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(57) Abstract: The present invention relates to an assay useful in characterizing the innate immune response associated with endometritis in animal subjects. The assay includes determining the likelihood of establishment of an inflammatory uterine disease in female equine animals which leads to the development of a protocol to manage a clinical pregnancy in female equine animals with inflammatory uterine disease.

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AN ASSAY AND METHOD OF TREATMENT

[0001] This application is associated with and claims priority from Australian Provisional Patent Application No. 2016904347, filed on 26 October, 2016, entitled "An assay and method of treatment", the entire contents of which, are incorporated herein by reference, in their entirety. This specification refers to a Sequence Listing. The "ST25.txt" file is in ANSI format. The file is hereby incorporated in its entirety by reference from AU 2016904347 into the subject specification.

FIELD

[0002] The present invention relates to an assay useful in characterizing the innate immune response associated with endometritis in animal subjects. The assay includes determining the likelihood of establishment of an inflammatory uterine disease in female equine animals which leads to the development of a protocol to manage a clinical pregnancy in female equine animals with inflammatory uterine disease.

BACKGROUND

[0003] Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

[0004] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgement or admission or any form of suggestion that the prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavor to which this specification relates.

[0005] The ability to determine the likelihood of a pregnancy being successfully initiated is of immense importance to animal natural reproduction and assisted pregnancy programs.

[0006] In particular, the successful breeding of horses is of significant commercial interest to the horse industry, especially for Thoroughbred, Warmblood, Quarter horse and Standardbred horses, as well as for horse breeding programs in general. Furthermore, the potential status of a mare's capacity to become pregnant forms part of the critical information for a potential buyer or insurer of the mare. Given the level of cost of natural cover by a stallion and horse semen, a failure of the horse to achieve pregnancy can have major adverse financial implications, including those due to delays to the breeding program. This is particularly important in the Thoroughbred and Standardbred breeding industry, since foals born early in the season have a competitive advantage in races and are sold at higher prices at yearling auctions. There is also a need to ensure breeding programs are successful in the zoological environment for other equine animals such as Przewalski horses, asses and zebras, as well as in farm animal and companion animal breeding programs. There is therefore a need to be able to monitor uterine receptivity to ensure achieving a clinical pregnancy.

[0007] Persistent mating-induced endometritis (PMIE) is a local inflammation of the superficial layers of the uterus after breeding and affects approximately 15% of Thoroughbred broodmares (Zent *et al.* (1998) *Proceedings of the Annual Convention of the American Association of Equine Practitioners*:64). PMIE is a major cause of subfertility in mares (Watson (2000) *Animal Reproduction Science 60-61*:2673-2681) leading to significant losses to the horse breeding industry (Riddle *et al.* (2007) *Theriogenology 68*:395-402).

[0008] Both natural breeding and assisted breeding protocols such as artificial insemination introduce seminal plasma, spermatozoa, cell debris and bacteria to the uterus which in turn induces transient physiological endometritis (Watson (2000) *supra)*; Troedsson *et al.* (2001) *Animal Reproduction Science 68(3-4)*:273-278). Similarly, the introduction of microorganisms into the uterine environment has also been shown to cause neutrophilia and an increase in the innate immune response (Christoffersen *et* al. (2010) *Vet Immunol Immunopathol* 138:95-105). In fact, microbial infection is found in 25-60%

of barren mares (Atli *et al.* (2010) *Anim 45*:58). This inflammatory response can last for more than 96 h after breeding in mares susceptible to PMIE. This contrasts to healthy mares which are able to clear these foreign materials from their uterus within 6 h (Troedsson *et al.* (1991) *Journal of Reproduction and Fertility 44(Supplement)*:283-288).

[0009] PMIE can be identified by persistent uterine fluid accumulation (Brinsko (2003) *American Association of Equine Practitioners*). However, cycle stage can affect the presence of fluid and thus the diagnostic value of fluid detection. For example, Adams *et al.* (1987) *Journal of Reproduction and Ferility 25(Supplement)*:445-454, found lower embryo recovery rates when fluid was detected in the dioestrus phase, however, another study showed no difference whether or not fluid was detected during the oestrus phase (Reilas *et al.* (1997) *Acta Veterinaria Scandinavica 38(1)*:69-78).

[0010] Pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors (NLRs) are responsible for the detection of pathogen-associated molecule patterns (PAMPs) such as lipopolysaccharides (LPS) as well as damage-associated molecular patterns (DAMPs) derived from injured body cells (Kawasaki and Kawai (2014) *Frontiers in Immunology 5*:461).

[0011] This induces an increased expression of cytokines and chemokines, whose main function is the regulation of transendothelial leucocyte migration (Zlotnik and Yoshie (2012) *Immunity 36(5)*:705-716). In addition, antimicrobial mechanisms have been detected for some chemokines, such as CXCL9, 10 and 11 (Cole *et al.* (2001) *Journal of Immunology 167(2)*:623-627; Yang *et al.* (2003) *Journal of Leukocyte Biology 74(3)*:448-455). Like other mucous membranes, the endometrium also uses antimicrobial peptides (AMPs) in its defence against bacteria. They can destabilize bacterial cell walls, a mechanism used by some defensins (Linde *et al.* (2008) *Journal of Veterinary Internal Medicine 22(2)*:247-268), lysozyme (Linde *et al.* (2008) *supra*) and secreted phospholipase A_2 ($sPLA_2$), which also stimulate neutrophils to release superoxide in a respiratory burst (Zallen *et al.* (1998) *Archives of Surgery 133(11)*:1229-1233). Other AMPs, such as

secretory leukoprotease inhibitor (SLPI), selectively inhibit microbial enzymes (Hiemstra *et al.* (1996) *Infection and Immunity 64(11)*:4520-4524). Lipocalin 2 (LCN2) [Flo *et* al. (2004) *Nature 432(7019)*:917-921] and lactoferrin (LFN) [Farnaud and Evans (2003) *Molecular Immunology 40(7)*:395-405] bind iron, an element essential for microbial metabolism, causing bacteriostasis, while LFN is also capable of binding to Lipid A of LPS (Appelmelk *et al.* (1994) *Infection and Immunity 62(6)*:2628-2632). Tissue inhibitor of metalloproteinases 1 (TIMP1) regulates the expression of matrix metalloproteinase 9 (MMP9), which has been shown to influence several components of the immune response (Van den Steen *et al.* (2000) *Blood 96(8)*:2673-2681, McQuidbban *et al.* (2002) *Blood 100(4)*:1160-1167).

[0012] There is a need to determine the clinical relevance of these innate immune response factors on inflammatory endometritis and in particular persistent inflammatory endometritis. A determination of the clinical relevance enables the development of protocols to maximize successful initiation of pregnancies in equine animal subjects.

SUMMARY

[0013] A list of abbreviations used throughout the subject specification is provided in Table 1.

[0014] Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO). The SEQ ID NOs correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 2. A sequence listing is provided after the claims.

[0015] The present specification teaches the establishment of an assay to determine the likelihood of establishment of an inflammatory uterine disease in female equine animals and this enables the stratification of female equine animals with respect to the likelihood of the occurrence of achieving a clinical pregnancy. The pregnancy may result from natural breeding or assisted breeding such as artificial insemination or embryo transfer. The likelihood of a successful clinical pregnancy is associated with the degree of inflammation in the uterus referred to as endometritis. Under natural conditions, female equine animals having a persistent form of endometritis including persistent mating-induced endometritis (PMIE), have a reduced success rate in initiating pregnancy. In the horse breeding industry, this represents a significant commercial risk factor for owners and breeders as well as for insurance companies. The present invention enables the detection of biomarkers associated with a chronic or persistent inflammatory endometritis, environment which is not conducive to the successful initiation of a pregnancy. The biomarkers do not necessarily determine that a pregnancy will go to term. However, knowledge of a female equine animals endometric inflammatory status can enable medical intervention to assist in pregnancy initiation and also provides valuable information to stakeholders.

[0016] The present invention is predicated in part on the development of an assay which defines whether a female equine animal is receptive for successfully initiating a pregnancy based on the duration of endometritis (persistent *versus* transient) in the uterus. The assay is based on distinguishing horses prone to develop persistent endometritis or transient

endometritis by use of uterine biomarkers of the innate immune system. Pathogen recognition receptors (PRRs), chemokines and cytokines and antimicrobial peptides (AMPs) are elevated during induced endometritis and are biomarkers of endometritis. In particular, the response to endometritis is an elevation of Toll-like receptors (TLRs), TLR2 and TLR4, NOD-like receptor (NLRC)5, tissue inhibitor of metallopeptidase 1 (TIMP1) and the chemokines CCL2, CXCL9, CXCL10 and CXCL11. The antimicrobial peptides (AMPs) equine β-defensin 1 (EBD1), lysozyme, secretory leukoprotease inhibitor (SLPI), lipcalin 2 (LCN2), lactoferrin and uteroferrin were also increased. Uterocalin P19 is elevated in dioestrus compared to oestrus. These biomarkers define a state of endometritis. Of these, elevated levels of EBD1, lysozyme and SLPI are found to distinguish female equine animals prone to the development of persistent endometritis from those that only develop a transient form of endometritis. It is proposed that persistent endometritis leads to a reduction in the rate of successful initiation of pregnancy compared to equine animals with none to transient endometritis.

[0017] Hence, an assay is developed to stratify a female equine animal on the basis of the likelihood to successfully initiate a pregnancy. Detection of elevated BD1, lysozyme and/or SLPI is indicative of the equine animals that are likely to develop a persistent endometritis after a breeding process. Sensitivity and specificity is improved by including the detection of elevated levels of 2 or all 3 biomarkers. Each of these biomarkers alone or in combination with one or more other of the biomarkers can be assayed. In addition, to any one or more of these biomarkers, elevation of any one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19 provides further evidence for a persistent endometritic environment which means any attempt at natural or assisted pregnancy is less likely to result in initiation of a pregnancy unless there is a significantly higher level of veterinary care. The levels of the biomarkers can be determined at the mRNA or corresponding cDNA level or by direct determination of protein levels in uterine biopsies, cytobrushes or uterine lavages. Hence, the assay determines the presence of an elevation of one or more of EBD1, lysozyme and/or SLPI in a uterine biopsy, cytobrush or lavage sample which is indicative of female equine animals prone to developing persistent endometritis and this equates to a reduced

likelihood or probability of achieving a clinical a clinical pregnancy. Furthermore, zero to basal levels of one or more of EBD1, lysozyme and/or SLPI is indicative of a reduced likelihood that endometritis will adversely affect the clinical pregnancy. There may, of course, be other unrelated factors which may adversely affect the potential for a pregnancy to successfully proceed to term.

[0018] Enabled herein is a method for the stratification of female equine animals with respect to whether there is a likelihood of an impairment of the establishment of a pregnancy due to persistent endometritis including PMIE. The stratification is based on an association between upregulated biomarkers in a uterine-derived tissue sample. The sample includes endometrial tissue collected by biopsy or cytobrush or uterine lavage. The association between the biomarkers and endometritis is validated through next generation sequencing (NGS) followed by qRT-PCR. A biomarker signature such as an NGS generated panel is used to predict the likelihood or probability of a successful clinical pregnancy or risk of a failed initiation of pregnancy. In an embodiment, provided heein is a method for determining the likelihood of a female equine animal establishing endometritis including persistent mating-induced endometritis, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of a biomarker selected from the list consisting of equine β-defensin1 (EBD1), lysozyme and secretory leukoprotein inhibitor (SLPI) wherein an elevated level of any one of these biomarkers relative to a control is indicative of subjects prone to develop persistent endometritis.

[0019] The biomarker expression profile is the level of expression of one or more mRNAs or corresponding cDNAs or protein concentrations. Conveniently, in relation to horse subjects forward and reverse primers selected from the list consisting of SEQ ID NOs:1 and 2 (EBD1), 3 and 4 (lysozyme) and 5 and 6 (SLPI) are used to determine the level of mRNA encoding one or more of the biomarkers to generate a profile associated with non-, transient or persistent endometric environments. This is compared to test data. The profile data (also referred to as a training data) represent the correlation of expression levels of the mRNAs encoding various biomarkers with female equine animals of known status with

respect to endometritis. In an embodiment, the profile data enable determination of the expression levels of particular mRNAs obtained using qPCR. Expression fold changes and levels of expression can also be measured of individual mRNAs and/or ratios determined of mRNA levels. The assay may or may not require control samples to be run side-by-side.

[0020] Hence, enabled herein is an assay based on the application of a comparison of levels of mRNA expression in a control sample or based on predetermined values. In another embodiment, the assay is based on application of statistical and machine learning algorithms. Such an algorithm uses the relationships between mRNA expression and endometritis status observed in training data (profile data with known endometritis status) to infer relationships which are then used to predict the status of the female equine animal with unknown status (test data) in relation to the likelihood or probability of the female equine animal becoming pregnant. A similar approach can be adopted for biomarker protein concentrations. Practitioners skilled in the art of data analysis recognize that many different forms of inferring relationships in the training data may be used without materially changing the scope of the present invention.

[0021] Hence, taught herein is a cost effective, low risk, minimally invasive test to stratify a female equine animal prior to natural or assisted pregnancy to ensure the greatest likelihood or probability of achieving a clinical pregnancy. The assay can also be used to monitor treatment protocols for the endometritis including PMEI. Where necessary, a female equine animal may first be treated with antagonists of EBD1, lysozyme and/or SLPI prior to breeding.

[0022] Further taught herein is an equine animal model for an innate immune response associated with endometritis. The equine animal model is defined by elevated biomarkers defining an innate immune response. The equine animal model is generated by infecting the uterus of a female equine animal with a microorganism. A transient environment of innate immunity biomarker expression comprising elevated levels of TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL9, CXCL10, CXCL11, EBD1, lysozyme, SLIPI, LCN2,

lactoferrin and uteroferrin as well as $sPLA_2$ and P19. A transient state of infectious endometritis ensues which provides a model to test medicaments for their ability to ameliorate the endometritic environment. Such medicaments are therefore useful prior to natural or assisted breeding to maximize the likelihood of a successful pregnancy.

