10 healthy cows were also grouped separately.

[0042] Discriminant analysis showed that the groups of samples that were most clearly different from the rest and thus easily discriminated were the normal milk samples from healthy cows and those characterised by culture as *Trueperella pyogenes* and *Streptococcus* spp. The mean prevalence, by different culture-based diagnosis, of bacterial
15 genera that were found to be significant for the discriminant analysis that used only groups of samples from mastitic cows is presented in Figure 1.

[0043] Tables 2-11 in the supplemental material, list the species-level information (with GenBank accession numbers and percentages of identity match) for each different group of samples. Prevalence in these tables is defined as the number of sequences that were
20 found to belong to each specific Operational Taxonomic Unit (OTU) out of the total number of sequences analyzed for each group of samples. The mastitis causative agent diagnosed by culture was generally among the organisms most frequently detected by pyrosequencing, and in three cases (*Escherichia coli*, *Klebsiella* spp. and *Streptococcus uberis* mastitis) it was the single most prevalent microorganism. *Trueperella pyogenes* sequences were the second most
25 prevalent sequences in mastitis cases diagnosed as *T. pyogenes* by culture, *Streptococcus dysgalactiae* sequences were the second most prevalent sequences in mastitis cases diagnosed as *S. dysgalactiae* by culture, and *Staphylococcus aureus* sequences were the third most prevalent in mastitis cases diagnosed as *S. aureus* by culture.

[0044] Shannon and Chao1 indices estimates for different distance cut-off values
30 (0.01, 0.03 and 0.05) are presented in Table 1.

[0045] Since species level results regarding samples diagnosed as *Staphylococcus* spp. by classical culture were unexpected, a phylogenetic tree of the ten most predominant sequences in the samples characterized as *Staphylococcus* spp. mastitis was built to provide

additional information and is presented in Figure 2.

[0046] The bacteria diagnosed by aerobic culture generally corresponded to the most frequent bacterial sequences detected by pyrosequencing, as shown in Tables 2-11.

However, along with this confirmation came important additional information.

5 [0047] Sequences classified by the RDP classifier as *Escherichia/Shigella* spp., *Raoultella* spp. and *Arcanobacterium* spp., were mainly prevalent in the groups of samples characterized by culture as *Escherichia coli*, *Klebsiella* spp. and *Trueperella pyogenes* respectively. *Streptococcus* spp. showed higher prevalence in the groups characterized as *Streptococcus* spp., *Streptococcus uberis* and *Streptococcus dysgalactiae*. However,
10 *Streptococcus* spp. was also prevalent in all other groups of samples, even in the milk samples derived from healthy cows. A similar observation was made for *Staphylococcus* spp. Interestingly, it has been reported that *Staphylococcus* spp. and *Streptococcus* spp. were part of the core microbiome of non-mastitic human milk samples.

[0048] Discriminant analysis showed that the microbiota of samples derived from
15 healthy cows was clearly different from the microbiota of the mastitic samples. Discriminant analysis performed using only the mastitic samples showed that the groups of samples that were most clearly different from the rest and thus easily discriminated were the ones characterised as *Trueperella pyogenes* and *Streptococcus* spp. These were also the groups of samples that showed the lowest diversity having the lowest Shannon and Chao1 indices.

20 *Streptococcus dysgalactiae* samples were found to group together with *Escherichia coli* samples, and culture negative samples grouped with *Staphylococcus* spp. samples. When *T. pyogenes* and *Streptococcus* spp. samples were not included in the discriminant analysis, *Staphylococcus* spp. samples grouped with culture negative samples and *Streptococcus dysgalactiae* samples with *E. coli* samples, while the remaining groups were well
25 discriminated. As shown in Figure 1, not all genera that were found to be highly prevalent in most samples were also significant for the discriminant analysis. While *Fusobacterium* spp. (prevalent in most samples) was highly significant for the discriminant analysis, *Asticcacaulis* spp. (a genus also shown to be prevalent in most samples, and highly prevalent in samples that were culture negative) was not. We were able to identify high numbers of anaerobic
30 bacterial sequences in all mastitis cases, regardless of the culture-based diagnosis. Milk samples from cases that were diagnosed as *T. pyogenes* mastitis in this study were also found to have a high prevalence of DNA sequences from *F. necrophorum subsp. funduliforme*. On the other hand, *F. necrophorum* sequences were practically absent in the 20 samples that were

derived from healthy, low somatic cell count quarters, while *Porphyromonas* spp. sequences were detected but in low prevalence comparing to their prevalence in the mastitic samples.

[0049] In the 33 samples that were identified by classical aerobic culture techniques as culture negative, pyrosequencing was able to detect sequences from bacteria that are known to cause bovine mastitis; this included *Streptococcus uberis*, *Trueperella pyogenes*, and *Escherichia coli*. *Streptococci* were prevalent in almost all the culture negative samples. Two of the culture negative samples showed a very high proportion of *Streptococci* and further evaluation of the individual sequences showed a predominance of *S. uberis* in these samples.

[0050] The most prevalent bacterial sequences in the culture negative samples were from *Caulobacter leidyia* in the family Sphingomonadaceae. The same bacterium was also prevalent (in lower numbers) in most of the milk samples with a positive bacterial identification, while it was also prevalent (in even lower numbers) in samples derived from healthy cows. Since *C. leidyia* is an aquatic bacterium its presence in milk samples might be a result of water contamination during the sampling process, or it may be present in high numbers in the milking parlor, where it may have opportunity to invade the udder when the cow's defences are compromised due to mastitis.

[0051] There were a number of samples where a mastitis pathogen was identified by culture, while pyrosequencing revealed the presence of other known mastitis pathogens. For example, in a number of samples diagnosed by bacterial culture as *Klebsiella* spp. or *Escherichia coli*, DNA of *Streptococcus uberis* was also detected by pyrosequencing. Pyrosequencing results regarding mastitis cases diagnosed by culturing as *Streptococcus* spp. showed that the most prevalent representative sequence belonged to *Streptococcus macedonicus*. On the other hand, *S. macedonicus* sequences were not detected in the milk samples obtained from healthy cows. This microorganism has not up to now been correlated with mastitis.

[0052] In samples characterized by culture as *Staphylococcus* spp., *Staphylococcus equorum* was detected by pyrosequencing. *Staph. equorum* sequences were not detected in the healthy milk samples. Surprisingly, only 1.8% of all identified sequences were associated with *Staphylococcus* spp. in this Example, and these were not necessarily the known dominant *Staphylococcus* spp. associated with bovine mastitis such as *Staph. chromogenes*. Results derived by discriminant analysis suggest that similarities exist between samples characterized as *Staphylococcus* spp. and culture negative samples. Evaluation of the genera

distribution in individual samples showed a remarkable similarity between culture negative samples and *Staphylococcus* species. This would suggest that the identified *Staphylococcus* species in classical culture form a small proportion of total bacterial DNA in the sample, resulting apparently in the minor pathology that is typically associated with this bacterial species.

[0053] *Helcococcus ovis* is a gram-positive, catalase-negative coccus that is associated with endocarditis in bovines. In the present study, *H. ovis* was prevalent in samples from culture negative, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus* spp. and *Streptococcus uberis* mastitis cases. However, *H. ovis* was also prevalent in the milk samples obtained from healthy cows.

[0054] As noted above, *Fusobacterium* spp. was highly significant for the discriminant analysis and *F. necrophorum* sequences were practically absent in the 20 samples that were derived from healthy, low somatic cell count quarters. We investigated the relationship between *F. necrophorum* and mastitis further using statistical analysis and unexpectedly determined that the presence and amount of *Fusobacterium necrophorum* in the milk is highly specific and sensitive for diagnosing the bovine mammal as having mastitis. Results of this analysis are shown in Figure 3, which depicts a receiver operating characteristic curve (ROC) illustrating the sensitivity (Y axis) and 100-specificity (X axis) of the % *Fusobacterium* spp. (percent of the overall bacterial population that was classified as *Fusobacterium* spp.) as a predictor of clinical mastitis. The data analyzed to produce the analysis summarized in Figure 3 was based on bacterial culture of milk samples from 136 mastitic dairy cows as described above, 16 cases were diagnosed as infected with *Streptococcus uberis*, 17 as *Escherichia coli*, 11 as *Klebsiella* spp., 17 as *Staphylococcus aureus*, 20 as *Staphylococcus* spp., 17 as *Streptococcus dysgalactiae*, 2 as *Streptococcus* spp., 3 as *Trueperella pyogenes*, and 33 samples were characterized as culture negative. Twenty milk samples obtained from healthy cows were also found to be culture negative. We analyzed the same 156 milk samples using metagenomic pyrosequencing of the bacterial 16S rRNA genes as also described in this Example. As can be seen from Figure 3, the results of this analysis reveal a threshold level of *F. necrophorum* to be highly predictive of clinical mastitis. Specifically, the data show that a % *Fusobacterium* spp. of ≥ 3.3363 is highly predictive of clinical mastitis with a Sensitivity of 83.8% and a Specificity of 100%. Thus, as will be recognized by those skilled in the art, 83.8% of the cows which were affected with mastitis had *Fusobacterium* spp. $\geq 3.3\%$ ($\geq 3.3363\%$ if determined to the fourth decimal

place), with an average of 8.96% (Standard deviation = 6.67). Further, 100% of the cows not affected with mastitis had % *Fusobacterium spp.* <3.3363. Thus, the Example provides a demonstration that clinical mastitis can be evaluated solely on the basis of determining an amount of *F. necrophorum* relative to the total bacteria in a milk sample.

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Example 3

[0055] This Example demonstrates stimulating an immune response in a bovine animal against *F. necrophorum* and *F. necrophorum* LKT, as well as other bacteria types and proteins. In particular, this Example discloses five vaccine formulations containing different combinations of proteins (FimH; leukotoxin, LKT; and pyolysin, PLO) and/or inactivated whole cells (*Escherichia coli*, *Fusobacterium necrophorum*, and *Trueperella pyogenes*).