[0023] Accordingly, enabled herein is an equine animal model for endometritis, the equine animal model comprising a female equine animal artificially subject to intrauterine infection with a microorganism for a time and under conditions sufficient to induce elevated expression of biomarkers of innate immunity, the biomarkers selected from the group consisting of TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL0, CXCL10, CXCL11, EBD1, lysozyme, SLIPI, LCN2, lactoferrin and uteroferrin as well as $sPLA_2$ and P19.

[0024] Reference to a "female equine animal" includes a horse, a Przewalski horse, zebra and an ass. A "horse" includes, but is not limited to a Thoroughbred, Warmblood, Quarter horse and Standardbred horse. Reference to an "assay" includes an assay for diagnostic, prognostic and/or determinative purposes. Hence, the assay may be referred to as an assay or a diagnostic assay, a prognostic assay or a determinative assay. An "assay" includes an assay to assess the likelihood of establishment of PMEI and determination of the probability of achieving a clinical pregnancy or otherwise.

[0025] Further taught herein is a method of conducting a business in relation to equine animals. The method is a business model comprising determining whether a female equine animal is prone to the development of a condition of persistent endometritis by the determination of levels of EBD1, lysozyme and/or SLPI, individually or together and optionally with one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19 wherein a decision to purchase the equine animal, use the equine animal in a breeding program and/or to insure the equine animal is based on the level of risk that persistent endometritis may impair the successful initiation of a pregnancy.

[0026] Kits and mechanical testing devices are also contemplated by the present invention.

Table 1

Abbreviations

ABBREVIATION	DESCRIPTION
AMP	An antimicrobial peptide
CFU	Colony forming units
CXCL	Chemokine
EBD1	Equine β-defensin1
eNAP-2	Equine neutrophil antimicrobial peptide 2
LCN2	Lipocalin 2
LFN	Lactoferrin
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
NLR	NOD receptor
NOD	Nucleotide-binding oligomerization domain
P19	Uterocalin P19
PAMP	Pathogen-associated molecular pattern
pi	Post inoculation
PMIE	Persistent mating-induced endometritis
PRR	Pathogen recognition receptor
SLPI	Secretory leukoprotease inhibitor
$sPLA_2$	Secreted phospholipase A_2
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
UFN	Uteroferrin

Table 2

Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	Forward primer EBD1
2	Reverse primer EBD1
3	Forward primer lysozyme
4	Reverse primer lysozyme
5	Forward primer SLPI
6	Reverse primer SLPI
7	Forward primer CCL2
8	Reverse primer CCL2
9	Forward primer CXCL9
10	Reverse primer CXCL9
11	Forward primer CXCL10
12	Reverse primer CXCL10
13	Forward primer CXCL11
14	Reverse primer CXCL11
15	Forward primer Lipocalin 2
16	Reverse primer Lipocalin 2
17	Forward primer P19
18	Reverse primer P19
19	Forward primer TLR2
20	Reverse primer TLR2
21	Forward primer TLR4
22	Reverse primer TLR4
23	Forward primer NLRC5
24	Reverse primer NLRC5
25	Forward primer sPLA2
26	Reverse primer sPLA2

SEQUENCE ID NO:	DESCRIPTION
27	Forward primer TIMP1
28	Reverse primer TIMP1
29	Forward primer GAPDH
30	Reverse primer GAPDH
31	Forward primer β-actin
32	Reverse primer β-actin
33	Forward primer RPL17
34	Reverse primer RPL17
35	Forward primer RPL27A
36	Reverse primer RPL27A
37	Forward primer RPL30
38	Reverse primer RPL30
39	Forward primer RPL32
40	Reverse primer RPL32
41	Forward primer RPS5
42	Reverse primer RPS5
43	Forward primer lactoferrin
44	Reverse primer lactoferrin
45	Forward primer uteroferrin
46	Reverse primer uteroferrin

BRIEF DESCRIPTION OF THE FIGURES

[0027] Some figures contain color representations or entities. Color photographs are available from the Patentee upon request or from an appropriate Patent Office. A fee may be imposed if obtained from a Patent Office.

[0028] Figure 1 is a graphical representation of levels of mRNA transcripts of endometrial TLR2, TLR4, NLRC5 and TIMP1 genes in resistant mares in dioestrus (RD) and oestrus (RE) and susceptible mares in dioestrus (SD) and oestrus (SE). Gene expression was measured in transcript copy numbers/ng RNA determined from standard curves for each gene. The line represents the median per group. Different letters indicate significant differences between resistant and susceptible mares at $P<0.05$. Different capitalisation of letters indicates significant differences between cycle stages at $P<0.05$.

[0029] Figure 2 is a graphical representation of levels of mRNA transcripts of endometrial chemokines CCL2, CXCL9, CXCL10 and CXCL11 genes in resistant horses in dioestrus (RD) and oestrus (RE) and susceptible horses in dioestrus (SD) and oestrus (SE). Gene expression was measured in transcript copy numbers/ng RNA determined from standard curves for each gene. The line represents the median per group. Different letters indicate significant differences between resistant and susceptible mares at $P<0.05$. Different capitalisation of letters indicates significant differences between cycle stages at $P<0.05$.

[0030] Figure 3 is a graphical representation of levels of mRNA transcripts of endometrial EBD1, lysozyme, sPLA2, SLPI, LCN2, lactoferrin, uteroferrin and uterocalin P19 genes in resistant horses in dioestrus (RD) and oestrus (RE) and susceptible horses in dioestrus (SD) and oestrus (SE). Gene expression was measured in transcript copy numbers/ng RNA determined from standard curves for each gene. The line represents the median per group. Different letters indicate significant differences between resistant and susceptible mares at $P<0.05$. Different capitalisation of letters indicates significant differences between cycle stages at $P<0.05$.

[0031] Figures 4a through c are graphical representations of the correlation between logarithmic copy numbers of transcripts of a) EBD1 and lysozyme (LYZ), b) EBD1 and SLPI, and c) LYZ and SLPI genes. Data from resistant mares are shown in grey and those from susceptible mares in black. Circles indicate mares in dioestrus and squares mares in oestrus. The R^2 values are the coefficients of determination.

[0032] Figures 5a through c are graphical representations of the receiver operating characteristic (ROC) curves of assays for transcripts of a) EBD1, b) lysozyme, and c) SLPI genes relating sensitivity and specificity values. The grey square indicates the optimum cut-off point - that with the highest Youden's index (Y = sensitivity + specificity – 1).

[0033] Figures 6a through f are graphical representations of the correlation between age and logarithmic copy numbers of transcripts of a-b) EBD1 c-d) lysozyme (LYZ), e-f) SLPI in resistant (a, c, e) and susceptible (b, d, f) mares. Data from resistant mares are shown in grey and those from susceptible mares in black. Circles indicate mares in dioestrus and squares mares in oestrus. The R^2 values are the coefficients of determination.

DETAILED DESCRIPTION

[0034] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or method step or group of elements or integers or method steps but not the exclusion of any other element or integer or method steps or group of elements or integers or method steps.

[0035] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a biomarker" includes a single biomarker, as well as two or more biomarkers; reference to "an inflammatory condition" includes a single inflammatory component, as well as multiple inflammatory components of the inflammatory condition; reference to "the disclosure" includes a single and multiple aspects taught by the disclosure; and so forth. Aspects taught and enabled herein are encompassed by the term "invention". All such aspects are enabled within the width of the present invention. Any variants and derivatives contemplated herein are encompassed by "forms" of the invention.

[0036] The present invention identifies a biomarker profile in a female equine animal indicative of an increased chance of developing a prolonged innate immune response associated with a form of endometritis including persistent mating-induced endometritis (PMEI). In addition, a subset of this profile can be used to identify female animals likely to develop persistent endometritis sufficient to decrease the likelihood or probability of the establishment of a successful clinical pregnancy in a female equine animal compared to a subject with none to transient endometritis where the risk of failure to achieve pregnancy is reduced.

[0037] The present specification teaches an assay which defines the state of innate immunity in response to a level of endometritis in a female equine animal. The endometritis provides an innate immune response similar to an endometritic condition after breeding in animals. This can then be used to distinguish between female equine animals

likely to develop only a transient form of endometritis which is less harmful for pregnancy compared to those from subjects who are likely to develop persistent endometritis which results in reduced rates of successful initiation of pregnancy. Hence, an assay is described herein which defines a profile of biomarkers comprising pathogen recognition receptors (PRRs), chemokines and cytokines and antimicrobial peptides (AMPs) as well as other biological regulators which are used to determine a state of endometritis in the female equine animal. These biomarkers comprise TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL9, CXCL10, CXCL11, EBD1, lysozyme, SLPI, LCN2, lactoferrin and uteroferrin as well as $sPLA_2$ and uterocalin P19. In an embodiment, the assay determines a selection of upregulated biomarkers, EBD1, lysozyme and/or SLPI, which define a state of persistent endometritis. Such a state provides an environment which is not conducive to successful initiation of pregnancy *via* either natural pregnancy protocols or assisted breeding (artificial insemination or embryo transfer). This assay enables the generation of a protocol to determine the risk of a clinical pregnancy not developing or the likelihood to achieve a clinical pregnancy. It may also be used in a method of treating the equine animal prior to initiation of a breeding program to maximize the likelihood of a successful pregnancy outcome. In particular, diagnosis of a female equine animal prone to developing a condition of persistent endometritis may need a higher level of animal veterinary care to manage the pregnancy. In addition, the assay enables business decisions to be made in relation to equine animals such as by owners, breeders and other stakeholders such as insurance companies. The assay may be referred to as an assay or a diagnostic assay, a prognostic assay or a determinative assay. Use of any such term is not to imply any limitation to the purpose of the assay. For example, an assay or a diagnostic, prognostic or determinative assay enables establishment of a probability or likelihood of one or other outcomes in relation to initiation of a pregnancy in an equine animal.

[0038] The assay is based on a profile data set (also referred to as training data) of the level of expression of mRNA or corresponding cDNA encoding the biomarkers or the concentration of biomarker protein in a female equine animal of known status with respect to the presence, absence or level of endometritis.

[0039] The term "endometritis" refers to an inflammatory condition or state in the endometrium which may be transient or persistent and includes PMEI. In general terms, persistent endometritis is used to encompass a level and duration of endometrial inflammation which renders conceptus or embryo establishment less likely. In an embodiment, the endometritis is referred to as inflammatory endometritis and can result from infection of the uterus. However, there are non-microbial components of endometritis such as cell debris and spermatozoa fluid. Encompassed herein is a method for detecting endometritis in a female equine animal. In an embodiment, the endometritis is transient endometritis. In an embodiment, the endometritis is persistent endometritis. It is proposed herein that transient endometritis is normal and not harmful to development of a successful clinical pregnancy compared to persistent endometritis and also infectious endometritis. Reference to "persistent endometritis" includes the persistence of the inflammatory condition or biomarkers associated with the inflammatory condition for but not limited to, from 20 h to about 100 h or longer. Reference to the range 20 to 100 h includes 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100 h. The term "transient endometritis" means an inflammatory condition or biomarker associated with an inflammatory condition which usually only lasts less than 6 to 12 h such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 h. The identification of a female equine animal with persistent endometritis does not necessarily mean that such a subject cannot become pregnant however the pregnancy will need to be monitored by animal health practitioners.

[0040] In an embodiment, the female equine animal is a horse, a Przewalski horse, zebra or an ass. In an embodiment, the equine animal is a horse such as but not limited to a Thoroughbred mare, a Warmblood mare, a Quarter Horse mare or a Standardbred mare. The term "mare" is used to describe any equine female animal. The present invention has application in the horse breeding industry, zoological animal husbandry and breeding of asses.

[0041] Accordingly, taught herein is a method for determining the likelihood of a successful pregnancy in a female equine animal, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI wherein an elevated level of any one of these biomarkers relative to a control is indicative of the female equine animal prone to develop persistent endometritis and an increased likelihood of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and an increased likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

[0042] In an embodiment, the biomarker assayed is EBD1. In an embodiment, the biomarker assayed is lysozyme. In an embodiment, the biomarker assayed is SLPI. In an embodiment, the biomarkers assayed are EBD1, lysozyme and SLPI. In an embodiment, the biomarker assays are lysozyme and SLPI. In an embodiment, the biomarkers assayed are BD1 and lysozyme. In an embodiment, the biomarkers assayed are BD1 and SLPI.

[0043] Where there is a finding of a likelihood of not achieving a clinical pregnancy the successful clinical pregnancy might nevertheless be achieved with veterinary intervention and management. Achieving a clinical pregnancy is subject to other factors unrelated to endometritis. A "pregnancy" means a clinical pregnancy but not necessarily to term.

[0044] In an embodiment, the female equine animal is a horse, a Przewalski horse, zebra or ass.

[0045] Accordingly, taught herein is a method for determining the likelihood of achieving a clinical pregnancy in an equine animal, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of EBD1 wherein an elevated level of EBD1 relative to a control is indicative of persistent endometritis and a likelihood of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and a likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

[0046] In an embodiment, enabled herein is a method for determining the likelihood of achieving a clinical pregnancy in an equine animal, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of EBD1 and lysozyme wherein an elevated level of EBD1 and lysozyme relative to a control is indicative of persistent endometritis and a likelihood of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and a likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

[0047] Further taught herein is a method for determining the likelihood of achieving a clinical pregnancy in an equine animal, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of EBD1 and SLPI wherein an elevated level of EBD1 and SLPI relative to a control is indicative of persistent endometritis and a likelihood of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and a likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

[0048] Still taught herein is a method for determining the likelihood of achieving a clinical pregnancy in an equine animal, the method comprising obtaining a uterine biopsy, cytobrush or lavage, sample determining the level of lysozyme and/or SLPI wherein an elevated level of lysozyme and/or SLPI relative to a control is indicative of persistent endometritis and a likelihood of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and a likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

[0049] In yet a further embodiment, the subject specification is instructional for a method for determining the likelihood of achieving a clinical pregnancy in an equine animal, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of EBD1, lysozyme and SLPI wherein an elevated level of EBD1, lysozyme and

SLPI relative to a control is indicative of persistent endometritis and a likelihood of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and a likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

[0050] Further taught herein is a method for determining the likelihood of achieving a clinical pregnancy in an equine animal, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of lysozyme wherein an elevated level of lysozyme relative to a control is indicative of persistent endometritis and a likelihood of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and a likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

[0051] Still taught herein is a method for determining the likelihood of a successful pregnancy in an equine animal, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of SLPI wherein an elevated level of SLPI relative to a control is indicative of persistent endometritis and a likelihood of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and a likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

[0052] The above aspects also refer to a method for determining the likelihood of establishment of endometritis in an equine animal, including PMEI. Such an assay includes the determination of EBD1, EBD1 and lysozyme, EBD1 and SLIPI, lysozyme and/or SLPI and/or EBD1, lysozyme and SLPI. In an embodiment, provided herein is a method for determining the likelihood of a female equine animal establishing endometritis including persistent mating-induced endometritis, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of a biomarker selected from the list consisting of equine β-defensin1 (EBD1), lysozyme and secretory leukoprotein inhibitor (SLPI) wherein an elevated level of any one of these biomarkers relative to a control is indicative of subjects prone to develop persistent endometritis.

[0053] Additional biomarkers may also be assayed such as but not limited to TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or uterocalin P19. The level of sensitivity and specificity can alter depending on the number biomarkers assayed. For example and without limiting the present invention to any particular levels, assessing levels of EBD1, lysozyme and/or SLPI results in a diagnostic sensitivity of at least 70% or greater which includes 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100% such as 81%, 71% and 76%, respectively. Specificity can be at least 80% or greater which includes 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100% such as 95%.

[0054] Enabled herein is a method for determining the likelihood of achieving a clinical pregnancy in a female equine animal subject, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI and optionally one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19 wherein an elevated level of any combination of EBD1, lysozyme and/or SLPI with one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19 relative to a control is indicative of persistent endometritis and a likelihood of an unsuccessful pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and a likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

[0055] Enabled herein is a method for determining the likelihood of establishment of endometritis in an equine animal, including PMEI, the method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI and optionally one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19 wherein an elevated level of any combination of EBD1, lysozyme

and/or SLPI with one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19 relative to a control is indicative of persistent endometritis.

[0056] As indicated above, reference to an equine animal includes a horse, a Przewalski horse, zebra or ass. In an embodiment, the equine animal is a horse. A horse includes a Thoroughbred, Standardbred, Quarter horse and a Warmblood horse.

[0057] The present invention is predicated in part on the development of an assay based on the level of one or more of EBD1, lysozyme and/or SLPI alone or a combination of two or more of EBD1, lysozyme and/or SLPI or all three of EBD1, lysozyme and SLPI optionally further in combination with one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or uterocalin P19, the level or levels of which is/are indicative of a state of endometritis such as but not limited to infectious endometritis or persistent breeding-induced endometritis. A level of persistent endometritis reduces the rate, likelihood or probability of a successful clinical pregnancy without at least some higher level of medical care. The rate of pregnancy in a mare without any indication of inflammation is approximately 60% per cycle. The rate of pregnancy in a mare having severe inflammation prior to breeding is approximately 7-14% per cycle (Riddle *et al.* (2007)). Hence, the present assay is useful in determining the profile of a successful pregnancy or an unsuccessful pregnancy wherein a rate of less than 50% is considered a high risk of an unsuccessful pregnancy and a rate of greater than or equal to 50% is considered a high chance of establishing clinical pregnancy. The present invention is not intended to predict a "successful" term pregnancy but rather conceptus or establishment of an embryo after transfer. The term "mare" is used to describe a female horse, a Przewalski horse, zebra or ass, notwithstanding its more common association with female horses.

[0058] The determination of level of any or all of the biomarkers may be based on level of expression of genes encoding the biomarkers such as *via* mRNA or the level of biomarker protein. For example, in relation to horses and EBD1, oligonucleotide primers as defined

by SEQ ID NO:1 (forward) and SEQ ID NO:2 (reverse) can be used to detect nucleic acid encoding this biomarker. For lysozyme, primers defined by SEQ ID NO:3 (forward) and SEQ ID NO:4 (reverse) may be employed. For SLPI, SEQ ID NO:5 (forward) and SEQ ID NO:6 (reverse) may be used. Other primers for biomarkers are shown in Tables 2 and 4. Encompassed herein are modified forms of these primers containing artificial nucleotides or labels such as labeled nucleotides or fluorescent markers. The primers may also contain one or more nucleotide substitutions, additions or deletions to take into account polymorphisms or natural variants in target mRNA sequence.

[0059] In terms of screening for the "level of expression" or changes in fold of expression of a nucleic acid such as mRNAs, this may be achieved in a variety of ways including screening directly for any of the forms of mRNA or cDNA generated therefrom. Accordingly, either RNA or DNA based screening may be employed. Changes to the absolute levels of any of these markers is indicative of changes to the expression of the subject mRNA. Furthermore, ratios of expression changes are also useful in determining a diagnosis. Still further, the mRNA which is identified and measured may be a whole molecule or a fragment thereof. For example, one may identify only fragments of mRNA from a uterine biopsy, cytobrush or lavage sample, depending on how it has been processed. Provided that the fragment comprises sufficient sequence to indicate its origin with a particular mRNA, fragmented mRNAs are useful in the context of the method of the subject assay.

[0060] Reference to "nucleic acid molecule" should be understood as a reference to both ribonucleic acid molecules and deoxyribonucleic acid molecules and fragments thereof. The subject assay extends, therefore, to both directly screening for mRNA levels in a uterine sample or screening for the complementary cDNA which has been reverse-transcribed from an mRNA population of interest. It is well within the skill of the person of skill in the art to design methodology directed to screening for either RNA or DNA.

[0061] The term "fragment" means a portion of the subject nucleic acid molecule. This is relevant with respect to screening for modulated mRNA levels in a sample. One may

actually be detecting fragments of the subject RNA molecule, which fragments are identified by virtue of the use of a suitably specific probe. Alternatively, one or more precursors of the mRNA may be targeted.

[0062] By "biological sample" is meant a uterine biopsy, cytobrush or lavage sample comprising endometrial tissue or cell extracts derived from a female equine animal. The mRNA may be located in fluid medium or in a medium which comprises cells or cellular debris. Hence, samples include, but are not limited to, a sample comprising or enriched from uterine tissue or fluid. The biological sample may be tested directly or undergo some form of pre-treatment prior to testing. For example, the sample may require the addition of a reagent, such as a buffer, to the addition of a solid support on which the mRNA can be immobilized. It should be further understood that the sample which is the subject of testing may be freshly isolated or it may have been isolated at an earlier point in time and subsequently stored or otherwise treated prior to testing. For example, the sample may have been collected at an earlier point in time and frozen or otherwise preserved in order to facilitate its transportation to the site of testing. In yet another example, the sample may be treated to neutralize any possible pathogenic infection, thereby reducing the risk of transmission of the infection to the technician.

[0063] To the extent that the subject biological sample is harvested from an equine animal should be understood to include a female horse, a Przewalski horse, zebra or ass of breeding age. Any female equine animal is encompassed by the term "mare". In an embodiment, the equine animal is a horse.

[0064] To the extent that it is sought to isolate and analyze the mRNA within the sample, it is necessary to lyse uterine cells in order to expose their nucleic acid content and to thereafter analyze the mRNA subpopulation of nucleic acid molecules. To this end, the analysis of RNA is based on isolation of total RNA followed by PCR amplification of specific transcripts of interest. Methods for isolating and analyzing total RNA are well known. Nucleic acids are exposed and extracted. Nucleic acids may also be free circulating or present in a uterine biopsy, cytobrush or lavage.

[0065] There is a wide variety of methods which can be and have been used to isolate total RNA from samples.

[0066] mRNA amplification or probing steps require use of primers. Reference to a "primer" or an "oligonucleotide primer" should be understood as a reference to any molecule comprising a sequence of nucleotides, or functional derivatives or analogs thereof, the function of which includes hybridization to a region of a nucleic acid molecule of interest. It should be understood that the primer may comprise non-nucleic acid components. For example, the primer may also comprise a non-nucleic acid tag such as a fluorescent or enzymatic tag or some other non-nucleic acid component which facilitates the use of the molecule as a probe or which otherwise facilitates its detection or immobilization. The primer may also comprise additional nucleic acid components, such as the oligonucleotide tag. In another example, the primer may be a protein nucleic acid which comprises a peptide backbone exhibiting nucleic acid side chains. The design and synthesis of primers suitable for use in the subject assay would be well known to those of skill in the art. Particular primers for horses are defined in Tables 2 and 4.

[0067] Various techniques can be used to analyze an amplification product in order to determine relative mRNA expression levels. Their operational characteristics, such as ease of use or sensitivity, vary so that different techniques may be useful for different purposes. They include but are not limited to sequencing, pyrosequencing, enzyme digestion, microarray analysis, denaturing gradient gel electrophoresis, agarose gel based separation, melt curve analysis on real-time PCR cyclers, quantitative real-time PCR (qPCR), denaturing high performance liquid chromatography, mass spectrometry, primer extension, oligonucleotide-ligation, mutation specific polymerase chain reaction, denaturing gradient, electrophoresis (DGGE), temperature gradient denaturing electrophoresis, constant denaturing electrophoresis, single strand conformational electrophoresis and denaturing high performance liquid chromatography (DHPLC).

[0068] It is well within the skill of the person of skill in the art to select and apply an

appropriate method of screening for the mRNA marker expression levels hereinbefore discussed.

[0069] Further enabled herein is an algorithm-based screening assay to screen biological samples from female equine animals for levels of selected mRNAs including a panel of mRNA. Generally, input data in the form of expression levels are collected based on mRNAs or precursor forms thereof or their corresponding cDNA forms and subjected to an algorithm to assess the statistical significance of any elevation or reduction in levels which information is then output data. Computer software and hardware for assessing input data are encompassed by the present invention.

[0070] Another aspect of the present invention contemplates a method of stratifying a female equine animal with respect to the relative risk that a pregnancy will be successful or unsuccessful, the method comprising subjecting the equine animal subject to an assay to determine the levels of one or more of mRNAs encoding EBD1, lysozyme and/or SLPI or their corresponding cDNA forms in a uterine sample to generate an index of probability of the equine animal having a duration of endometritis detrimental or non-detrimental to achieving a clinical pregnancy.

[0071] The present invention further provides the use of the levels of one or more mRNAs encoding one or more of EBD1, lysozyme and/or SLPI or their corresponding cDNA forms in the generation of an index of probability for use in an assay to predict the presence of a duration of endometritis detrimental or non-detrimental to achieving a clinical pregnancy in an equine animal.

[0072] The assay of the present invention permits integration into existing or newly developed veterinary pathology architecture or platform systems. For example, the present invention contemplates a method of allowing a user to determine the status of female equine animal with respect to the likelihood or otherwise of establishing endometritis including PMEI and achieving a clinical pregnancy, the method including:

(a) receiving data in the form of levels of expression of one or more mRNAs

encoding one or more of EBD1, lysozyme and/or SLPI and/or from the user *via* a communications network;

(b) processing the subject data *via* an algorithm which provides a likelihood index value of persistent endometritis;

(c) determining the status of the equine animal in accordance with the results of the likelihood index value in comparison with predetermined values; and

(d) transferring an indication of the status of the equine animal to the user *via* the communications network.

[0073] Reference to an "algorithm" or "algorithmic functions" as outlined above includes an analysis function to determine the level of expression of an mRNA or corresponding cDNA. A range of different architectures and platforms may be implemented in addition to those described above. It will be appreciated that any form of architecture suitable for implementing the present invention may be used. However, one beneficial technique is the use of distributed architectures. In particular, a number of end stations may be provided at respective geographical locations. This can increase the efficiency of the system by reducing data bandwidth costs and requirements, as well as ensuring that if one base station becomes congested or a fault occurs, other end stations could take over. This also allows load sharing or the like, to ensure access to the system is available at all times.

[0074] It will also be appreciated that in one example, the end stations can be hand-held devices, such as PDAs, mobile phones, or the like, which are capable of transferring the subject data to the base station *via* a communications network such as the Internet, and receiving the reports.

[0075] In the above aspects, the term "data" means the levels or concentrations of the biomarkers. The "communications network" includes the internet. When a server is used, it is generally a client server or more particularly a simple object application protocol (SOAP).

[0076] In another embodiment, the present invention contemplates an assay for

determining the level of risk for failure to achieve pregnancy in an equine animal, the assay comprising determining the concentration of one or more of EBD1, lysozyme and/or SLPI in a biological sample from the uterus of the female equine animal wherein an elevated concentration of EBD1, lysozyme and/or SLPI is indicative of the equine animal having a high risk of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and a likelihood of a successful pregnancy following natural breeding, artificial insemination or embryo transfer. In accordance with this embodiment, levels of BD1, lysozyme and/or SLPI may be screened alone or in combination with other biomarkers such as one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19.

[0077] In another embodiment, the present invention contemplates an assay for determining the level of risk of establishing endometritis in an equine animal including PMEI, the assay comprising determining the concentration of one or more of EBD1, lysozyme and/or SLPI in a biological sample from the uterus of the female equine animal wherein an elevated concentration of EBD1, lysozyme and/or SLPI is indicative of the equine animal having a high risk of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis.