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[0056] Inactivated whole cells were produced using two genetically distinct strains of each bacterial species (*E. coli*, *F. necrophorum*, and *T. pyogenes*). FimH and PLO subunits were produced using recombinant protein expression, and LKT was recovered from culturing a wild *F. necrophorum* strain. Three subcutaneous vaccines were formulated: Vaccine 1 was composed of inactivated bacterial whole cells and proteins; Vaccine 2 was composed of proteins only; and Vaccine 3 was composed of inactivated bacterial whole cells only. Two intravaginal vaccines were formulated: Vaccine 4 was composed of inactivated bacterial whole cells and proteins; and Vaccine 5 was composed of PLO and LKT. In general, vaccination induced a significant increase in serum IgG titers against all antigens, with subcutaneous vaccination again being more effective. Results for *F. necrophorum* are presented in Figure 3, which depicts the effect of vaccination on ELISA-detected serum IgG against *F. necrophorum*. The X-axis represents days relative to calving, while the Y-axis represents OD₆₅₀ of ELISA-detected serum IgG against. Standard errors of the means are represented by the error bars. The following Materials and Methods were used to obtain the data presented in this Example.

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[0057] Inactivated bacterial components. *E. coli* strains 4612-2 and 12714-2 were used for this Example. Strains were grown aerobically on Luria-Bertani (LB) broth (Sigma-Aldrich) at 37°C. They were inoculated with 1% of an overnight culture and grown in 800 ml of medium, with agitation (150 rpm). For strain 12714-2, cells were harvested at 4 h, with an OD₆₀₀ of 0.432 and 1.0×10^9 CFU/ml; for strain 4612-2, cells were harvested at 3.5 h, OD₆₀₀ of 0.473 and 1.2×10^9 CFU/ml. The cultures were inactivated with 0.1% formalin for 12 h, and the cells were concentrated 4-fold (final volume of 200 ml), so 0.25 ml of each strain would be present in the final vaccine formulation, with approximately 10^9 CFU per dose.

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[0058] *Trueperella pyogenes* strains 10481-8 and 6375-1 were isolated from the uterine lumen of dairy cows. Strains were grown on VersaTREK REDOX 1 (Trek Diagnostic Systems, OH) in 7% CO₂ at 37°C. Cells were harvested at 48 h, with 1.3×10^8 and 0.5×10^8 CFU/ml for strains 10481-8 and 6375-1, respectively. The cultures were inactivated with 0.1% formalin for 12 h, and 1 ml of each strain was added to the final vaccine formulation, with approximately 10^8 CFU per dose.

[0059] *Fusobacterium necrophorum* strains 5663 and 513 were isolated from the uterine lumen of dairy cows. Strains were grown on VersaTREK REDOX 2 (Trek Diagnostic Systems, OH) anaerobically at 37°C. All cultures were inactivated with 0.1% formalin for 12 h before the cells were concentrated. Cells were harvested at 12 h, with 1.6×10^{12} and 1.8×10^{12} CFU/ml for strains 513 and 5663, respectively. The cultures were inactivated with 0.1% formalin for 12 h, and 0.01 ml of each strain was added to the final vaccine formulation, with approximately 10^{10} CFU per dose.

[0060] **Recombinant protein expression and purification.** *Bacterial strain growth and induction conditions.* *E. coli* TOP10 (Invitrogen, NY) was grown either on LB agar or in LB broth (Sigma-Aldrich, MO) at 37°C. Ampicillin (50 µg/ml) was added as appropriate. *T. pyogenes* 49698 (American Type Culture Collection, VA) was grown on brain heart infusion (BHI) agar or in BHI broth (BD BBL, MD) supplemented with 5% defibrinated horse blood at 37°C and 7% CO₂.

[0061] For the preparation of His-tagged proteins (His-PLO or FimH₁₋₁₅₆-His), appropriate *E. coli* cultures were grown at 37°C with agitation (200 rpm) to an optical density at 600 nm of ~0.6. At this point, isopropyl 1-thio-β-D-galactopyranoside (IPTG; Sigma) was added to the cultures to 1 mM, which were further incubated with agitation for at least 3 h.

[0062] *DNA manipulation and constructs.* Standard procedures for *E. coli* transformation and plasmid extraction, DNA restriction, ligation, and agarose gel electrophoresis were performed. Primers were synthesized by IDT, and PCRs were performed in a GeneAmp PCR System 9700 (Applied Biosystems, CA). To confirm that no mutations were introduced by PCR, all DNA constructs were sequenced using an automated DNA sequencer (Cornell Biotechnology Resource Center, NY) and analyzed using LaserGene software (DNASTAR, WI).

[0063] *Cloning and purification of recombinant His-PLO.* The *PLO* gene, lacking the coding region for the predicted signal sequence, was amplified from *A. pyogenes* ATCC49698 genomic DNA by PCR with a 5' primer containing an *Xho*I site (5' -

ACAGCATCCTCGAGTGCCGGATTGGGAAAC-3' (SEQ ID NO:3) and a 3' primer containing an *EcoRI* site (5'-TGGAATTCCCTAGGATTTGACATTGT-3' (SEQ ID NO:4)). The 50- μ l reaction contained 1 $\times$ Pfx amplification buffer (Invitrogen) with 1 mM MgSO₄ (Invitrogen, NY), 0.3 mM of each dNTP, 0.3 μ M of each primer, 1 U of Platinum Pfx DNA polymerase (Invitrogen, NY), and approximately 50 ng of template DNA. The cycling parameters for amplification were: initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation (94°C for 1 min), annealing (58°C for 1 min), extension (72°C for 3 min), and a final extension at 72°C for 7 min. The 1.5-kb amplicon was digested with *XhoI-EcoRI* and cloned into *XhoI-EcoRI*-digested pTrcHisB (Invitrogen, NY).

[0064] After 3 h of induction, the cells were harvested by centrifugation at 10,000 $\times g$ for 10 min and the pellet was resuspended in 1 X Extraction/Wash Buffer (50 mM sodium phosphate, 300 mM NaCl) (pH 7.0). Lysozyme was added to a final concentration of 0.75 mg/ml and the mixture was incubated at 4°C with shaking for 30 min. The cells were disrupted by two passages through a French pressure cell (Amicon) at 20,000 psi (138 Mpa), and the insoluble material was removed by centrifugation at 12,000 $\times g$ for 30 min. His-PLO was purified from the soluble fraction with TALON metal affinity resin (Clontech, CA) according to the manufacturer's instructions. Isolated pure protein fraction was concentrated using a fiber concentration/desalting system using a filter with a molecular weight exclusion of 10 kDa (Amicon ultra 100K, Millipore, MA) and subjected to SDS-PAGE (15%) using the Mini-PROTEAN Tetra Cell electrophoresis system (Bio-Rad, CA), following standard protocols.

[0065] Protein concentration was determined by the Bradford method. A total of 30 liters of culture was grown to produce a total of 321.24 mg of His-PLO. The final volume of His-PLO was 41 ml and the final concentration was 7.83 mg/ml.

[0066] *Cloning and purification of recombinant FimH₁₋₁₅₆-His.* The portion of the *FimH* gene encoding the signal peptide and the first 156 amino acids (the mannose-binding lectin domain, LD) of the mature protein was amplified from plasmid pET-22b(+)-F3-LD, provided by Dr. Evgeni Sokurenko, University of Washington, WA. The 5' primer used contained a *BamHI* site (5'-CGCGGATCCATGAAACGTGTTATTACCCTG-3' (SEQ ID NO:5)) and the 3' primer contained a *HindIII* site (5'-CCCAAGCTTCTAGTGATGGTGATGGTGATGGCCGCCAGTAGGCACCAC-3' (SEQ ID NO:6)) and a six-histidine tag following the authentic sequence of the protein. The PCR components were as described for *PLO* gene amplification. The cycling parameters for

amplification were: initial denaturation for 5 min at 94°C, followed by 25 cycles of denaturation (94°C for 1 min), annealing (61°C for 1 min), extension (72°C for 3 min), and a final extension at 72°C for 7 min. The amplicon, approximately 0.6 kb, was digested with *Bam*HI-*Hind*III and cloned into *Bam*HI-*Hind*III-digested pTrcHisA (Invitrogen). After 5 h of induction, FimH₁₋₁₅₆-His purification was performed as described for *PLO*. A total of 92 liters of culture was grown to produce 216.34 mg of FimH₁₋₁₅₆-His. The final volume of FimH₁₋₁₅₆-His was 172.5 ml and the concentration was 1.25 mg/ml.

[0067] *Culture concentrated supernatant and affinity purification of Leukotoxin. F.*

necrophorum strain 6586 was grown in VersaTREK REDOX 2 for 12 h anaerobically at 37°C. The culture supernatant was concentrated at 4°C in a hollow fiber concentration/desalting system using a filter with a molecular weight exclusion of 100 kDa (Amicon ultra 100K, Millipore, MA). Affinity purification of LKT was performed to evaluate the concentration of LKT in the *F. necrophorum* 6586 culture concentrated supernatant. Briefly, purified mAb F7B10 (3.5 mg) was coupled to 5 ml of Affi-Gel 10 affinity support (Bio-Rad, CA) and packed in a 1 × 20 cm column. The *F. necrophorum* 6586 culture concentrated supernatant was applied to the column, and non-binding materials were removed by passing 15 mL of 0.5 M NaCl in PBS through the column. Purified LKT was eluted with 0.2 M glycine-HCl (pH 3.0), immediately neutralized with NaOH, and washed and concentrated using an Amicon ultra 10K. Purity of the toxin was determined by SDS-PAGE.

[0068] A total of 10 L of *F. necrophorum* 6586 was grown to produce 220 mL of concentrated supernatant containing 0.186 mg/ml of LKT. The presence and concentration of LKT in the concentrated supernatant was determined by affinity purification.