[0078] The determination of the concentrations or levels of the biomarkers enables an assay based on the concentrations relative to controls. Alternatively, the assay is based on the application of a statistical and machine learning algorithm. Such an algorithm uses relationships between biomarkers and risk status of not achieving a clinical pregnancy observed in training data (with known endometritis status) to infer relationships which are then used to predict the status of a female equine animal with unknown status with respect to endometritis.

[0079] Hence, the present invention contemplates the use of a knowledge base of training data comprising levels of biomarkers from a female equine animal to generate training data which, upon input of a second knowledge base of data comprising levels of the same

biomarkers from a female equine animal with an unknown endometritis condition, provides an index of probability that predicts the probability of the subject falling pregnant.

[0080] In a particular embodiment, the present invention contemplates the use of a knowledge base of training data comprising levels of biomarkers from an equine animal to generate training data which, upon input of a second knowledge base of data comprising levels of the same biomarkers from a female equine animal with an unknown endometritis condition, provides an index of probability that predicts the probability of the subject developing or establishing endometritis including PMEI. The establishment of endometritis including PMEI affects the likelihood of the equine animal having a successful pregnancy.

[0081] The term "training data" includes knowledge of levels of biomarkers relative to a control. A "control" includes a comparison to levels of biomarkers in an animal devoid of endometritis or may be a statistically determined level based on trials. The term "levels" also encompasses ratios of levels of biomarkers.

[0082] The "training data" also include the concentration of one or more of the biomarkers. The data may comprise information on an increase or decrease in a biomarker concentration.

[0083] Hence, the biomarker levels can be determined at the protein level. The agents which specifically bind to the biomarker proteins generally include an immunointeractive molecule such as an antibody or hybrid, derivative including a recombinant or modified form thereof or an antigen-binding fragment thereof. The agents may also be a receptor or other ligand. These agents assist in determining biomarker protein.

[0084] Hence, the present invention further provides a panel of immobilized ligands to one or more of EBD1, lysozyme and/or SLPI. Optionally, the panel may also comprise immobilized ligands to one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10,

CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19.

[0085] The ligands, such as antibodies specific to each of the biomarkers, enable the quantitative or qualitative detection or determination of the level of one or more biomarkers. Reference to "level" includes concentration as weight per volume, activity per volume or units per volume or other convenient representative as well as ratios of levels.

[0086] As indicated above, the "ligand" or "binding agent" and like terms, refers to any compound, composition or molecule capable of specifically or substantially specifically (that is with limited cross-reactivity) binding to an epitope on the biomarker. The "binding agent" generally has a single specificity. Notwithstanding, binding agents having multiple specificities for two or more biomarkers are also contemplated herein. The binding agents (or ligands) are typically antibodies, such as monoclonal antibodies, or derivatives or analogs thereof, but also include, without limitation: Fv fragments; single chain Fv (scFv) fragments; Fab' fragments; F(ab')2 fragments; and multivalent versions of the foregoing. Multivalent binding reagents also may be used, as appropriate, including without limitation: monospecific or bispecific antibodies; such as disulfide stabilized Fv fragments, scFv tandems [$(scFv)_2$ fragments], diabodies, tribodies or tetrabodies, which typically are covalently linked or otherwise stabilized (i.e. leucine zipper or helix stabilized) scFv fragments. "Binding agents" also include aptamers, as are described in the art.

[0087] Methods of making antigen-specific binding agents, including antibodies and their derivatives and analogs and aptamers, are well-known in the art. Polyclonal antibodies can be generated by immunization of an animal. Monoclonal antibodies can be prepared according to standard (hybridoma) methodology. Antibody derivatives and analogs, can be prepared recombinantly by isolating a DNA fragment from DNA encoding a monoclonal antibody and subcloning the appropriate V regions into an appropriate expression vector according to standard methods. Phage display and aptamer technology is described in the literature and permit *in vitro* clonal amplification of antigen-specific binding reagents with very low affinity cross-reactivity. Phage display reagents and systems are available commercially, and include the Recombinant Phage Antibody System (RPAS),

commercially available from Amersham Pharmacia Biotech, Inc. of Piscataway, New Jersey and the pSKAN Phagemid Display System, commercially available from MoBiTec, LLC of Marco Island, Florida. Aptamer technology is described for example and without limitation in US Patent Nos. 5,270,163; 5,475,096; 5,840,867 and 6,544,776.

[0088] ECLIA, ELISA and Luminex LabMAP immunoassays are examples of suitable assays to detect levels of the biomarkers. In one example a first binding reagent/antibody is attached to a surface and a second binding reagent/antibody comprising a detectable group binds to the first antibody. Examples of detectable-groups include, for example and without limitation: fluorochromes, enzymes, epitopes for binding a second binding reagent (for example, when the second binding reagent/antibody is a mouse antibody, which is detected by a fluorescently-labeled anti-mouse antibody), for example an antigen or a member of a binding pair, such as biotin. The surface may be a planar surface, such as in the case of a typical grid-type array (for example, but without limitation, 96-well plates and planar microarrays) or a non-planar surface, as with coated bead array technologies, where each "species" of bead is labeled with, for example, a fluorochrome (such as the Luminex technology described in U. S. Patent Nos. 6,599, 331,6, 592,822 and 6,268, 222), or quantum dot technology (for example, as described in U. S. Patent No. 6,306. 610). Such assays may also be regarded as laboratory information management systems (LIMS).

[0089] In the bead-type immunoassays, the Luminex LabMAP system can be utilized. The LabMAP system incorporates polystyrene microspheres that are dyed internally with two spectrally distinct fluorochromes. Using precise ratios of these fluorochromes, an array is created consisting of 100 different microsphere sets with specific spectral addresses. Each microsphere set can possess a different reactant on its surface. Because microsphere sets can be distinguished by their spectral addresses, they can be combined, allowing up to 100 different analytes to be measured simultaneously in a single reaction vessel. A third fluorochrome coupled to a reporter molecule quantifies the biomolecular interaction that has occurred at the microsphere surface. Microspheres are interrogated individually in a rapidly flowing fluid stream as they pass by two separate lasers in the Luminex analyzer. High-speed digital signal processing classifies the microsphere based on its spectral

address and quantifies the reaction on the surface in a few seconds per sample.

[0090] As used herein, "immunoassay" refers to immune assays, typically, but not exclusively sandwich assays, capable of detecting and quantifying a desired biomarker, namely one of EBD1, lysozyme and/or SLPI.

[0091] The present invention further contemplates a method for facilitating a successful pregnancy outcome in an equine animal, the method comprising assaying a uterine biopsy, cytobrush or lavage sample from the equine animal for levels of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI wherein an elevation in any one or more of these biomarkers is an indication of persistent endometritis wherein a equine animal diagnosed with persistent endometritis is either prevented from undergoing natural or assisted breeding or can only undergo breeding with significantly increased management and veterinary care or the animal is administered with one or more antagonists of EBD1, lysozyme and/or SLPI or an antagonist of another biomarker of innate immunity prior to breeding.

[0092] The present invention further contemplates a method for determining whether an equine animal will establish endometritis including MEIR, the method comprising assaying a uterine biopsy, cytobrush or lavage sample from the equine animal for levels of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI wherein an elevation in any one or more of these biomarkers is an indication of persistent endometritis.

[0093] An equine animal includes a horse, a Przewalski horse, zebra and an ass. In an embodiment, the equine animal is a horse. Hence, the present invention has application to the horse breeding industry as well as zoo breeding programs and development of working equine animals.

[0094] Taught herein a method for facilitating a successful pregnancy outcome in a female horse, the method comprising assaying a uterine biopsy, cytobrush or lavage sample from

the female horse for levels of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI wherein an elevation in any one or more of these biomarkers is an indication of likelihood to develop persistent endometritis wherein a female horse diagnosed with persistent endometritis is either prevented from undergoing natural or assisted breeding or can only undergo breeding with significantly increased management and veterinary care or the animal is administered with one or more antagonists of EBD1, lysozyme and/or SLPI or other biomarkers of innate immunity prior to breeding.

[0095] Taught herein a method for determining whether an equine animal will establish endometritis, the method comprising assaying a uterine biopsy, cytobrush or lavage sample from the female horse for levels of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI wherein an elevation in any one or more of these biomarkers is an indication of likelihood to develop persistent endometritis wherein a female horse diagnosed with persistent endometritis is either prevented from undergoing natural or assisted breeding or can only undergo breeding with significantly increased management and veterinary care or the animal is administered with one or more antagonists of EBD1, lysozyme and/or SLPI or other biomarkers of innate immunity prior to breeding.

[0096] The biomarkers of innate immunity include TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL9, CXCL10, CXCL11, EBD1, lysozyme, SLPI, LCN2, lactoferrin and uteroferrin as well as $sPLA_2$ and P19. Antagonists of any or all of these may be employed.

[0097] Specific antagonists to EBD1, lysozyme and/or SLPI are of particular use prior to undergoing breeding. The aim is to ameliorate the inflammatory conditions associated with an innate immune response.

[0098] The antagonists are also referred to as medicaments. Hence, contemplated herein is the use of an antagonist of an innate immunity biomarker in the manufacture of a medicament to treat endometritis in a female equine animal prior to that subject undergoing natural or assisted pregnancy protocol.

[0099] In an embodiment, the medicament targets one or more of TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL9, CXCL10, CXCL11, EBD1, lysozyme, SLPI, LCN2, lactoferrin and uteroferrin as well as $sPLA_2$ and P19. In an embodiment, the medicament targets EBD1, lysozyme and/or SLPI. The "medicament" may comprise multiple antagonists, i.e. one for each biomarker targeted.

[0100] Veterinary pharmaceutical compositions are therefore encompassed by the present invention.

[0101] The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the antagonists of the biomarkers of the present invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

[0102] The pharmaceutical compositions of the present invention may be administered in any number of ways. Administration may be topical (including ophthalmic and to mucous membranes including vaginal, uterine and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion. Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

[0103] The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or

both, and then, if necessary, shaping the product.

[0104] The formulation of therapeutic compositions and their subsequent administration (dosing) is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the endometritic condition is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the female equine animal. Medical practitioners of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models.

[0105] Alternatively, rather than antagonists of the biomarkers, another treatment regime is implemented to reduce the endometritis. Such alternative regimes include antibiotics, antibodies to potential microbial pathogens and anti-inflammatory agents.

[0106] Accordingly, taught herein a method for facilitating a successful pregnancy outcome in a female horse, the method comprising assaying a uterine biopsy, cytobrush or lavage sample from the female horse for levels of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI wherein an elevation in any one or more of these biomarkers is an indication of indication of likelihood to develop persistent endometritis wherein a female horse diagnosed with persistent endometritis is either prevented from undergoing natural or assisted breeding or can only undergo breeding with significantly increased management and veterinary care or the animal is administered with one or more anti-inflammatory agents or anti-microbial agents prior to breeding.

[0107] Further taught herein is a diagnostic device which enables detecting the elevation of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI in a uterine sample from an equine animal, the device comprising a sample end, the sample end comprising an immobilized ligand of the biomarker protein or nucleic acid complementary

to a targeted mRNA species or amplified form thereof to be measured and means to determine if the biomarker is bound to its ligand wherein an elevated amount of the biomarker compared to a statistically validated level or control is indicative that the equine animal is not receptive for achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and a likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

[0108] Taught herein is an equine animal model for an innate immune response associated with endometritis. The equine animal model is defined by elevated biomarkers defining an innate immune response. The equine animal model is generated by infecting the uterus of a female equine animal with a microorganism. A transient environment of innate immunity biomarker expression comprising elevated levels of TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL9, CXCL10, CXCL11, EBD1, lysozyme, SLIPI, LCN2, lactoferrin and uteroferrin as well as $sPLA_2$ and P19. A transient state of endometritis ensues which provides a model to test medicaments for their ability to ameliorate the endometritic environment. Such medicaments are therefore useful prior to natural or assisted breeding to maximize the likelihood of a successful pregnancy.

[0109] Accordingly, enabled herein is an equine animal model for endometritis, the animal model comprising a female equine animal artificially subject to intrauterine infection with a microorganism for a time and under conditions sufficient to induce elevated expression of biomarkers of innate immunity, the biomarkers selected from the group consisting of TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL0, CXCL10, CXCL11, EBD1, lysozyme, SLIPI, LCN2, lactoferrin and uteroferrin as well as $sPLA_2$ and P19. In an embodiment, the equine animal is selected from a horse, a Przewalski horse, zebra and ass. In an embodiment, the microorganism is *E. coli* or a species of *Streptococcus* such as *Streptococcus equi* including sub-species *zooepidemicus*.

[0110] In an embodiment, the equine animal is a horse in particular a female horse.

[0111] The present invention extends to kits comprising reagents useful in measuring the levels of biomarkers such as EBD1, lysozyme, SLPI, TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, $sPLA_2$ and/or P19. Such reagents may include immobilizing reagents, solid supports such as beads, antibodies and fluorescent or chemiluminescent agents. The kit may further comprise a receptacle adapted to receive a uterine sample.

[0112] Further taught herein is a method of conducting a business in relation to equine animals. The method is a business model comprising determining whether a equine female animal is prone to the development of a condition of persistent endometritis by the determination of levels of EBD1, lysozyme and/or SLPI, individually or together and optionally with one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19 wherein a decision to purchase the animal, use the animal in a breeding program and/or to insure the animal is based on the level of risk that persistent endometritis may impair the successful initiation of a pregnancy. In an embodiment, the equine animal such as a horse, a Przewalski horse, zebra or ass.

[0113] Taught herein is an assay for detecting EBD1, lysozyme and/or SLPI individually or together and optionally with one or more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19. In an embodiment, the assay tests a uterine biopsy, cytobrush or lavage for these biomarkers. In an embodiment, the assay determines an elevation in these biomarkers. In an embodiment, the results of the assay are correlated to a likelihood of successful initiation of a pregnancy or otherwise in equine animals.