[0069] *Vaccine formulation.* Five different vaccine formulations were made: three subcutaneous vaccines (Vaccines 1-3) and two intravaginal vaccines (Vaccine 4-5). Vaccine 1 was composed of inactivated bacterial whole cells (*E. coli*, *T. pyogenes* and *F. necrophorum*) and proteins (FimH, PLO and LKT); Vaccine 2 was composed only of proteins (FimH, PLO and LKT); and Vaccine 3 was composed only of inactivated bacterial whole cells (*E. coli*, *T. pyogenes* and *F. necrophorum*). Vaccine 4 was composed of inactivated bacterial whole cells (*E. coli*, *T. pyogenes* and *F. necrophorum*) and proteins (FimH, PLO and LKT), and Vaccine 5 was composed only of proteins (PLO and LKT). The adjuvant for the subcutaneous vaccines was aluminum hydroxide (Rehydragel HPA, General Chemical, NJ). The adjuvant volume used in the subcutaneous vaccines was 25% of the final

vaccine volume. Aluminum hydroxide was added to each component separately, and it was gently stirred overnight. The adjuvant for the intravaginal vaccines was 20 µg/dose of Cholera toxin (List Biological Laboratories, Inc., CA).

[0070] *Treatment groups and Case definition.* Late pregnant heifers were enrolled on a weekly basis; inclusion criteria for enrollment were: 230 ± 3 days of pregnancy, 629 to 734 days of age and body condition score (BCS) greater than 2.5. Heifers that were visually lame were not included in the study. A total randomized field trial study design was used; heifers were randomly allocated into one of six different treatment groups using the random number function of Excel (Microsoft, Redmond, MA). A total of 371 pregnant heifers were enrolled in the study; 105, 54, 54, 53, 53 and 53 heifers were randomly allocated to the control, Vaccine 1, Vaccine 2, Vaccine 3, Vaccine 4 and Vaccine 5 groups, respectively. Heifers assigned to the vaccine groups received two doses of vaccine: at 230 ± 3 days of pregnancy and 260 ± 3 days of pregnancy.

[0071] To obtain serum samples, blood was collected from a coccygeal vein/artery using a Vacutainer tube without anticoagulant and a 20 gauge $\times$ 2.54 cm Vacutainer needle (Becton, Dickinson and Company, Franklin Lakes, NJ). All blood samples were transported to the laboratory on ice and spun in a centrifuge at $2,000 \times g$ for 15 min at 4°C; serum was harvested and frozen at - 80°C. Serum samples were collected at 230 ± 3 days of gestation, 260 ± 3 days of gestation, 1 ± 2 DIM, 6 ± 1 DIM and 35 ± 3 DIM.

[0072] *Enzyme-linked immunosorbant assays (ELISAs).* Portions of the antigens produced for preparation of vaccines were used in ELISAs. *E. coli* strains were pooled together as a single antigen. The same was done for *F. necrophorum* and *T. pyogenes* strains. Bovine serum samples were thawed and mixed before analysis. The selected ELISA protocols were as follows. ELISA micro-titer plates (Greiner Bio-One, Germany) were coated with PBS (Phosphate-Buffered Saline 10X, pH 7.4, Ambion®) containing either 0.295 µg/ml of FimH₁₋₁₅₆-His, 0.036 µg/ml of His-PLO, 0.186 µg/ml of LKT, 10^7 cells/ml of *E. coli*, 10^{10} cells/ml of *F. necrophorum*, and 10^7 cells/ml of *T. pyogenes* for anti-FimH, anti-LKT, anti-PLO, anti-*E. coli*, anti-*F. necrophorum*, and anti-*T. pyogenes* IgG assays, respectively. Binding of antigen to microtiter wells was carried out overnight at 4°C, non-specific binding sites were blocked with PBS containing 1% casein (Thermo Scientific, Rockford, IL). Dilutions of bovine serum samples were then added to the ELISA plates; serum samples were diluted in proportions of 1:1000, 1:5000, 1:5000, 1:150, 1:500, and 1:150 for anti-FimH, anti-LKT, anti-PLO, anti-*E. coli*, anti-*F. necrophorum*, and anti-*T.*

pyogenes IgG assays, respectively. The optimal antigen and antibody concentrations were determined by performing the quantitative ELISA protocol with varying concentrations. The serotype-specific antibody bound to the ELISA plate was detected with anti-bovine IgG antibody conjugated with horseradish peroxidase, diluted according to the manufacturer's instructions (Sigma Aldrich, St. Louis, MO), followed by addition of the substrate, 3,3',5,5'-tetramethylbenzidine –TMB (Sigma Aldrich, St. Louis, MO). The optical density of each well was measured after 20 min at 650 nm using an ELISA plate reader (Synergy HTmicroplate reader BioTek Instruments, VT). The amount of color produced was proportional to the amount of primary antibody bound to the proteins on the bottom of the wells. Between each step of the assay, the microtiter wells were aspirated and rinsed 3 times with washing solution (1X Phosphate Buffered Saline Tween-20).

Table 1. Chao1 and Shannon indices for different cutoff distances (0.01, 0.03 and 0.05) and for different group of samples

	Distance cutoff	Number of samples/ sequences analyzed	Clusters	Chao1	Shannon Index (H')
<i>Trueperella pyogenes</i>	0.01		75	144.46	3.30
	0.03	3/304	49	82.55	2.97
	0.05		44	74.30	2.84
<i>Escherichia coli</i>	0.01		597	2311.78	5.29
	0.03	20/1473	343	1207.39	4.51
	0.05		283	846.89	4.23
<i>Klebsiella pneumoniae</i>	0.01		304	909.36	4.94
	0.03	11/674	188	422.00	4.19
	0.05		163	279.91	4.04
<i>Culture negative</i>	0.01		1176	3399.11	5.64
	0.03	33/3008	591	1337.23	4.52
	0.05		472	916.54	4.35
<i>Staphylococcus aureus</i>	0.01		719	2453.41	5.36
	0.03	17/1862	386	892.95	4.48
	0.05		312	609.56	4.28
<i>Staphylococcus spp.</i>	0.01		676	2374.15	5.33
	0.03	20/1555	411	1015.20	4.55
	0.05		339	627.02	4.37
<i>Streptococcus dysgalactiae</i>	0.01		489	1754.64	5.13
	0.03	17/1263	280	599.22	4.42
	0.05		237	480.29	4.21
<i>Streptococcus spp.</i>	0.01		57	140.25	3.43
	0.03	2/151	46	104.00	3.11

	0.05		43	118.60	2.90
	0.01		461	1601.00	4.17
<i>Streptococcus uberis</i>	0.03	16/1487	273	681.49	3.57
	0.05		235	468.93	3.45
	0.01		641	2096.00	5.52
<i>Normal milk samples</i>	0.03	20/1307	427	864.27	4.98
	0.05		376	704.91	4.85

5 **Table 2.** Species level information (with GenBank Accession number, and identity match) for the predominant representative sequences in samples characterized as culture negative

Species	Accession No	Prevalence	Identity (%)
<i>Caulobacter leidyia</i>	GQ891704	20.55	98.9
<i>Uncultured Fusobacteria</i>	EF704825	5.49	100
<i>Geobacillus pallidus</i>	FJ808716	5.09	100
<i>Streptococcus uberis</i>	HQ326694	4.82	100
<i>Uncultured bacterium</i>	JF643239	3.32	100
<i>Propionibacterium acnes</i>	CP002409	2.76	100
<i>Uncultured bacterium</i>	EU289919	2.46	100
<i>Porphyromonas levii</i>	AB547664	2.09	100
<i>Uncultured Porphyromonas</i>	HM754526	1.46	99
<i>Staphylococcus equorum</i>	AB334773	1.40	99.7
<i>Swine manure</i>	AF445295	1.33	99.7
<i>Bacteroides heparinolyticus</i>	GQ422742	1.30	100
<i>Ureaplasma diversum</i>	NR_025878	1.23	99
<i>Paenibacillus borealis</i>	HM563046	1.03	99.4
<i>Uncultured Porphyromonas</i>	HM754526	1.00	100
<i>Uncultured bacterium</i>	EU290110	0.96	99.7
<i>Uncultured Prevotella</i>	GU905978	0.96	99.2
<i>Uncultured bacterium</i>	AM183009	0.70	95.6
<i>Prevotella spp.</i>	FJ848548	0.70	100
<i>Uncultured bacterium</i>	EU290098	0.66	100
<i>Histophilus somni</i>	AB176902.1	0.66	99
<i>Uncultured bacterium</i>	AB107461	0.63	99.3
<i>Bacillus spp.</i>	FR749853	0.63	100
<i>Uncultured bacterium</i>	EF205694	0.60	100
<i>Ochrobactrum pseudogrignonense</i>	FJ859687	0.57	99.6
<i>Helcococcus ovis</i>	NR_027228	0.53	99.7
<i>Corynebacterium falsenii</i>	AF537594	0.53	100
<i>Uncultured bacterium</i>	HM316969	0.47	99.7
<i>Trueperella pyogenes</i>	JN578133	0.43	100
<i>Uncultured bacterium</i>	GU601118	0.40	100
<i>Delftia spp.</i>	CP002735	0.40	100
<i>Escherichia coli</i>	CP001671	0.37	100
<i>Uncultured Ruminococcaceae</i>	EU794142	0.37	99.7
<i>Mycoplasma bovis</i>	AY121098	0.37	99.7
<i>Xanthomonas campestris</i>	CP002789	0.33	100
<i>Uncultured bacterium</i>	HM257593	0.33	95.1
<i>Uncultured Bacteroidetes</i>	FM252970	0.33	100
<i>Brevibacillus parabrevis</i>	JN315628	0.33	99.7
<i>Uncultured Staphylococcus</i>	JN082690	0.33	100

Table 3. Species level information (with GenBank Accession number, and identity match) for the predominant representative sequences in samples characterized as *Trueperella pyogenes* mastitis

Species	Accession No	Prevalence	Identity (%)
<i>Fusobacterium necrophorum</i> subsp. <i>Funduliforme</i>	AB525413.1	21.05	100
Uncultured bacterium	EU290118.1	17.76	100
Uncultured bacterium	EU290137.1	13.16	99
<i>Trueperella pyogenes</i>	JN578141.1	11.20	100
Uncultured bacterium	GQ467006.1	4.28	99
Uncultured bacterium	JF643239.1	3.29	100
<i>Porphyromonas</i> sp.	FJ848565.1	2.63	99
Uncultured alpha proteobacterium	EU810967.1	2.30	100
<i>Ureaplasma diversum</i>	NR_025878.1	2.30	99
<i>Staphylococcus equorum</i>	AB334773.1	1.97	100
<i>Mycoplasma bovis</i>	AY121109.1	1.64	100
<i>Geobacillus pallidus</i>	HM030740.1	1.64	99
Uncultured <i>Prevotella</i> spp.	GU905978.1	1.32	99
<i>Paenibacillus caespitius</i>	AM745263.1	1.32	98
<i>Porphyromonas levii</i>	FJ822532.1	1.32	100
<i>Bacteroides heparinolyticus</i>	GQ422742.1	0.99	100
Uncultured bacterium	JF212531.1	0.66	100
<i>Paenibacillaceae</i> bacterium	FM173657.1	0.66	99
<i>Prevotella</i> spp.	FJ848548.1	0.66	100
Uncultured bacterium	EU466510.1	0.66	98
Uncultured bacterium	AM183009.1	0.66	94
Uncultured bacterium	JF532102.1	0.66	86
<i>Bacteroides pyogenes</i>	AB542769.1	0.66	99
Uncultured bacterium	DQ791376.1	0.66	99
Uncultured bacterium	GU608976.1	0.33	97
Uncultured <i>Peptostreptococcus</i> spp.	EU029258.1	0.33	99
<i>Pseudomonas fluorescens</i>	AM779082.1	0.33	99
Uncultured bacterium	EU472381.1	0.33	99
Uncultured bacterium	AB107469.1	0.33	100
Uncultured bacterium	FJ685469.1	0.33	99
Uncultured bacterium	JN021854.1	0.33	99
<i>Paenibacillus</i> spp.	AB505863.1	0.33	99
<i>Corynebacterium falsenii</i>	AF537594.1	0.33	99
Uncultured bacterium	GQ449199.1	0.33	99
Uncultured bacterium	GU605695.1	0.33	99
Uncultured bacterium	GU602554.1	0.33	99
Uncultured bacterium	FM253079.1	0.33	99
<i>Psychrobacter marincola</i>	AY292940.1	0.33	98
Uncultured bacterium	EF205694.1	0.33	100
Uncultured bacterium	HM318928.1	0.33	95
<i>Histophilus somni</i>	AB176902.1	0.33	99
Uncultured <i>Porphyromonas</i> spp.	HM754526.1	0.33	96
Uncultured bacterium	EU290137.1	0.33	94
Uncultured bacterium	GU612267.1	0.33	100
Uncultured bacterium	GU616751.1	0.33	100
Uncultured <i>Clostridia</i> bacterium	HM111527.1	0.33	87
Uncultured bacterium	GQ466866.1	0.33	99

Table 4. Species level information (with GenBank Accession number, and identity match) for the predominant representative sequences in samples characterized as *Escherichia coli* mastitis

Species	Accession No	Prevalence	Identity (%)
<i>Escherichia coli</i>	CP003034.1	10.12	100
<i>Fusobacterium necrophorum</i> subsp. <i>funduliforme</i>	AB525413.1	8.21	99
<i>Caulobacter leidyia</i>	GQ891705.1	7.67	99
<i>Porphyromonas levii</i>	AB547664.1	3.87	100
<i>Streptococcus uberis</i>	HQ326695.1	3.73	100
Uncultured bacterium	JF643239.1	2.78	99
Uncultured bacterium	JF663845.1	2.78	98
Uncultured bacterium	EF205686.1	2.58	99
<i>Staphylococcus equorum</i> subsp. <i>equorum</i>	FR691468.1	2.51	100
Uncultured <i>Porphyromonas</i> spp.	HM754526.1	2.31	100
Uncultured bacterium	AM183009.1	2.24	95
Uncultured <i>Porphyromonas</i> spp.	HM754526.1	2.17	99
<i>Ureaplasma diversum</i>	NR_025878.1	1.97	99
Uncultured bacterium	EU290118.1	1.97	100
<i>Ureaplasma diversum</i>	NR_025878.1	1.97	99
Uncultured bacterium	GU629689.1	1.22	100
<i>Bacteroides heparinolyticus</i>	GQ422742.1	1.15	100
Uncultured <i>Prevotella</i> spp.	GU905978.1	1.15	99
<i>Rumen bacterium</i>	HM597702.1	0.95	100
Uncultured <i>Propionibacterium</i> spp.	HQ891164.1	0.95	100
Uncultured bacterium	HM318928.1	0.88	95
Uncultured bacterium	FJ675143.1	0.81	99
<i>Paenibacillus</i> spp.	GU733397.1	0.81	99
<i>Clostridium perfringens</i>	AB627081.1	0.68	100
<i>Ochrobactrum pseudogrignonense</i>	FJ859687.2	0.68	99
<i>Helcococcus ovis</i>	AB542088.1	0.54	100
<i>Mycoplasma bovis</i>	AY121109.1	0.54	98
<i>Histophilus somni</i>	AB176910.1	0.48	100
<i>Peptostreptococcus anaerobius</i>	AB640695.1	0.48	97
Uncultured bacterium	EU458333.1	0.48	99
Uncultured bacterium	FJ682454.1	0.48	99
Uncultured <i>Firmicutes</i>	FR749723.1	0.48	93
Uncultured <i>Paludibacter</i> spp.	EU794247.1	0.41	100
Uncultured bacterium	FJ683872.1	0.41	100
<i>Prevotella</i> spp.	FJ848548.1	0.41	99
Uncultured bacterium	HM317008.1	0.41	100
Uncultured bacterium	JN575965.1	0.41	100
<i>Streptococcus pasteurianus</i>	JN581988.1	0.41	99
<i>Corynebacterium falsenii</i>	AF537594.1	0.34	99
Uncultured bacterium	AM183108.1	0.34	92
Uncultured bacterium	GU629031.1	0.34	100
Uncultured bacterium	JF905989.1	0.34	99

Table 5. Species level information (with GenBank Accession number, and identity match) for the predominant representative sequences in samples characterized as *Klebsiella pneumoniae* mastitis

Species	Accession No	Prevalence	Identity (%)
<i>Klebsiella pneumoniae</i>	AB675600.1	13.20	100
<i>Streptococcus uberis</i>	HQ326695.1	10.39	100
<i>Caulobacter leidyia</i>	GQ891705.1	5.19	100
<i>Fusobacterium necrophorum</i> subsp. <i>Funduliforme</i>	AB525413.1	4.45	100
<i>Uncultured Bacteroides</i> spp.	GU145757.2	3.12	100
<i>Uncultured bacterium</i>	FJ682454.1	3.12	99
<i>Geobacillus pallidus</i>	HM030740.1	2.37	99
<i>Uncultured bacterium</i>	GU629689.1	2.37	100
<i>Uncultured bacterium</i>	JF663845.1	2.08	98
<i>Uncultured Porphyromonas</i> spp.	HM754526.1	1.93	99
<i>Porphyromonas levii</i>	AB547664.1	1.93	100
<i>Paenibacillus borealis</i>	HM563046.1	1.78	99
<i>Staphylococcus equorum</i> subsp. <i>equorum</i>	FR691468.1	1.63	100
<i>Uncultured bacterium</i>	JF643239.1	1.48	100
<i>Uncultured bacterium</i>	FJ675143.1	1.34	99
<i>Prevotella</i> spp.	FJ848548.1	1.34	99
<i>Uncultured bacterium</i>	EU290118.1	1.34	100
<i>Bacteroides heparinolyticus</i>	GQ422742.1	1.04	100
<i>Ureaplasma diversum</i>	NR_025878.1	0.89	99
<i>Lactobacillus reuteri</i>	JN092132.1	0.89	99
<i>Uncultured Clostridium</i> spp.	HM235660.1	0.89	98
<i>Uncultured bacterium</i>	HQ701538.1	0.89	99
<i>Uncultured Bacteroides</i> spp.	EU289111.1	0.89	100
<i>Uncultured bacterium</i>	JN575965.1	0.74	100
<i>Clostridium perfringens</i>	FJ215342.1	0.74	100
<i>Clostridiales bacterium</i>	HQ452852.1	0.59	99
<i>Bacteroides fragilis</i>	FQ312004.1	0.59	100
<i>Fusobacterium necrophorum</i> subsp. <i>funduliforme</i>	AB525413.1	0.45	99
<i>Uncultured bacterium</i>	JF794810.1	0.45	98
<i>Uncultured Gram-positive bacterium</i>	AB191022.1	0.45	100
<i>Uncultured bacterium</i>	JN559616.1	0.45	92
<i>Uncultured bacterium</i>	FJ683872.1	0.45	100
<i>Uncultured bacterium</i>	JN575958.1	0.45	99
<i>Bacillus</i> spp.	AM491453.2	0.45	100
<i>Uncultured bacterium</i>	JF237709.1	0.45	99
<i>Uncultured bacterium</i>	FJ683700.1	0.45	99
<i>Uncultured bacterium</i>	EU460092.1	0.45	97
<i>Enterococcus faecalis</i>	JN644614.1	0.45	100
<i>Uncultured Prevotella</i> spp.	GU905979.1	0.45	98
<i>Uncultured bacterium</i>	GU614672.1	0.45	98
<i>Uncultured Ruminococcus</i> spp.	HM235655.1	0.45	99
<i>Uncultured bacterium</i>	GQ449096.1	0.45	100
<i>Streptococcus dysgalactiae</i> subsp. <i>dysgalactiae</i>	EF151154.1	0.45	99