EXAMPLES

[0114] Aspects disclosed herein are further described by the following non-limiting Examples.

EXAMPLES 1 TO 4

[0115] Many broodmares are affected by persistent endometritis, a disease that has been identified as the third most common medical problem in American horses (Traub-Dargatz *et al.* (1991) *J. Am Vet Med Assoc 198*:1745-1747), and results in reduced pregnancy rates (Riddle *et al.* (2007)). Both breeding and the introduction of bacteria have been shown to induce an inflammatory response in the equine uterus. Thus, the first step to understanding the pathophysiological mechanisms underlying the diseases is to analyze the precise timeline of the inflammatory response in the equine uterus during infectious endometritis in healthy mares. This is the subject of Examples 1 to 4. In addition, the evaluation of the impact the different hormonal environments associated with the oestrous cycle have on this process, provides further insights into the use of experimental inoculation with *E. coli* as a model of infectious endometritis. As the uterine bacterial load during PMIE is unknown, a relatively high dose is used in these Examples in order to reliably induce a measurable immune response in mares resistant to PMIE. The bacterial growth detected during these studies showed that infection was reliably induced in both cycle stages and that it was cleared within the 72 h study period (Table 3), indicating an appropriate dose of *E. coli* was used.

Table 3

Bacterial growth from uterine biopsies and swabs before (0 h) and 3, 12, 24, 48 and 72 h after intrauterine inoculation of E. coli.

Mare ID	Cycle	Biopsy Sample						Swab Sample					
		0 h	3 h	12 h	24 h	48 h	72 h	0 h	3 h	12 h	24 h	48 h	72 h
B	oestrus	1	6	1	1*	0	2*	0	>50	12	0	0	0
E	oestrus	0	0	1	4	1	0	0	>50	2	2	0	0
S	oestrus	0	20	2	0	0	24*	1*	16	0	0	0	0
U	oestrus	0	0	0	1*	0	3*	0	6	0	0	0	0
V	oestrus	0	4	0	1*	0	2	0	3	1	0	>50*	0
B	dioestrus	0	32	>50	16	8	0	0	>50	>50	0	18	0
E	dioestrus	1*	>50	>50	7	0	0	0	>50	>50	>50	0	0
S	dioestrus	0	>50	50	28	11	0	0	>50	>50	51	9	0
U	dioestrus	0	>50	>50	8	4	0	0	>50	>50	43	14	2
V	dioestrus	0	>50	15	2	3	0	0	>50	3	0	0	0

* Other uterine pathogen and/or contamination (lactose negative on MacConkey agar).

Methods

Selection of experimental animals

[0116] Endometrial biopsies were obtained from five Standardbred mares aged between 3 and 4 years. They were maintained on pasture at the facilities of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia.

[0117] For this study, clinically healthy mares were confirmed to be resistant to PMIE based on histopathological evaluation of the endometrium and by insemination with frozen stallion semen during oestrus. This reliably induces uterine inflammation (Kotilainen *et al.* 91994) *Theriogenology 41*:629-636). There were no clinical signs of persistent inflammatory oedema or intrauterine fluid 24 h after insemination. A histopathological evaluation by two independent examiners classified samples from all five mares in category I according to Kenney and Doig's classification (Kenny and Doig (1986) *Current Therapy in Theriogenology 2*:723-729). The absence of any pre-existing bacterial infection

in the uterus was confirmed by conventional bacteriological culture of uterine swabs collected from each mare prior to insemination.

Preparation of E. coli inocula

[0118] An *E. coli* strain (EC_CM1) isolated from the reproductive tract of a mare susceptible to post-breeding endometritis was stored at −80°C. To prepare inocula the strain was streaked onto a Mueller-Hinton agar plate and incubated for 24 h at 37°C. A single colony was transferred to 2 mL of Mueller-Hinton broth and the broth incubated overnight at 37°C. The overnight broth culture was diluted to achieve a final concentration of 10^5 colony forming units (CFU) per inoculum. The inocula were kept on ice for a maximum of 1 h before use.

Inoculation of E. coli and collection of endometrial tissue and swab samples

[0119] Follicle development, intrauterine fluid, development of uterine oedema and cervical and uterine tone were monitored daily by trans-rectal palpation and ultrasonographic examination to determine the stage of the oestrous cycle. Rectal temperature, heart rate and respiratory rate were monitored daily or at each sample collection.

[0120] A five tier oedema score system (E0-E4), with E0 representing the absence of any uterine oedema and E4 representing pathological inflammatory oedema, was used to evaluate uterine oedema indicative of cycle stage and/or inflammation.

[0121] Mares were inoculated with *E. coli* in two consecutive oestrous cycles in a crossover study design. The three randomly assigned mares in group 1 were inoculated with *E. coli* during oestrus, as indicated by the presence of a dominant follicle ≥35 mm in diameter, uterine oedema and decreased uterine and cervical tone. A sample for uterine culture was collected using a double-guarded swab (Minitube Australia, Ballarat, Vic, Australia), before endometrial tissue samples were obtained by trans-cervical biopsy before (0 h) and 3, 12, 24, 48 and 72 h post inoculation (pi) using an alligator jaw biopsy punch (Jorvet, Loveland, CO, USA). Care was taken to obtain biopsies from different sites

at the base of the uterine horns alternating the left and right horn at each time point.

[0122] During the following oestrus, the absence of inflammation was established by the lack of pathological oedema, as well as the lack of neutrophilia or detectable bacterial growth. The same mares were then inoculated with the same strain of *E. coli* during dioestrus, as indicated by detection of a functional corpus luteum and the absence of uterine oedema five days after ovulation. In addition, serum samples were obtained to evaluate plasma progesterone prior to collection. Endometrial biopsies and swabs were again taken before (0 h) and 3, 12, 24, 48 and 72 h pi.

[0123] The two mares assigned to the second group were subjected to the same procedures in reverse order, being initially inoculated during dioestrus and subsequently during oestrus. Samples were taken at the same time points using the same methods as described above.

[0124] After collection, all endometrial biopsies were divided into three sections with a sterile scalpel blade. One section was used immediately for microbiological culture. One section was snap-frozen in liquid nitrogen in OCT embedding medium (Tissue-Tek, Olympus Australia Pty. Ltd, Mount Waverly, VIC, Australia) and stored at −80°C until further processing. The final section was placed in RNAlater (Life Technologies Australia, Mulgrave, VIC, Australia) and incubated overnight on a rocking platform at room temperature before being stored at −80°C until further processing.

Preparation of samples for microbiology

[0125] The portion of the biopsy used for microbiological culture and the uterine swab sample were used to inoculate Mueller-Hinton-Agar plates, which were then incubated aerobically at 37 °C for 24 h to quantify and identify the bacteria in the uterus. Colony counts were scored as: no growth (<5 colony forming units (CFU)); mild growth (5-10 CFU); moderate growth (11-50 CFU); and heavy growth (>50 CFU), as previously described (Christoffersen *et al.* (2010) *supra*).

[0126] Representative colonies from each plate were replated onto MacConkey agar to confirm their identity as *E. coli* by assessing their capacity to ferment lactose and to produce indole from tryptophan. Results were recorded as *E. coli* or other uterine pathogen/contaminant.

Histopathology

[0127] The OCT embedded sections of the 0, 3, 12 and 24 h samples were mounted on a cryostat (Leica, North Ryde, Australia) to cut 7-10 µm thick tissue sections, which were stained with haematoxylin and eosin (H&E). Histopathological assessments of the endometrium were made on 0 h samples according to Kenney and Doig's classification (Kenny and Doig (1986) *supra*).

[0128] Classification of the extent of inflammation was based on the total number of neutrophilic leukocytes present in 3 fields at a magnification of 400 ×, with the categories assigned being: no neutrophilia (<10 neutrophils in total in the three fields), moderate neutrophilia (10-99 neutrophils in total in the three fields), severe neutrophilia (100-149 neutrophils in total in the three fields) and very severe neutrophilia (>150 neutrophils in total in the three fields). In addition, the presence of neutrophils in the epithelial layer and in the endometrial glands was determined for each field and the total number of eosinophil leukocytes was counted.

[0129] The endometrial biopsy samples placed in RNAlater were homogenized in Trizol (Qiagen, Chadstone, Vic, Australia) using a Polytron homogeniser (IKA Works, Selangor, Malaysia) and the total RNA was purified using the RNeasy Universal Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The total RNA was resuspended in 70 µL RNAse-free water and the nucleotide concentration and purity was assessed for each sample by spectrophotometry using a NanoDrop ND-1000 (Thermo Fisher Scientific Australia Pty Ltd, Scoresby, Vic, Australia). All samples had A_{260}/A_{280} ratios greater than 1.99 and A_{260}/A_{230} ratios greater than 1.82. RNA samples were then reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Gladesville, Australia) according to the manufacturer's instructions and the resulting cDNA diluted to a concentration equivalent

to 2 ng total original RNA/ L with RNAse-free water.

Quantitative real-time PCR (qPCR)

[0130] Genes were chosen for further analysis by PCR based on the analysis of next-generation sequencing data previously published (Marth *et al.* (2015) *BMC Genomics 16*:34). These included the genes for the Toll-like receptors TLR2 and 4, the NOD-like receptor NLRC5, the chemokines CCL2, CXCL9, CXCL10 and CXCL11, as well as the antimicrobial peptides equine -defensin 1 (EBD 1), lysozyme, secretory leukoprotease inhibitor (SLPI), secreted phospholipase A_2 ($sPLA_2$), lipocalin 2 (LCN 2), lactoferrin, uteroferrin and the uterocalin precursor (P19). In addition, seven genes with little variation were selected from the sequencing data: -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the ribosomal proteins (RP) RPL17, RPL27A, RPL30, RPL32 and RPS5.

[0131] Primers used to detect the EBD 1 (Davis *et al.* (2004) *Vet Immunol Immunopathol 99*:127-132) and -actin (Christoffersen *et al.* (2010)) genes were sourced from the literature, while specific primer pairs for all other genes of interest were designed using the NCBI nucleotide blast website (Ye *et al.* (2012) *BMC Bioinformatics 13*:1-11). Where possible, primer pairs spanned at least one intron to prevent amplification of contaminating genomic DNA (Table 4). The oligonucleotide sequences are shown in Table 4 and were commercially synthesized by Geneworks (Hindmarsh, SA, Australia).

[0132] Standard curves were created to quantify absolute copy numbers for each gene. Briefly, positive PCR samples were pooled, DNA purified, a ligation reaction set up using the pGEM-T Easy Vector System (Promega) and -Select Bronze Efficiency competent cells (Bioline, Alexandria, Australia) were transformed, inoculated onto LB agar and incubated overnight. A single colony was selected and cultured overnight in LB broth. Plasmid DNA was extracted from the overnight culture. After extraction, the plasmid was sent for sequencing to Monash Micromon (Melbourne, Australia) to confirm the sequence of the insert before dilution series were generated based on plasmid DNA concentration and size. Standard curves containing ten-fold dilutions from 30 000 000 to 300 copies per

5 L were created for each gene.

[0133] Quantitative real-time PCRs (qPCRs) were performed using the Rotor-Gene Q PCR machine (Qiagen). Each 20 L reaction was set up with 10 L iTaq Universal SYBR Green Supermix (Bio-Rad), 1 L of each primer (forward and reverse, 10 M), 3 1 RNAse-free water and 5 L cDNA. A no-template control (RNAse-free water) and the appropriate standard curves were included in each run. All samples were run in triplicate. The reactions were incubated through an initial activation and denaturation step at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. A melt curve analysis was performed between 65 and 95°C in 0.5°C increments to confirm the identity of the amplicons.

Data analysis and statistical methods

[0134] All statistical analyzes were performed using Stata release 14 (StataCorp, College Station, TX, USA). Results were considered statistically different if the value of *P* was <0.05. To assess the distribution of all variables, we generated frequency distributions for all categorical variables and histograms and summary measures for all continuous variables. For comparison of the presence of bacteria, a multi-level mixed-effects generalized linear model (with logit link function) was used with horse id entered as a random effect to account for repeated measurement and individual variability by horse. Data were log-transformed where appropriate.

[0135] Cycle stage comparisons were performed using interaction terms to combine time and cycle stage. The effect of the introduction of *E. coli* on clinical parameters and the expression of several genes associated with the immune response were analyzed using a repeated measures ANOVA applying the Bonferroni correction for multiple comparisons. The interaction model was then used to estimate the fold change of gene expression at different time points in comparison to 0 h, which was designated as reference category.