Table 6. Species level information (with GenBank Accession number, and identity match) for the predominant representative sequences in samples characterized as *Staphylococcus aureus* mastitis

Species	Accession No	Prevalence	Identity (%)
<i>Caulobacter leidyia</i>	GQ891705.1	11.12	99
<i>Fusobacterium necrophorum</i> subsp. <i>funduliforme</i>	AB525413.1	6.44	100
<i>Staphylococcus aureus</i> subsp. <i>aureus</i>	FR821779.1	6.39	100
<i>Uncultured bacterium</i>	JF643239.1	5.96	100
<i>Uncultured bacterium</i>	FJ657725.1	4.19	100
<i>Porphyromonas levii</i>	FJ822532.1	3.28	100
<i>Uncultured bacterium</i>	JF663845.1	3.11	98
<i>Uncultured Prevotella</i> spp.	GU905978.1	2.15	99
<i>Propionibacterium acnes</i>	CP003084.1	2.15	100
<i>Uncultured Porphyromonas</i> spp.	HM754526.1	2.15	98
<i>Uncultured Porphyromonas</i> spp.	HM754526.1	1.99	100
<i>Uncultured bacterium</i>	AM183009.1	1.88	95
<i>Streptococcus uberis</i>	HQ326695.1	1.61	100
<i>Swine manure pit bacterium</i>	AF445295.1	1.40	99
<i>Staphylococcus equorum</i> subsp. <i>linens</i>	NR_041926.1	1.40	100
<i>Bacteroides heparinolyticus</i>	GQ422742.1	1.34	100
<i>Uncultured Bacteroides</i> spp.	EU289111.1	1.29	100
<i>Uncultured Clostridiales</i> bacterium	HM080230.1	1.24	94
<i>Paenibacillus borealis</i>	HM563046.1	1.18	98
<i>Ureaplasma diversum</i>	NR_025878.1	1.13	99
<i>Prevotella</i> spp.	FJ848548.1	0.97	100
<i>Uncultured bacterium</i>	HM317008.1	0.86	100
<i>Uncultured bacterium</i>	EU290118.1	0.86	100
<i>Helcococcus ovis</i>	AB542088.1	0.70	100
<i>Mycoplasma bovis</i>	AY121109.1	0.64	99
<i>Uncultured bacterium</i>	EU290005.1	0.59	100
<i>Histophilus somni</i>	AB176913.1	0.54	100
<i>Halomonas desiderata</i>	AB362300.1	0.48	100
<i>Corynebacterium falsenii</i>	AF537594.1	0.43	100
<i>Ochrobactrum pseudogrignonense</i>	FJ859687.2	0.43	100
<i>Trueperella pyogenes</i>	JN578112.1	0.43	99
<i>Uncultured bacterium</i>	JN230113.1	0.38	99
<i>Bacillus psychrodurans</i>	GU385871.1	0.38	99
<i>Escherichia coli</i>	JN180967.1	0.38	100
<i>Uncultured bacterium</i>	GU614651.1	0.38	99
<i>Uncultured bacterium</i>	GU617155.1	0.38	100
<i>Uncultured bacterium</i>	FJ684368.1	0.38	99
<i>Uncultured bacterium</i>	EU458333.1	0.32	98
<i>Uncultured bacterium</i>	GU600588.1	0.32	98
<i>Streptococcus dysgalactiae</i> subsp. <i>dysgalactiae</i>	EF151154.1	0.32	100

Table 7. Species level information (with GenBank Accession number, and identity match) for the predominant representative sequences in samples characterized as *Staphylococcus* spp. mastitis

Species	Accession No	Prevalence	Identity (%)
<i>Caulobacter leidyia</i> (OTU1)	GQ891705.1	18.91	98
<i>Fusobacterium necrophorum</i> subsp. <i>Funduliforme</i> (OTU2)	AB525413.1	5.14	100
Uncultured bacterium (OTU3)	JF643239.1	4.82	100
<i>Geobacillus pallidus</i> (OTU4)	HM030740.1	3.86	99
Uncultured bacterium (OTU5)	JF663845.1	3.60	98
<i>Porphyromonas levii</i> (OTU6)	AB547664.1	2.25	100
Uncultured bacterium (OTU7)	FJ675143.1	1.93	99
Uncultured <i>Prevotella</i> spp. (OTU8)	GU905979.1	1.80	98
<i>Staphylococcus equorum</i> subsp. <i>linens</i> (OTU9)	NR_041926.1	1.80	100
<i>Propionibacterium acnes</i> (OTU10)	CP003084.1	1.74	100
Uncultured <i>Porphyromonas</i> spp.	HM754526.1	1.41	100
<i>Bacteroides heparinolyticus</i>	GQ422742.1	1.41	100
<i>Paenibacillus borealis</i>	HM563046.1	1.35	98
<i>Rumen bacterium</i>	HM597702.1	1.16	100
Uncultured <i>Porphyromonas</i> spp.	HM754526.1	1.09	98
<i>Ureaplasma diversum</i>	NR_025878.1	1.03	98
Uncultured bacterium	AM183009.1	0.90	95
Uncultured bacterium	EU845721.1	0.84	99
<i>Ochrobactrum pseudogrignonense</i>	FJ859687.2	0.84	99
<i>Prevotella</i> spp.	FJ848548.1	0.84	100
Uncultured bacterium	HM317008.1	0.71	100
Uncultured bacterium	HM318928.1	0.64	95
<i>Streptococcus uberis</i>	HQ326695.1	0.58	100
Uncultured bacterium	EU290118.1	0.58	100
Uncultured bacterium	FN436078.1	0.51	96
<i>Escherichia coli</i>	CP003034.1	0.51	100
Uncultured bacterium	GU616649.1	0.51	99
Uncultured bacterium	GU608491.1	0.51	99
Uncultured bacterium	GU602110.1	0.45	98
Uncultured bacterium	EU464157.1	0.45	99
<i>Histophilus somni</i>	AB176913.1	0.45	100
<i>Helcococcus ovis</i>	AB542088.1	0.45	99
<i>Mycoplasma bovis</i>	AY121109.1	0.39	100
Uncultured <i>Bacteroides</i>	FM252970.1	0.39	100
Uncultured bacterium	JF810468.1	0.39	100
<i>Corynebacterium falsenii</i>	AF537594.1	0.32	100
<i>Paenibacillus caespitis</i>	AM745263.1	0.32	99
Uncultured bacterium	GQ449221.1	0.32	98
<i>Pseudomonas saccharophila</i>	AF368755.1	0.32	100
<i>Enterococcus</i> spp.	AF445307.2	0.32	99
Uncultured bacterium	FN994146.1	0.32	93
<i>Psychrobacter aquaticus</i>	NR_042206.1	0.32	100
Uncultured bacterium	EU458333.1	0.32	99
Uncultured <i>Bacteroidales</i>	EU794077.1	0.32	98
Uncultured bacterium	GU608708.1	0.32	99
<i>Arthrobacter</i> spp.	AM260537.1	0.32	100
Uncultured bacterium	AM982606.1	0.32	98
Uncultured <i>Lactococcus</i> spp.	GQ464389.1	0.32	98

Table 8. Species level information (with GenBank Accession number, and identity match) for the predominant representative sequences in samples characterized as *Streptococcus dysgalactiae* mastitis

Species	Accession No	Prevalence	Identity (%)
<i>Streptococcus uberis</i>	HQ326695.1	6.97	100
<i>Streptococcus dysgalactiae subsp. dysgalactiae</i>	EF151154.1	6.25	100
<i>Uncultured bacterium</i>	JF643239.1	5.46	99
<i>Porphyromonas levii</i>	AB547664.1	5.07	99
<i>Uncultured bacterium</i>	FJ657725.1	4.35	100
<i>Uncultured Fusobacteria</i>	EF704825.1	4.35	100
<i>Caulobacter leidyia</i>	GQ891705.1	4.12	100
<i>Uncultured Porphyromonas spp.</i>	HM754526.1	4.04	100
<i>Uncultured bacterium</i>	JF663845.1	3.01	98
<i>Propionibacterium acnes</i>	CP003084.1	2.69	100
<i>Uncultured bacterium</i>	AM183009.1	2.45	95
<i>Uncultured Bacteroides spp.</i>	EU289070.1	2.06	99
<i>Uncultured Porphyromonas spp.</i>	HM754526.1	1.98	99
<i>Uncultured Prevotella spp.</i>	GU905979.1	1.82	98
<i>Uncultured bacterium</i>	HM318928.1	1.74	94
<i>Uncultured bacterium</i>	JF810468.1	0.71	100
<i>Veillonella dispar</i>	GU404460.1	0.63	89
<i>Histophilus somni</i>	AB176910.1	0.63	100
<i>Ochrobactrum pseudogrignonense</i>	FJ859687.2	0.63	99
<i>Paenibacillus borealis</i>	HM563046.1	0.55	99
<i>Helcococcus ovis</i>	AB542088.1	0.55	100
<i>Uncultured bacterium</i>	JN575965.1	0.55	100
<i>Bacteroides fragilis</i>	FQ312004.1	0.55	100
<i>Halomonas spp.</i>	AJ302088.1	0.48	99
<i>Uncultured bacterium</i>	JN575958.1	0.48	100
<i>Uncultured bacterium</i>	EU460092.1	0.48	97
<i>Staphylococcus equorum subsp. linens</i>	NR_041926.1	0.40	99
<i>Uncultured Gram-positive bacterium</i>	AB191022.1	0.40	98
<i>Trueperella pyogenes</i>	JN578141.1	0.40	100
<i>Uncultured Corynebacterium spp.</i>	JN082697.1	0.32	100
<i>Clostridium perfringens str.</i>	BA000016.3	0.32	100
<i>Uncultured Ruminococcus spp.</i>	HM235655.1	0.32	100
<i>Psychrobacter marincola</i>	AY292940.1	0.32	98
<i>Mycoplasma bovis genitalium</i>	AY121109.1	0.32	100
<i>Uncultured bacterium</i>	GU608534.1	0.32	99
<i>Bacillus spp.</i>	FR774960.1	0.32	100

Table 9. Species level information (with GenBank Accession number, and identity match) for the predominant representative sequences in samples characterized as *Streptococcus* spp. mastitis

Species	Accession No	Prevalence	Identity (%)
<i>Streptococcus macedonicus</i>	AF459431.1	18.54	100
<i>Uncultured Porphyromonas spp.</i>	HM754526.1	11.92	99
<i>Uncultured Fusobacteria</i>	EF704825.1	9.27	100
<i>Uncultured Streptococcus spp.</i>	GU145660.2	8.61	100
<i>Porphyromonas levii</i>	AB547664.1	6.62	100
<i>Caulobacter leidyia</i>	GQ891705.1	3.97	100
<i>Uncultured Bacteroides spp.</i>	EU289070.1	3.97	100
<i>Uncultured bacterium</i>	GQ179047.1	3.31	98
<i>Geobacillus pallidus</i>	HM030740.1	2.65	99
<i>Uncultured bacterium</i>	AM183009.1	1.99	95
<i>Uncultured Bacteroidetes bacterium</i>	GU954712.1	1.99	99
<i>Uncultured bacterium</i>	HM318928.1	1.32	95
<i>Uncultured bacterium</i>	EU290135.1	1.32	99
<i>Uncultured bacterium</i>	EU458333.1	1.32	99
<i>Staphylococcus equorum subsp. linens</i>	NR_041926.1	1.32	100
<i>Uncultured bacterium</i>	HQ420215.1	1.32	99
<i>Paenibacillus borealis</i>	HM563046.1	1.32	98
<i>Ureaplasma diversum</i>	NR_025878.1	0.66	99
<i>Uncultured Ruminococcaceae bacterium</i>	EU794228.1	0.66	99
<i>Thermoanaerobacterales bacterium</i>	GU797851.1	0.66	99
<i>Bacillus firmus</i>	EF636895.1	0.66	100
<i>Swine manure pit bacterium</i>	AF445295.1	0.66	99
<i>Uncultured bacterium</i>	GU615111.1	0.66	99
<i>Uncultured bacterium</i>	FN994146.1	0.66	99
<i>Arthrobacter spp.</i>	AM260537.1	0.66	100
<i>Uncultured bacterium</i>	JF553154.1	0.66	94
<i>Uncultured bacterium</i>	GU609709.1	0.66	99
<i>Uncultured bacterium</i>	AY816889.1	0.66	87
<i>Uncultured bacterium</i>	HM327761.1	0.66	85
<i>Uncultured bacterium</i>	EU290118.1	0.66	100
<i>Uncultured bacterium</i>	AB200302.1	0.66	95
<i>Uncultured bacterium</i>	GU613627.1	0.66	99
<i>Uncultured bacterium</i>	FJ368239.1	0.66	98
<i>Uncultured bacterium</i>	JF643239.1	0.66	99
<i>Clostridium perfringens</i>	AB627081.1	0.66	99
<i>Uncultured bacterium</i>	EU773780.1	0.66	96
<i>Uncultured organism</i>	JF782021.1	0.66	97
<i>Uncultured bacterium</i>	DQ793282.1	0.66	95
<i>Uncultured bacterium</i>	FJ682049.1	0.66	99
<i>Uncultured Prevotella spp.</i>	GU905979.1	0.66	98
<i>Clostridium ramosum</i>	AB595128.1	0.66	100
<i>Uncultured bacterium</i>	HQ319439.1	0.66	94
<i>Uncultured bacterium</i>	GU615040.1	0.66	91
<i>Uncultured bacterium</i>	GU609321.1	0.66	95
<i>Uncultured bacterium</i>	FJ507484.1	0.66	99
<i>Halomonas spp.</i>	AJ302088.1	0.66	100

Table 10 Species level information (with GenBank Accession number, and identity match) for the predominant representative sequences in samples characterized as *Streptococcus uberis* mastitis

Species	Accession No	Prevalence	Identity (%)
<i>Streptococcus uberis</i>	HQ326695.1	34.84	100
<i>Caulobacter leidyia</i>	GQ891705.1	5.11	100
<i>Porphyromonas levii</i>	AB547664.1	4.77	100
<i>Fusobacterium necrophorum</i> subsp. <i>Funduliforme</i>	AB525413.1	3.50	100
<i>Uncultured Porphyromonas</i> spp.	HM754526.1	3.16	100
<i>Uncultured bacterium</i>	AM183009.1	2.89	95
<i>Geobacillus pallidus</i>	HM030740.1	2.69	99
<i>Uncultured Porphyromonas</i> spp.	HM754526.1	2.62	99
<i>Uncultured bacterium</i>	JF643239.1	2.22	100
<i>Uncultured bacterium</i>	JF663845.1	1.88	98
<i>Rumen bacterium enrichment culture</i>	HM597702.1	1.61	100
<i>Uncultured bacterium</i>	HM318928.1	1.41	94
<i>Propionibacterium acnes</i>	CP003084.1	1.08	100
<i>Staphylococcus equorum</i> subsp. <i>linens</i>	NR_041926.1	1.08	100
<i>Bacteroides heparinolyticus</i>	GQ422742.1	0.81	100
<i>Swine manure pit bacterium</i>	AF445295.1	0.81	99
<i>Uncultured bacterium</i>	FJ682454.1	0.67	99
<i>Uncultured bacterium</i>	EU290118.1	0.67	100
<i>Uncultured bacterium</i>	EU458333.1	0.67	98
<i>Prevotella</i> spp.	FJ848548.1	0.54	100
<i>Peptostreptococcus anaerobius</i>	AB640695.1	0.54	98
<i>Helcococcus ovis</i>	AB542088.1	0.47	100
<i>Uncultured Prevotella</i> spp.	GU905979.1	0.47	98
<i>Staphylococcus epidermidis</i>	JN644588.1	0.40	100
<i>Corynebacterium falsenii</i>	AF537594.1	0.40	100
<i>Ochrobactrum pseudogrignonense</i>	FJ859687.2	0.40	99
<i>Halomonas</i> spp.	AJ302088.1	0.34	99
<i>Bacteroides vulgatus</i>	JN084208.1	0.34	100
<i>Uncultured bacterium</i>	GU603837.1	0.34	98
<i>Ureaplasma diversum</i>	NR_025878.1	0.34	98
<i>Paenibacillus borealis</i>	HM563046.1	0.34	99

Table 11. Species level information (with GenBank Accession number, and identity match) for the predominant representative sequences in samples obtained from healthy cows and had a low SCC

Species	Asseccion No	Prevalence	Identity (%)
<i>Uncultured Propionibacterium sp.</i>	JQ288555.1	14.00	100
<i>Uncultured bacterium</i>	EF205691.1	8.03	100
<i>Staphylococcus epidermidis</i>	JQ795860.1	2.07	100
<i>Uncultured proteobacterium</i>	GU956128.1	2.07	100
<i>Uncultured bacterium</i>	GU634642.1	1.99	99
<i>Bacteroides heparinolyticus</i>	GQ422742.1	1.84	99
<i>Frateruia sp.</i>	AY495959.1	1.61	99
<i>Uncultured bacterium</i>	FJ682300.1	1.53	100
<i>Streptococcus uberis</i>	HQ326695.1	1.53	100
<i>Uncultured bacterium</i>	AY511727.1	1.38	100
<i>Bacteroides vulgatus</i>	JN084208.1	1.15	100
<i>Uncultured Bacteroides sp.</i>	JQ083405.1	1.07	100
<i>Uncultured bacterium</i>	EU772991.1	0.99	99
<i>Uncultured bacterium</i>	EU290137.1	0.99	99
<i>Bacteroides fragilis</i>	AB618792.1	0.84	99
<i>Uncultured Porphyromonas sp.</i>	JN167617.1	0.84	99
<i>Uncultured bacterium</i>	JX108076.1	0.84	99
<i>Rhodanobacter terrae</i>	FJ405366.1	0.61	99
<i>Uncultured bacterium</i>	JX108142.1	0.61	100
<i>Pelistega europaea</i>	FJ999734.1	0.61	98
<i>Uncultured bacterium</i>	JX108447.1	0.61	99
<i>Clostridiales bacterium</i>	HQ452852.1	0.61	100
<i>Uncultured bacterium</i>	JF086910.1	0.54	100
<i>Uncultured bacterium</i>	FN658975.1	0.54	100
<i>Uncultured bacterium</i>	JX013206.1	0.54	100
<i>Staphylococcus equorum</i>	JN969599.1	0.54	100
<i>Uncultured bacterium</i>	JF194563.1	0.46	100
<i>Uncultured Corynebacterium sp.</i>	JN584700.1	0.46	100
<i>Uncultured Helcococcus sp.</i>	JN167606.1	0.46	100
<i>Clostridium sp.</i>	AB739698.1	0.46	99
<i>Uncultured bacterium</i>	EF205694.1	0.46	99
<i>Uncultured bacterium</i>	JX106677.1	0.46	99
<i>Uncultured bacterium</i>	FN658980.1	0.46	100
<i>Uncultured Clostridiales bacterium</i>	HM076511.1	0.46	100
<i>Uncultured Bacteroides sp.</i>	JN167633.1	0.46	100
<i>Uncultured Firmicutes bacterium</i>	HE583207.1	0.46	100
<i>Uncultured bacterium clone</i>	AY511727.1	0.46	100
<i>Uncultured bacterium clone</i>	EU474921.1	0.46	97
<i>Uncultured bacterium</i>	JX107110.1	0.38	99
<i>Propionibacterium granulosum</i>	AB638444.1	0.38	100
<i>Trueperella pyogenes</i>	JN578141.1	0.38	100
<i>Uncultured bacterium</i>	JF137212.1	0.38	100
<i>Uncultured Lachnospiraceae bacterium</i>	EU794239.1	0.38	100
<i>Uncultured bacterium</i>	JX108364.1	0.38	99
<i>Uncultured Porphyromonas sp.</i>	HM754526.1	0.38	99
<i>Uncultured bacterium</i>	EU464106.1	0.38	99
<i>Uncultured bacterium</i>	GQ136816.1	0.38	99
<i>Caulobacter leidyia</i>	GQ891705.1	0.38	100