Table 4

Primer sequences used for quantitative reverse transcriptase PCR

Gene name	Forward Primer Sequence	Reverse Primer Sequence	Insert Size (bp)	Inter-exonic	Accession number/ Reference
EBD1	ATTTTCTCCTTGCCTTCCTCAT (SEQ ID NO:1)	GATACAAGTGCCGATCTGT (SEQ ID NO:2)	148	yes	(Davis (2004))
Lysozyme	TGGGTCTGTTTGGCCAGATG (SEQ ID NO:3)	GTGAGGTCGTGGTTCTGACA (SEQ ID NO:4)	284	yes	XM_001494130.2
SLPI	CTTCCCCCTCGTGCTTCTTG (SEQ ID NO:5)	CAATGGGGTCCAGGCATTTG (SEQ ID NO:6)	209	yes	XM_005604654.1
CCL2	CAACAACTCTCAGGCCGAA (SEQ ID NO:7)	ATCTCCTTGGCCAATATGGTCT (SEQ ID NO:8)	262	yes	NM_001081931.1
CXCL9	AACAGTTTGCTCCAAGCCCT (SEQ ID NO:9)	CTTTTGACGAGGACGTTGCC (SEQ ID NO:10)	221	yes	NM_001130078.1
CXCL10	CCTCTCTCTAGAACTGCACGC (SEQ ID NO:11)	GACGGTCTTGGACTCTGGATT (SEQ ID NO:12)	180	yes	NM_001114940.1
CXCL11	GGCCCTGGAGTAAAAGCAGT (SEQ ID NO:13)	AACAGGCCGGAGAAAGTCAG (SEQ ID NO:14)	301	yes	NM_001278930.1
Lipocalin 2	GACCACAGCTACAACGTCAC (SEQ ID NO:15)	TCAGCTCCTTGGTCCTCCTAT (SEQ ID NO:16)	253	yes	XM_005605817.1
P19	TGATGACGGCTCACAAAACG (SEQ ID NO:17)	GCACCGATCAGTTTGGGTCAG (SEQ ID NO:18)	269	yes	NM_001082509.2

Gene name	Forward Primer Sequence	Reverse Primer Sequence	Insert Size (bp)	Inter-exonic	Accession number/ Reference
TLR2	GTGGACGGTGTGGGTCTTAG (SEQ ID NO:19)	TGATGTCATTGGACCCCAGC (SEQ ID NO:20)	254	no	NM_001081796.1
TLR4	CTGTTACGGTGCGTCATGCT (SEQ ID NO:21)	ACCTGCAGTTCTGGGAAGTT (SEQ ID NO:22)	301	yes	NM_001099769.1
NLRC5	CAGCTCCAGCACGGTATCAA (SEQ ID NO:23)	GCTAGTGTGGTCTTGCCCAT (SEQ ID NO:24)	377	yes	XM_005608529.1
sPLA2	GTTATGGCTTCTACGGTTGCCACT (SEQ ID NO:25)	ACACCCACGTTTCTGCAGACGATA (SEQ ID NO:26)	119	yes	NM_001100113.2
TIMP1	CTACACCCCCGCTATGGAGA (SEQ ID NO:27)	CTGGTCCGTCCACAAGCAAT (SEQ ID NO:28)	268	yes	NM_001082515.1
GAPDH	GCTTCCCTTCCGCACTGCTA (SEQ ID NO:29)	CTCGGCCTTGACTGTGCCAT (SEQ ID NO:30)	222	yes	NM_001163856.1
-Actin	CGTGGGCCGCCCTAGGCACCA (SEQ ID NO:31)	TTGGCCTTAGGGTTCAGGGGGG (SEQ ID NO:32)	243	yes	(Christoffersen (2010))
RPL17	TACAAAGTCATGCAAATCAAGAGGT (SEQ ID NO:33)	TTGGGAGCTTTGTTCACCTGG (SEQ ID NO:34)	339	yes	NC_009151.2
RPL27A	AGGAAGACCCGGAAACTTCG (SEQ ID NO:35)	TTTGTAGTAGCCCGATCGCACC (SEQ ID NO:36)	315	yes	NC_009150.2
RPL30	GGCCGTCCCGCACCTAAG (SEQ ID NO:37)	ATGACCAGTTTCGCTTTGCCT (SEQ ID NO:38)	166	yes	XM_001491150.4

Gene name	Forward Primer Sequence	Reverse Primer Sequence	Insert Size (bp)	Inter-exonic	Accession number/ Reference
RPL32	TGGTCCACAATGTCAAGGAGC (SEQ ID NO:39)	TCGTCTATTCGTTTTCTTCGCTGC (SEQ ID NO:40)	180	no	XM_001500029.4
RPS5	TGCCATCATCAACAGTGGTCC (SEQ ID NO:41)	AGGTTTATTGGGGCTGTGGTCG (SEQ ID NO:42)	301	yes	XM_001495360
Lactoferrin	TGGCTGAACTCCAAGGCAAA (SEQ ID NO:43)	GCGAGCATCACTCTCAGGAA (SEQ ID NO:44)	216	yes	NM_001163974.1
Uteroferrin	CCAATATGGTCCATCGCGGA (SEQ ID NO:45)	GGGGTCCATGAAGTTCCCAG (SEQ ID NO:46)	180	yes	NM_001246672

EXAMPLE 1

Clinical and gynaecological examination

[0136] All mares identified as being in the dioestrus phase of the cycle were found to have plasma progesterone concentrations between 25 and 33 nmol/L, as expected.

[0137] The heart and respiratory rates of all mares remained within physiological limits throughout the experiment. Rectal temperatures were increased at 12 h post inoculation (pi) compared to before and 3 h pi ($P<0.001$;). During dioestrus, the mares had higher rectal temperatures at 3, 12 and 24 h pi compared to their rectal temperatures after inoculation during oestrus at the same time points ($P<0.05$). The rectal temperatures of the mares ranged between 38.7 and 39.7°C at 12 h pi in dioestrus, above the physiological range (37.5-38.5°C). Temperatures were within the normal range in oestrus in the same mares at the same time point.

[0138] Oedema scores prior to inoculation with *E. coli* were in category 2 and 3 in oestrus, while no oedema was detected in dioestrus. Three h after inoculation with *E. coli*, an oedema score of 4 was detected in all horses, irrespective of cycle stage. At 12 h pi, oedema scores ranged from 2-4 in both cycle stages, before decreasing to a range between 1 and 3 at 48 h pi and between 1 and 2 at 72 h pi.

EXAMPLE 2

Bacteriological culture

[0139] No growth was detected prior to inoculation of *E. coli* in any of the cultures. Three h afterwards, growth from biopsy sample cultures ranged from no to moderate growth during oestrus and from moderate to heavy growth in dioestrus, with more than 50 CFU/sample cultured from four of five mares (Table 3). From 12 h until 72 h pi, no growth was detected from any biopsies taken in oestrus, except for one horse with moderate growth of bacteria other than *E. coli* at 72 h pi. In dioestrus, the numbers of bacteria remained high, with all samples yielding moderate to heavy growth 12 h pi. In the period from one to two days after the introduction of *E. coli*, culture yielded no to moderate growth in dioestrus samples and no growth was detected in any cultures from 72 h samples. Cultures of swab samples yielded comparable results, with slightly higher numbers of bacteria detected (Table 3). Overall, 8.1 times more samples obtained in dioestrus were culture positive in comparison to oestrus ($P = 0.008$) and significantly more samples obtained between 3 and 24 h pi were culture positive in comparison to the baseline (0 h).

EXAMPLE 3

Histopathology

[0140] One sample taken in dioestrus at 24 h was missing and omitted from the analysis. No inflammation was detected prior to the introduction of *E. coli* in any of the samples. Three h afterwards, the level of neutrophilia ranged from moderate to severe in dioestrus samples, while all oestrus samples had very severe levels of neutrophilia. After 12 h, samples in both cycle stages showed signs of severe to very severe neutrophilia. At 24 h, oestrus samples were classified as having moderate to severe neutrophili and dioestrus samples moderate to very severe neutrophilia, with more neutrophils present in all dioestrus samples when paired with the oestrus samples from the same time point in the same horse. Neutrophils were detected in the epithelium of most samples within the first 12 h, regardless of cycle stage, while there were fewer seen in oestrus at 24 h pi in two of five samples. All but one 3 h sample in dioestrus had neutrophils inside endometrial glands, while two oestrus samples did not have any neutrophils in this location 12 h pi with *E. coli*. Eosinophil counts ranged from 0 to 16 at 3 h, 1 to 56 at 12 h and 0 to 33 at 24 h (Table 5).

Table 5

Leukocyte counts (neutrophils and eosinophils) before (0 h) and 3, 12, and 24 h after intrauterine inoculation of E. coli

Mare ID	Cycle	Neutrophils				Eosinophils			
		0 h	3 h	12 h	24 h	0 h	3 h	12 h	24 h
B	oestrus	7(Ep1, G0)	>150(Ep3, G1)	110(Ep2, G0)	60(Ep1, G0)	2	10	1	0
E	oestrus	1(Ep0, G0)	>150(Ep3, G2)	110(Ep3, G0)	75(Ep2, G0)	3	15	10	22
S	oestrus	7(Ep0, G0)	>150(Ep3, G1)	>150(Ep3, G3)	80(Ep3, G2)	0	16	1	4
U	oestrus	5(Ep0, G0)	>150(Ep3, G3)	>150(Ep3, G1)	130(Ep3, G0)	1	14	23	8
V	oestrus	8(Ep1, G0)	>150(Ep3, G2)	>150(Ep3, G2)	130(Ep3, G3)	1	16	2	1
B	dioestrus	2(Ep0, G0)	80(Ep1, G0)	120(Ep3, G1)	90(Ep3, G0)	0	0	56	7
E	dioestrus	4(Ep0, G0)	125(Ep3, G3)	140(Ep3, G2)	140(Ep3, G0)	1	6	15	8
S	dioestrus	7(Ep0, G0)	130(Ep3, G1)	>150(Ep3, G3)	>150(Ep3, G2)	2	0	35	28
U	dioestrus	6(Ep2, G0)	145(Ep3, G3)	>150(Ep3, G3)		0	14	21	
V	dioestrus	7(Ep0, G1)	145(Ep3, G3)	>150(Ep3, G3)	>150(Ep3, G3)	4	6	14	33

Ep, presence of neutrophils in epithelium in 1, 2 or 3 fields

G, presence of neutrophils in endometrial glands in 1, 2 or 3 field

EXAMPLE 4

Endometrial gene expression

[0141] Seven genes were tested to identify the most stable genes under the experimental conditions used for the study. Of these, GAPDH, RPL30, RPL32 and RPS5 showed no significant variation between any of the time points or cycle stages. This also confirmed that the efficiency of the cDNA synthesis was consistent between samples, allowing for gene-specific absolute quantification.

[0142] The target genes were divided into several groups based on their function in the immune system.

Pathogen recognition receptors

[0143] The mRNA expression levels of the genes for the pathogen recognition receptors TLR2, TLR4 and NLRC5 were two to four times higher at 3 h pi compared to levels before inoculation. The levels of expression of these genes decreased to baseline between 12 and 24 h pi (Table 6).

Chemokines

[0144] There was an increase in expression of all four chemokine genes analyzed at 3 h pi, with levels starting to decrease between 12 and 24 h pi and reaching pre-inoculation levels at 72 h pi. Peak expression levels ranged between 126 and 443 times that of the respective baseline levels and were reached at 3 h pi for CCL2, CXCL10 and CXCL11 and at 12 h pi for CXCL9 (Table 6).

Tissue inhibitors of metallopeptidases

[0145] The levels of expression of the gene for the metallopeptidase inhibitor TIMP-1 increased to 32 times baseline levels at 3 h after inoculation of *E. coli* and remained elevated throughout the experimental period (Table 6).

Table 6

Linear mixed regression coefficients and confidence intervals for all statistically significantly difference comparisons between time points after inoculation with E. coli and baseline (0 h) for pathogen recognition receptors, chemokines and tissue inhibitor of metallopeptidase 1

Group	Gene	Time (h)	*P* value	Contrast	(95% CI)
pathogen recognition receptors	TLR2	3	<0.001	4.3	(2.7,7)
		12	0.005	2.0	(1.2,3.2)
	TLR4	3	<0.001	5.5	(3.4,9.1)
		12	0.006	2.0	(1.2,3.3)
		48	0.007	0.5	(0.3,0.8)
		72	0.022	0.6	(0.3,0.9)
	NLRC5	3	0.018	2.3	(1.2,4.5)
		12	0.010	2.5	(1.2,4.9)
chemokines	CCL2	3	<0.001	257.3	(126.2,524.3)
		12	<0.001	13.9	(6.8,28.3)
		24	<0.001	4.4	(2.2,9)
		48	0.001	3.3	(1.6,6.8)
	CXCL9	3	<0.001	25.5	(12.6,51.5)
		12	<0.001	126.6	(62.7,255.6)
		24	<0.001	41.0	(20.3,82.8)
		48	<0.001	6.6	(3.3,13.4)
	CXCL10	3	<0.001	442.7	(189.2,1035.4)
		12	<0.001	228.1	(97.5,533.5)
		24	<0.001	17.2	(7.4,40.2)
		48	<0.001	4.6	(2,10.9)
	CXCL11	3	<0.001	116.4	(45.1,301)
		12	<0.001	388.7	(150.4,1004.6)
		24	<0.001	23.0	(8.9,59.5)
		48	<0.001	8.5	(3.3,22)
Tissue inhibitor of matrix metallo-peptidases	TIMP1	3	<0.001	31.7	(14.1,71.5)
		12	<0.001	27.8	(12.4,62.7)
		24	<0.001	7.4	(3.3,16.7)
		48	0.002	3.6	(1.6,8.2)
		72	<0.001	10.9	(4.9,24.6)

Contrasts represent back-transformed regression coefficients for multiplicative interpretation. Time, time point in h (h) after inoculation of *E. coli*. CI: confidence interval

Antimicrobial peptides

[0146] Expression profiles of genes for antimicrobial peptides (AMPs) exhibited greater variability in response to *E. coli* (Table 7). While most genes were expressed at higher levels between 3 and 12 h after inoculation compared to baseline, expression of P19 and $sPLA_2$ remained unchanged. The greatest difference between oestrus and dioestrus expression profiles was seen at 12 h pi. Genes for LCN2, UFN and SLPI were expressed at higher levels in dioestrus, while the gene for LFN was expressed at higher levels in oestrus at this time point.

[0147] EBD-1 gene expression did not differ between cycle stages. There was a very rapid increase from levels of between 0 and 5 copies/ng of RNA prior to inoculation of *E. coli* to levels of between 3488 and 96 657 copies/ng of RNA at 12 h after inoculation.

[0148] The expression of the lysozyme gene was increased to 42 times baseline at 3 h pi and remained at a similar level until 24 h pi, after which levels decreased.

[0149] No significant difference in expression was detected for the $sPLA_2$ gene either over time or between cycle stages, with levels averaging 109 409 copies/ ng of RNA.

[0150] SLPI gene expression increased to more than 15 000 times baseline at 12 h pi, before slowly decreasing. Gene expression started with higher levels in oestrus compared to dioestrus, but dioestrus levels were higher than oestrus levels at 12 and 24 h pi. At 48 h pi the level of expression was similar in both oestrus and dioestrus samples, but oestrus levels were higher at 72 h pi.