<i>Halomonas sp.</i>	AJ302088.1	0.31	100
<i>Uncultured Lachnospiraceae bacterium</i>	EF698785.1	0.31	99
<i>Uncultured Clostridiales bacterium</i>	JQ083415.1	0.31	99
<i>Uncultured bacterium</i>	JX108113.1	0.31	100
<i>Ornithinibacillus sp.</i>	GQ903473.1	0.31	100
<i>Uncultured bacterium</i>	HM339654.1	0.31	99
<i>Uncultured bacterium</i>	JX120473.1	0.31	100
<i>Geobacillus tepidamans</i>	FJ823100.2	0.31	99
<i>Uncultured bacterium</i>	JX087346.1	0.31	100
<i>Uncultured bacterium</i>	JX108142.1	0.31	99
<i>Uncultured Lactobacillus</i>	JQ083429.1	0.31	100
<i>Uncultured bacterium clone</i>	GQ448802.1	0.31	100
<i>Flavobacterium sp.</i>	HQ836451.1	0.31	100
<i>Uncultured bacterium</i>	JN834156.1	0.31	98
<i>Uncultured bacterium</i>	JQ825064.1	0.31	92
<i>Uncultured organism</i>	HQ766342.1	0.31	100
<i>Uncultured Firmicutes bacterium</i>	HE583206.1	0.31	100

- 5 [0073] While the invention has been described through specific embodiments, routine modifications will be apparent to those skilled in the art and such modifications are intended to be within the scope of the present invention.

We claim:

1. A method for diagnosis of mastitis in a female ruminant, comprising: (a) testing a milk sample from the ruminant to determine *Fusobacterium necrophorum* as a percentage of
5 bacteria in the milk sample, and b) diagnosing the ruminant as having mastitis if the percentage of *Fusobacterium necrophorum* is $\geq 3.3\%$ of the bacteria in the milk sample, or diagnosing the ruminant as not having mastitis if the percentage of *Fusobacterium necrophorum* is $< 3.3\%$ of the total bacteria in the milk sample.
2. The method of claim 1, wherein the testing the milk sample comprises determining
10 prevalence of a plurality of bacteria types in the milk sample by determining polynucleotide sequences which are unique to each of the bacteria types.
3. The method of claim 2, wherein the determining the polynucleotide sequences comprises determining the sequence of 16S rRNA for the bacteria types.
4. The method of claim 1, wherein the ruminant is a bovine animal.
- 15 5. The method of claim 4, wherein the bovine mammal is a dairy cow.
4. A method for determining that a sample of milk was obtained from a ruminant which does not have clinical mastitis comprising (a) testing a milk sample from the ruminant to determine *Fusobacterium necrophorum* as a percentage of bacteria in the milk sample, and b) identifying the sample of milk as having been obtained from a ruminant that does not have
20 mastitis if the *Fusobacterium necrophorum* is less than 3.3% of the bacteria in the milk sample.
5. The method of claim 4, wherein the testing the milk sample comprises determining prevalence of a plurality of bacteria types in the milk sample by determining polynucleotide sequences which are unique to each of the bacteria types.
- 25 6. The method of claim 5, wherein the determining the polynucleotide sequences comprises determining the sequence of 16S rRNA for the bacteria types.
7. The method of claim 4, wherein the ruminant is a bovine animal.
8. The method of claim 4, wherein the bovine mammal is a dairy cow.

9. A method for determining that a sample of milk was obtained from a ruminant which has clinical mastitis comprising (a) testing a milk sample from the ruminant to determine *Fusobacterium necrophorum* as a percentage of bacteria in the milk sample, and b) identifying the sample of milk as having been obtained from a ruminant that has mastitis if the *Fusobacterium necrophorum* is more than 3.3% of the bacteria in the milk sample.
10. The method of claim 9, wherein the testing the milk sample comprises determining prevalence of a plurality of bacteria types in the milk sample by determining polynucleotide sequences which are unique to each of the bacteria types.
11. The method of claim 10, wherein the determining the polynucleotide sequences comprises determining the sequence of 16S rRNA for the bacteria types.
12. The method of claim 9, wherein the ruminant is a bovine animal.
13. The method of claim 9, wherein the bovine mammal is a dairy cow.

Figure 1.

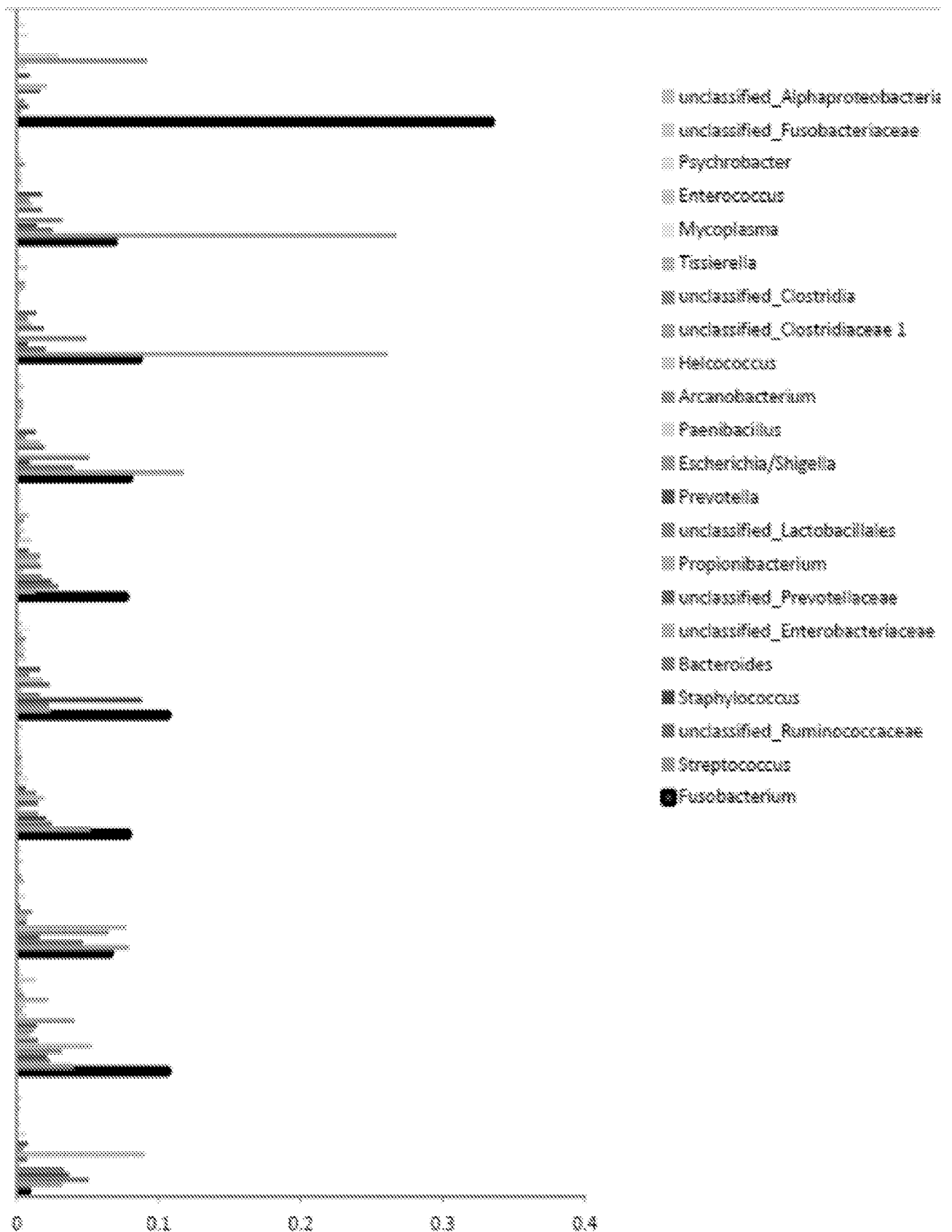


Figure 2.