[0151] Expression of the LCN2 gene increased to 72 times baseline levels at 12 h pi, before slowly declining. The levels of expression were higher in dioestrus at 12 h pi, compared to in oestrus.

[0152] The increase in gene expression of the LFN gene was continuous, reaching 14.7 times baseline levels at 72 h pi. While LFN gene expression in oestrus was higher

throughout the first 48 h pi, dioestrus levels were higher at 72 h pi.

[0153] UFN gene expression increased to 7.2 times higher than baseline levels at 3 h pi in both cycle stages. At 12 h after the introduction of *E. coli*, expression remained at similar levels in dioestrus samples. By 24 h, oestrus and dioestrus levels of mRNA for the UFN gene were similar and did not differ from those in baseline samples.

[0154] Expression of the P19 gene did not change over the first 48 h pi, but then decreased to levels 43 times lower than baseline. Dioestrus levels higher before and at 3 and 12 h pi.

Table 7

Linear mixed regression coefficients and confidence intervals for all statistically significantly different comparisons between time points after inoculation with E. coli and baseline (0 h) for antimicrobial peptides

Group	Gene	Time (h)	*P* value	Contrast	(95% CI)
antimicrobial peptides	EBD1	3	<0.001	1185.8	(239.8,5863)
		12	<0.001	65950.5	(13339,326071.6)
		24	<0.001	7309.0	(1478.3,36137.1)
		48	<0.001	580.3	(117.4,2869.3)
		72	<0.001	102.3	(20.7,505.6)
	Lysozyme	3	<0.001	41.5	(19.8,87)
		12	<0.001	23.6	(11.3,49.4)
		24	<0.001	40.1	(19.2,84.1)
		48	<0.001	19.4	(9.3,40.7)
		72	0.001	3.6	(1.7,7.5)
	SLPI	3	<0.001	510.8	(173.1,1507.9)
		12	<0.001	15661.6	(5305.8,46229.3)
		24	<0.001	1283.8	(434.9,3789.6)
		48	<0.001	408.6	(138.4,1206)
		72	<0.001	197.9	(67,584.1)
	LCN2	3	<0.001	9.4	(3.7,23.8)
		12	<0.001	71.6	(28.2,182.2)
		24	<0.001	64.4	(25.3,163.8)
		48	<0.001	20.4	(8,51.9)
		72	<0.001	19.3	(7.6,49)
	LFN	24	<0.001	6.3	(2.7,15.1)
		48	<0.001	8.1	(3.4,19.4)
		72	<0.001	14.7	(6.2,34.9)
	UFN	3	<0.001	7.2	(2.8,18.6)
		12	<0.001	9.2	(3.6,23.7)
		24	0.035	2.8	(1.1,7.1)
		72	0.014	3.3	(1.3,8.4)
	P19	12	0.006	0.2	(0,0.6)
		24	0.002	0.1	(0,0.5)
		48	<0.001	0.1	(0,0.3)
		72	<0.001	0.0	(0,0.1)

Contrasts represent back-transformed regression coefficients for multiplicative interpretation. Time, time point in h (h) after inoculation of *E. coli.* CI: confidence interval.

[0155] Examples 1 to 4 demonstrate the very rapid response of innate immune processes to the introduction of *E. coli* into the uterine environment. The number of neutrophils in the uterus increased by over 8-fold in three hours. Similarly, selected immune response genes reach peak levels of expression within the first 3 to 12 h pi, and often decreased again by 24 to 48 h pi. From a physiological perspective such an immediate response is likely to be required for effective removal of *E. coli*, a common uterine pathogen, which has a generation time of 20 minutes (Albihn *et al.* (2003) *Acta Vet Scandinavia 44*:121-129). Many of the genes analyzed in this study have not previously been implicated in uterine immune responses.

[0156] The surprisingly small differences in gene expression between oestrus and dioestrus in response to *E. coli* indicate that the hormonal environment has a smaller impact on the innate immune response genes investigated than might be anticipated. This is in contrast to the results anticipated due to the difference in bacterial clearance, neutrophil counts, fluid accumulation and systemic inflammation detected in this study.

[0157] These Examples identify several markers of acute endometritis in the equine uterus.

EXAMPLES 5 TO 9

[0158] As indicated above, persistent mating-induced endometritis is one of the main causes for lower fertility in the mare, with pregnancy rates per cycle decreasing from 60% in mares without signs of inflammation or bacterial infection to as low as 7–14% in mares with severe uterine inflammation prior to breeding (Riddle *et al.* (2007)). Examples 5 to 9 describe the identification of diagnostic markers to predict susceptibility to persistent endometritis in mares based on expression levels of genes associated with the innate immune response. PMIE is used as a model for persistent endometritis.

[0159] All samples were taken prior to breeding or in a cycle in which mares were not bred. Thus, any inflammation, including any up-regulation of gene expression associated with innate immunity, could not have been an immediate transient response to contamination during breeding. Therefore, it can reasonably be assumed that the differences in the expression of immune response genes were directly correlated with differences in the uterine environment between mares resistant and susceptible to PMIE.

Materials and Methods

Selection of mares included in the study

[0160] Endometrial biopsies were obtained from mares of mixed breed and age. These included:

(1) Paired biopsy samples (in oestrus and dioestrus) collected from five mares maintained at the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia, that were confirmed to be resistant to PMIE based on histopathological evaluation of the endometrium and negative bacteriological culture results. In addition, they showed no signs of inflammatory fluid or oedema 24 hours after insemination with frozen stallion semen. These mares have already been described as part of a previous study leading to Examples 1 to 4:

(2) Single biopsy samples from eight mares at a local knackery (Victoria, Australia), taken immediately after slaughter. These samples were categorized by horse

age, histopathological evaluation and bacteriological culture; mares in this group were part of the control group;

(3) Single biopsies from six mares maintained at the facilities of the Department of Veterinary Science, University of Kentucky, Lexington, USA. These horses were categorized as susceptible based on their inability to clear fluid and inflammatory cells from the uterus within 96 hours after breeding. If horses were bred in the same cycle, all biopsies were obtained prior to breeding;

(4) Single biopsies from ten client-owned mares in the care of Matamata Veterinary Services, New Zealand. These horses were categorized as susceptible based on uterine fluid accumulation 48 hours after natural breeding. If horses were bred in the same cycle, all biopsies were obtained prior to breeding.

[0161] For all samples, determination of oestrous cycle stage was based on uterine and ovarian findings and progesterone levels (where available). A summary of the samples can be found in Table 8.

[0162] Mares were determined to be susceptible to PMIE, if they had shown delayed uterine clearance with detectable fluid still present until at least 48 hours after breeding in a previous cycle. Mares in the control group were identified by histopathology (category I) in combination with no growth detectable on bacterial culture. Two 1-year old mares were included in the control group due to their very young age, despite not having been histopathologically evaluated. One mare with an unknown bacteriological status was included in the control group, because no signs of inflammation were detected on histopathology.

[0163] Based on our prior Examples 1 to 4 it was estimated that a minimum sample size of 24 would be required to have 95% confidence and 80% power of detecting a significant difference between two groups that differed by a factor of 2, assuming the geometric mean concentration was 1000 and an equivalent SD in each group of 700, applying Student's t-test on log-transformed data.

Clinical and gynaecological examination

[0164] Trans-rectal palpation and ultrasonographic examination were used to identify functional bodies on the ovaries, uterine oedema, cervical and uterine tone and intrauterine fluid accumulation.

[0165] A five tier oedema score system (E0-E4) was used to evaluate uterine oedema. The absence of any uterine oedema is represented by E0 and pathological inflammatory oedema is represented by E4. Uterine fluid scores were evaluated from F0 to F5, representing the measured fluid depth in centimetres.

[0166] Horse cycle stage was determined using ultrasound findings of the ovaries and the uterus and progesterone levels. Horses with progesterone levels < 3 nmol/mL (equivalent to < 1 ng/ mL) were categorized as being in oestrus, while horses with higher progesterone levels were categorized as being in dioestrus, according to previously established thresholds.

Microbiology and histopathology

[0167] Uterine swabs (collected *ante mortem*) or samples from endometrial biopsies (collected *post mortem*) were used to inoculate sheep blood and MacConkey agar plates, which were then incubated aerobically at 37°C for 24 h. Pathogens were identified using routine microbiological procedures.

[0168] Histopathological assessments of the endometrium were performed according to a previously established classification system (Kenney and Doig (1986) *supra*).

Quantitative real-time PCR

[0169] Total RNA was extracted and reverse transcribed as described for Examples 1 to 4. In brief, RNA was homogenized in Trizol (Qiagen, Chadstone, Vic, Australia), purified using the RNeasy Universal Plus Mini Kit (Qiagen) and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Gladesville, Australia). All cDNA was diluted to a concentration equivalent to 2 ng total original RNA/ 1 with RNAse-free water.

[0170] Primers and quantitative PCR (qPCR) protocols were as described for Examples 1 to 4. Briefly, standard curves were created to quantify absolute copy numbers for each gene. PCR products were ligated into pGEM-T (Promega, Alexandria, Australia) and these ligation products were used to transform -Select Bronze Efficiency competent cells (Bioline, Alexandria, Australia). Plasmid DNA was extracted and dilution series were generated based on plasmid DNA concentration and size. Standard curves containing ten-fold dilutions from 30,000,000 to 300 copies per 5 1 were created for each gene. Quantitative real-time PCRs (qPCRs) were performed in the Rotor-Gene Q PCR machine (Qiagen) using the iTaq Universal SYBR Green Supermix (Bio-Rad) and a melt curve analysis was performed to confirm the identity of the amplicons. The concentrations of transcripts encoding ribosomal proteins RPS5, RPL30 and RPL32 were identified as stable between samples and thus were used to confirm that the efficiency of cDNA synthesis was consistent between samples, allowing gene-specific absolute quantification.

Data analysis and statistical methods

[0171] Statistical analyzes were performed using Stata release 14 (StataCorp, College Station, TX, USA). To assess the distribution of all variables, histograms and summary measures were created. Based on these findings, all data were log-transformed.

[0172] For each of 17 genes previously identified to be associated with acute endometritis for Examples 1 to 4, robust linear regression models were constructed to test their association with PMIE status, age and cycle stage with robust standard errors based on horse ID to account for repeated measurement and individual variability by horse.

[0173] Receiver operating characteristic (ROC) curve analyzes were estimated for all genes that were found to differ significantly (at $P<0.05$) in concentration in regression modelling based on susceptibility to PMIE. Diagnostic sensitivity (Se) and specificity (Sp) were calculated for each possible cut-off threshold and the cut-off with the highest Youden's index ($Y = Se + Sp - 1$) was considered the optimal threshold for each gene. Analyzes were repeated for each individual gene. If Youden's index was >50%, genes

underwent further analyzes including the combinations of several genes considered in series and in parallel.

[0174] The raw data were dichotomized at the optimal cut-off for each gene, 2×2 diagnostic contingency tables were constructed and confidence intervals (CI) for sensitivity and specificity estimated using the library epiR version 0.9-69 (Stevenson *et al.* (2015) *Analysis of Epidemiological Data 0.9*:69) in R version 3.2.3 (R Development Core Team 2015).

[0175] Pairwise correlations were performed to compare mRNA levels between these genes based on the coefficient of determination (R^2). Log transformed mRNA expression levels were also tested for correlation with age, for resistant and susceptible mares separately.

Table 8

Summary of individual traits of the mares entering the study

Horse ID	Breed	Age	Group	Cycle	Prog (nmol/L)	Culture	Histology grade	Origin
RD03	TB	4	R	D	14.3	no growth	I	K
RD04	SB	4	R	D	-	no growth	I	K
RD06	-	5–6	R	D	-	no growth	I	K
RD07	SB	3–4	R	D	29	no growth	I	UoM
RD08	SB	3–4	R	D	25	no growth	I	UoM
RD09	SB	3–4	R	D	32	no growth	I	UoM
RD10	SB	3–4	R	D	33	no growth	I	UoM
RD11	SB	3–4	R	D	27	no growth	I	UoM
RE04	SB	>13	R	E	-	no growth	I	K
RE06	Brumby	1	R	E	0.9	no growth	-	K
RE07	Brumby	1	R	E	1	no growth	-	K
RE08	Pony	1	R	E	<0.6	no growth	I	K
RE09	-	-	R	E	-	-	I	K
RE10	SB	3–4	R	E	-	no growth	I	UoM
RE11	SB	3–4	R	E	-	no growth	I	UoM
RE12	SB	3–4	R	E	-	no growth	I	UoM
RE13	SB	3–4	R	E	-	no growth	I	UoM
RE14	SB	3–4	R	E	-	no growth	I	UoM
SD01	-	-	S	D	-	no growth	-	UK
SD02	-	20	S	D	-	no growth	-	UK
SD03	-	13	S	D	-	no growth	-	UK
SD04	-	22	S	D	-	no growth	II a	NZ
SE01	-	24	S	E	-	no growth	-	UK
SE02	-	23	S	E	-	no growth	-	UK
SE03	-	18	S	E	-	no growth	-	UK
SE04	-	19	S	E	-	-	II b	NZ
SE05	-	15	S	E	-	*Streptococcus*	II b	NZ
SE06	-	13	S	E	-	*Corynebacterium (light growth)*	II b	NZ
SE07	-	16	S	E	-	*Enterobacter (light growth)*	II b	NZ
SE08	-	11	S	E	-	*Streptococcus*	III	NZ
SE09	-	18	S	E	-	no growth	II a	NZ
SE10	-	11	S	E	-	*E. coli*	II b	NZ
SE11	-	10	S	E	-	*E. coli*	II b	NZ
SE12	-	8	S	E	-	no growth	II a	NZ

TB, Thoroughbred

SB, Standardbred
WB, Warmblood
-, unknown/ not collected
R, resistant
S, susceptible
D, dioestrus
E, oestrus
K, knackery
UoM, The University of Melbourne
UK, University of Kentucky
NZ, New Zealand (Matamata Veterinary Clinic)

EXAMPLE 5

Comparison of biomarker levels between PMIE resistant and sensitive horses

[0176] PMIE is a persistent endometritis. A total of 10 samples were collected from resistant mares during dioestrus and 11 samples during oestrus. Five samples were collected from susceptible mares in dioestrus and 14 during oestrus. The median age of resistant mares was 4 years (range: 1 to 13 years), while the median age of susceptible mares was 15 years (range: 5 to 24 years).