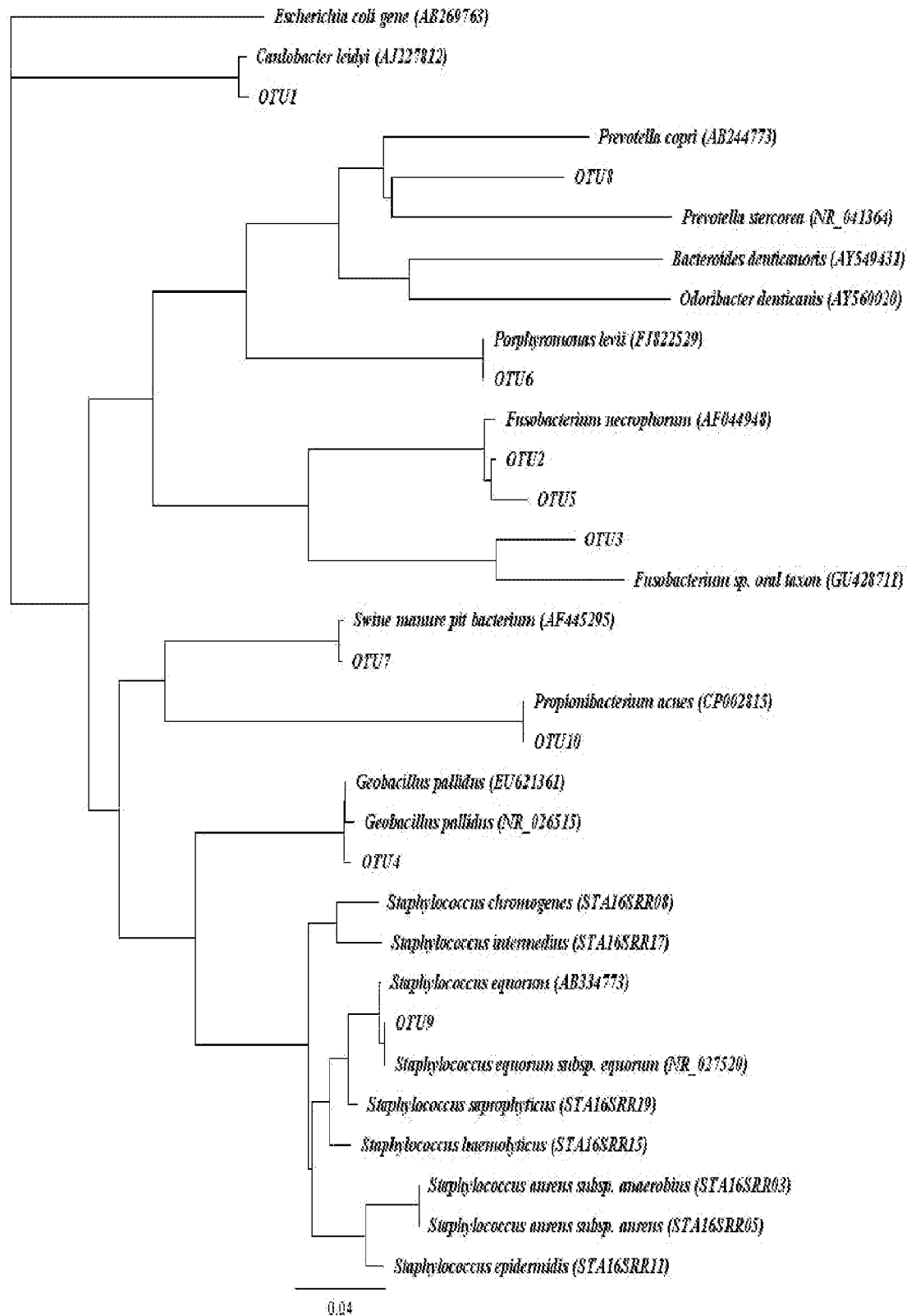
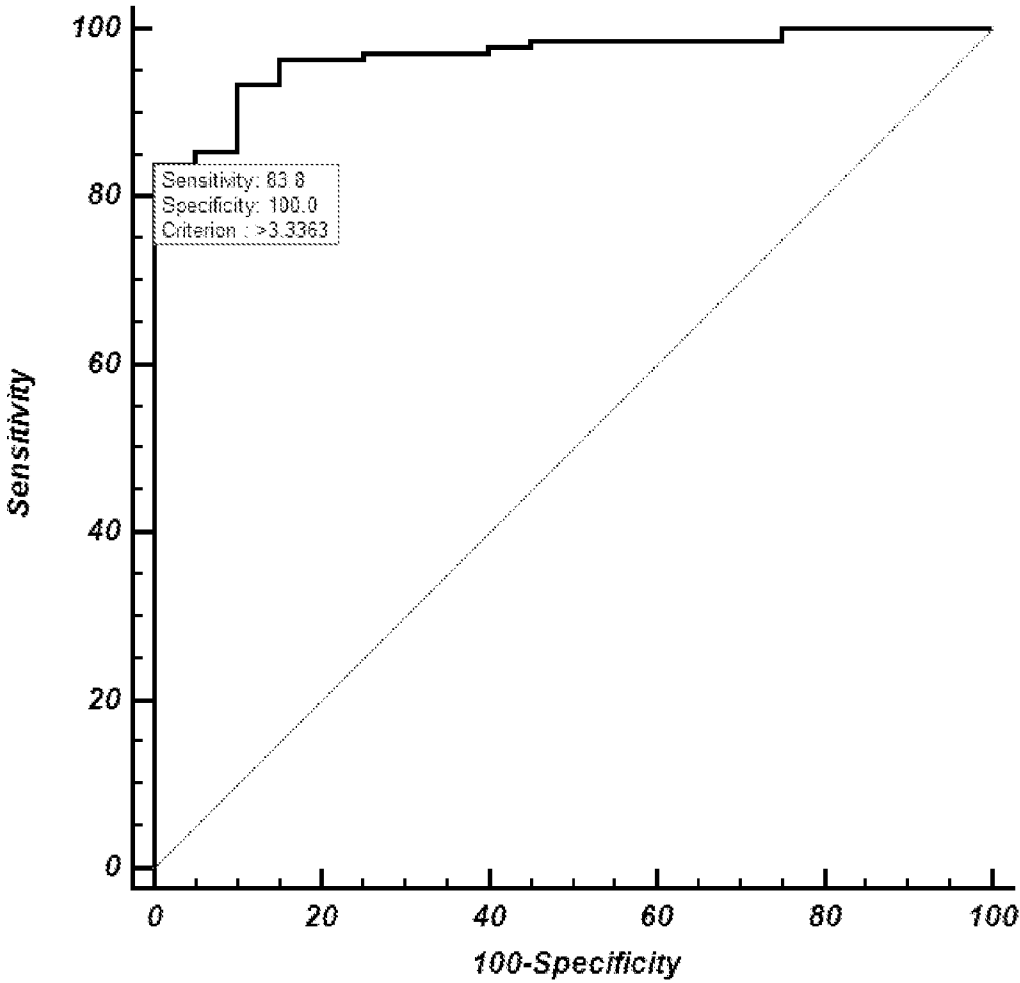
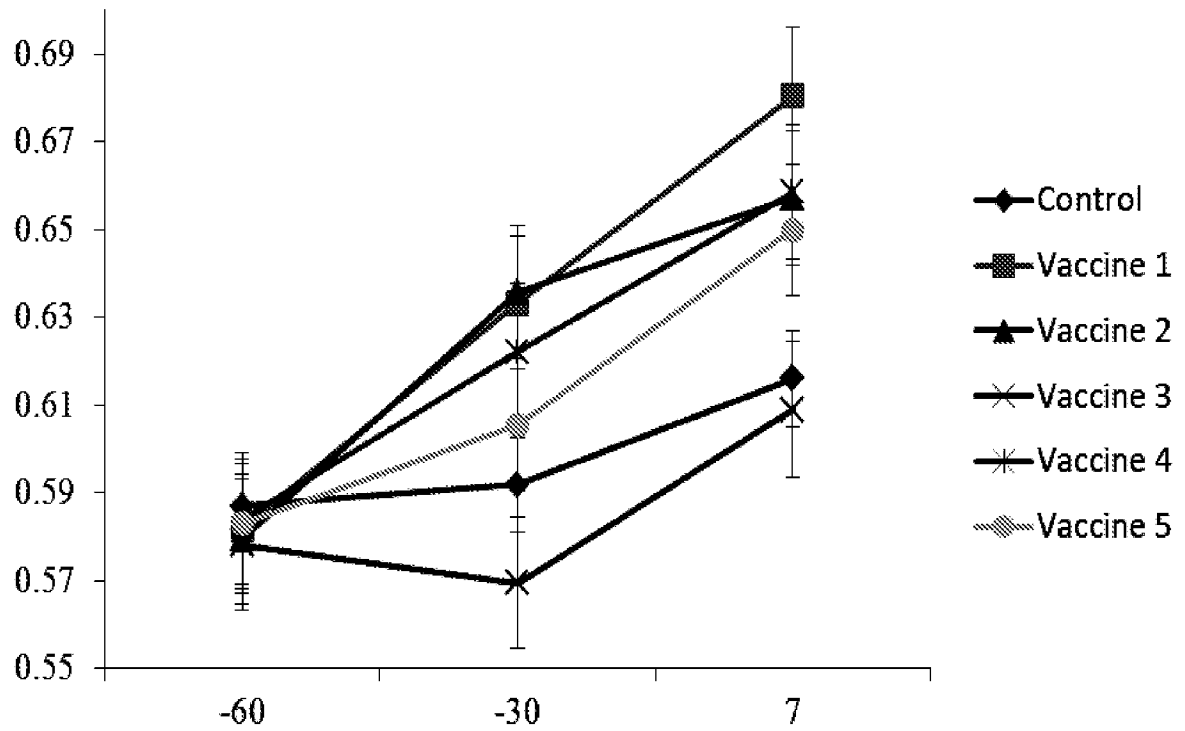


Figure 3.



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Figure 4.



INTERNATIONAL SEARCH REPORT

2013/063874 24.01.2014
International application No.

PCT/US13/63874

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01N 63/00 (2014.01)

USPC - 424/93.4, 93.1; 435/4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A01N 63/00 (2014.01)

USPC: 424/93.4, 93.1; 435/4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; PubMed; ProQuest; testing, ruminant, mammal, cow, cattle, Mastitis, 'breast inflammation,' 'milk sample,' determine, 'Fusobacterium necrophorum,' percentage

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	OLIVER, SP et al. Heifer Mastitis: Prevalence, Risk Factors, And Control Strategies. NMC Annual Meeting Proc. 2004, Vol. 43, No. 83' pages 83-99; page 83, paragraph 1; page 84, paragraph 2; page 85, paragraphs 2, 3; page 86, paragraph 1; page 95, paragraph 2.	1, 4(A), 4(B), 5(A), 7-9, 12, 13 — 2, 3, 5(B), 6, 10, 11
Y	SANTOS, TMA et al. Metagenomic Analysis Of The Uterine Bacterial Microbiota In Healthy And Metritic Postpartum Dairy Cows. J. Dairy Sci. 2011, Vol. 94, pages 291-302; abstract; page 292, column 2, paragraph 2.	2, 3, 5(B), 6, 10, 11

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

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Date of the actual completion of the international search

13 January 2014 (13.01.2014)

Date of mailing of the international search report

24 JAN 2014

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