Clinical and gynaecological examination

[0177] A corpus luteum was detected by ultrasonography or macroscopic inspection *post mortem* in the ovaries of all mares in dioestrus. No uterine oedema or intrauterine fluid was detected in the uterus of any of these mares. Progesterone levels ranged from 14 to 49 nmol/L.

[0178] Oestrus samples were collected from mares with at least one follicle larger than 30 mm and uterine oedema scores between 1 and 3 in resistant mares and between 3 and 4 in susceptible mares. No fluid was found in any of the resistant mares, while the susceptible mares had fluid scores of between 0 and 4. Where progesterone was measured, it was less than 1.3 nmol/L (Tables 9a and 9b).

Bacteriological culture and histopathology

[0179] No growth was detected in any bacteriological cultures taken from mares in the resistant group. Several Gram-negative and the Gram-positive bacterial species were detected in bacteriological cultures taken from some susceptible mares, while others yielded no growth (Tables 9a and 9b).

Endometrial gene expression

[0180] RPS5, RPL30 and RPL32 expression was comparable between groups and confirmed the consistent efficiency of cDNA synthesis, allowing for absolute quantification using gene-specific standard curves.

[0181] Three samples were excluded from the analysis due to unknown age (Tables 9a and 9b).

[0182] Detailed robust regression outputs for all tested comparisons are presented in Tables 9a and 9b. Sensitivity and specificity of genes analyzed are shown in Tables 10 and 11.

Table 9a

Robust linear regression results for all analyzed genes and comparisons

Gene transcript (log_{10})	Variable	Coeff	SE	z	P>z	95% CI (Coeff)		Contr	95% CI (Contr)	
GAPDH	susc	0.45	0.23	1.94	0.06	-0.03	0.93	2.84	0.94	8.60
	bact	-0.28	0.15	-1.85	0.08	-0.60	0.03	0.52	0.25	1.08
	cycle	-0.04	0.08	-0.48	0.63	-0.19	0.12	0.92	0.64	1.32
	age	-0.01	0.01	-0.58	0.57	-0.04	0.02	0.98	0.92	1.05
TLR2	susc	0.17	0.19	0.91	0.37	-0.22	0.57	1.49	0.60	3.68
	bact	-0.10	0.14	-0.71	0.49	-0.39	0.19	0.79	0.41	1.55
	cycle	-0.04	0.10	-0.40	0.69	-0.24	0.16	0.91	0.58	1.45
	age	0.00	0.01	-0.16	0.87	-0.03	0.02	1.00	0.94	1.06
TLR4	susc	0.44	0.21	2.13	0.04	0.01	0.86	2.74	1.03	7.27
	bact	-0.35	0.16	-2.14	0.04	-0.69	-0.01	0.44	0.20	0.97
	cycle	-0.20	0.09	-2.29	0.03	-0.39	-0.02	0.62	0.41	0.95
	age	-0.02	0.01	-1.54	0.14	-0.05	0.01	0.95	0.89	1.02
NLRC5	susc	0.37	0.35	1.08	0.29	-0.34	1.09	2.37	0.46	12.25
	bact	0.15	0.29	0.51	0.62	-0.45	0.75	1.41	0.35	5.61
	cycle	-0.21	0.12	-1.72	0.10	-0.45	0.04	0.62	0.35	1.10

Gene transcript (log_{10})	Variable	Coeff	SE	z	P>z	95% CI (Coeff)		Contr	95% CI (Contr)	
	age	-0.02	0.02	-0.80	0.43	-0.06	0.03	0.96	0.86	1.07
TIMP1	**susc**	0.75	0.51	1.46	0.16	-0.30	1.80	5.60	0.50	63.16
	bact	-0.26	0.31	-0.86	0.40	-0.89	0.37	0.55	0.13	2.33
	cycle	0.05	0.16	0.33	0.74	-0.28	0.39	1.13	0.53	2.43
	age	-0.03	0.03	-1.00	0.33	-0.09	0.03	0.93	0.81	1.08
CCL2	**susc**	0.82	0.39	2.11	0.05	0.02	1.62	6.63	1.05	42.01
	bact	0.19	0.36	0.53	0.60	-0.55	0.94	1.56	0.28	8.68
	cycle	-0.07	0.12	-0.61	0.55	-0.32	0.17	0.85	0.48	1.50
	age	-0.04	0.02	-1.66	0.11	-0.08	0.01	0.92	0.83	1.02
CXCL9	**susc**	0.54	0.41	1.33	0.20	-0.30	1.38	3.48	0.51	23.92
	bact	0.21	0.36	0.59	0.56	-0.52	0.94	1.62	0.30	8.76
	cycle	-0.01	0.12	-0.12	0.91	-0.27	0.24	0.97	0.54	1.74
	age	-0.02	0.02	-0.94	0.35	-0.07	0.03	0.95	0.85	1.06
CXCL10	**susc**	0.55	0.35	1.58	0.13	-0.17	1.26	3.51	0.68	18.05
	bact	0.31	0.41	0.76	0.45	-0.53	1.16	2.06	0.30	14.32
	cycle	0.21	0.15	1.44	0.16	-0.09	0.51	1.62	0.81	3.24
	age	-0.03	0.02	-1.44	0.16	-0.07	0.01	0.94	0.85	1.03

Gene transcript (log_{10})	Variable	Coeff	SE	z	P>z	95% CI (Coeff)		Contr	95% CI (Contr)	
CXCL11	susc	0.54	0.33	1.62	0.12	-0.15	1.23	3.46	0.71	16.81
	bact	0.41	0.38	1.09	0.29	-0.36	1.19	2.58	0.43	15.46
	cycle	0.07	0.16	0.42	0.68	-0.26	0.39	1.16	0.55	2.46
	age	-0.02	0.02	-0.73	0.47	-0.06	0.03	0.97	0.87	1.07
EBD1	susc	2.70	0.96	2.82	0.01	0.73	4.67	501.34	5.36	46870.87
	bact	0.09	0.61	0.15	0.89	-1.16	1.34	1.23	0.07	22.00
	cycle	-0.72	0.40	-1.78	0.09	-1.55	0.11	0.19	0.03	1.29
	age	-0.01	0.06	-0.24	0.82	-0.13	0.11	0.97	0.74	1.28
LYZ	susc	1.34	0.47	2.83	0.01	0.36	2.32	21.92	2.32	207.53
	bact	-0.12	0.34	-0.34	0.74	-0.82	0.59	0.76	0.15	3.91
	cycle	-0.20	0.12	-1.59	0.12	-0.45	0.06	0.63	0.35	1.14
	age	-0.04	0.03	-1.66	0.11	-0.10	0.01	0.90	0.79	1.03
sPLA2	susc	0.88	0.39	2.30	0.03	0.09	1.68	7.67	1.23	47.76
	bact	-0.52	0.29	-1.76	0.09	-1.12	0.09	0.31	0.08	1.22
	cycle	0.02	0.18	0.10	0.93	-0.34	0.38	1.04	0.45	2.38
	age	-0.02	0.02	-0.85	0.40	-0.07	0.03	0.96	0.86	1.07
SLPI	susc	2.13	0.99	2.15	0.04	0.09	4.18	135.91	1.23	14962.84
	bact	-0.02	0.56	-0.03	0.98	-1.16	1.13	0.97	0.07	13.49

Gene transcript (log_{10})	Variable	Coeff	SE	z	P>z	95% CI (Coeff)		Contr	95% CI (Contr)	
	cycle	-0.06	0.36	-0.17	0.87	-0.80	0.68	0.87	0.16	4.79
	age	-0.02	0.06	-0.25	0.80	-0.14	0.11	0.96	0.72	1.30
LCN2	**susc**	1.38	0.65	2.12	0.04	0.04	2.73	24.21	1.09	538.37
	bact	-0.18	0.45	-0.41	0.69	-1.10	0.74	0.66	0.08	5.46
	cycle	-0.20	0.27	-0.73	0.47	-0.75	0.36	0.64	0.18	2.27
	age	-0.02	0.05	-0.46	0.65	-0.11	0.07	0.95	0.77	1.18
LFN	**susc**	1.80	0.73	2.48	0.02	0.30	3.29	62.83	2.02	1955.85
	bact	-0.53	0.53	-0.99	0.33	-1.62	0.57	0.30	0.02	3.70
	cycle	-0.13	0.29	-0.45	0.66	-0.73	0.47	0.74	0.19	2.95
	age	-0.04	0.04	-1.00	0.33	-0.13	0.05	0.90	0.73	1.11
UFN	**susc**	0.59	0.38	1.58	0.13	-0.18	1.37	3.93	0.66	23.40
	bact	-0.05	0.29	-0.17	0.87	-0.64	0.54	0.90	0.23	3.47
	cycle	-0.08	0.14	-0.58	0.57	-0.38	0.21	0.82	0.42	1.63
	age	0.00	0.03	0.00	1.00	-0.06	0.06	1.00	0.88	1.14
P19	**susc**	-0.56	0.51	-1.10	0.28	-1.60	0.49	0.28	0.03	3.06
	bact	0.59	0.43	1.37	0.18	-0.30	1.48	3.90	0.50	30.39
	cycle	-1.01	0.32	-3.15	0.00	-1.66	-0.35	0.10	0.02	0.45
	age	0.07	0.04	1.74	0.09	-0.01	0.15	1.17	0.97	1.41

susc, comparison between susceptible (susc = 1) and resistant mares (susc = 0)
bact, comparison between samples from which bacteria were cultured (bact = 1) and those from which no bacteria were cultured (bact = 0)
origin, comparison between samples originating from the following sources and selected by slightly different criteria, Knackery (origin = 1), New Zealand (origin = 2),

University of Kentucky (origin = 3) and The University of Melbourne (origin = 4)
cycle, comparison between oestrus (cycle = 1) and dioestrus (cycle = 0)
age, effect of a unit increase (i.e. 1 year) in age
Coeff, regression coefficient on the log10 scale contrasting groups on the basis of presence and absence of the respective variable.
SE, standard error of Coeff
CI, confidence interval
Contr, contrast, represented as the back-transformed coefficient (i.e. $10^{(Coeff)}$) for multiplicative interpretation. A back-transformed Coeff resulting in a Contr = 5 implies a 5-fold increase in the level of mRNA when the variable is positive (i.e. susc = 1)
Highlighted lines, $P < 0.05$

Table 9b

Robust linear regression coefficients and confidence intervals for all statistically significantly different comparisons between resistant and susceptible mares in oestrus and dioestrus

Group	Gene	comparison	P value	Contrast	(95% CI)
		susc	**0.04**	2.74	(1.03,7.27)
PRR	**TLR4**	**bact**	0.04	0.44	(0.2,0.97)
		cycle	0.03	0.62	(0.41,0.95)
chemokine	**CCL2**	**susc**	0.05	6.63	(1.05,42)
	EBD1	**susc**	0.01	501.34	(5.36,46870)
	LYZ	**susc**	0.01	21.92	(2.32,207)
	sPLA2	**susc**	0.03	7.67	(1.23,47)
AMP	**SLPI**	**susc**	0.04	135.91	(1.23,14962)
	LCN2	**susc**	0.04	24.21	(1.09,538)
	LFN	**susc**	0.02	62.83	(2.02,1955)
	P19	**cycle**	0.00	0.1	(0.02,0.45)

susc, comparison between susceptible (susc = 1) and resistant mares (susc = 0)
cycle, comparison between oestrus (cycle = 1) and dioestrus (cycle = 0)
age, effect of a unit increase (i.e. 1 year) in age
Contrasts represent back-transformed regression coefficient for multiplicative interpretation. A contrast of 5 implies a 5-fold increase in the level of mRNA when the variable is positive (i.e. susc = 1).
CI, confidence interval

Table 10

Sensitivity and specificity at estimated optimal cut point for gene expression assays with Youden's indices greater 60%

Gene	optimal cp	Sn	(95% CI)	Sp	(95% CI)	Y	(95% CI)
EBD1	2.322	1.00	(0.79, 1)	0.83	(0.59,0.96)	0.83	(0.38, 0.96)
SLPI	2.841	1.00	(0.79, 1)	0.78	(0.52, 0.94)	0.78	(0.32, 0.94)
LYZ	3.468	0.94	(0.7, 1)	0.78	(0.52, 0.94)	0.72	(0.22, 0.93)
LCN2	4.305	0.75	(0.48, 0.93)	0.89	(0.65, 0.99)	0.64	(0.13, 0.91)
LFN	4.435	0.69	(0.41, 0.89)	0.94	(0.73, 1)	0.63	(0.14, 0.89)

Optimal cp, optimal cut point
Sn, sensitivity; Sp, specificity; Y, Youden's index (Y=Sp+Sn-1)
CI, confidence interval

Table 11

Sensitivity and specificity of genes analyzed in series with EBD1 gene assay

Step	Gene	Sn	(95% CI)	Sp	(95% CI)	Y	(95% CI)
1	EBD1	1.00	(0.79, 1)	0.83	(0.59,0.96)	0.83	(0.38, 0.96)
2	EBD1+SLPI	1.00	(0.79,1)	0.83	(0.59,0.96)	0.83	(0.38,0.96)
	EBD1+LYZ	0.94	(0.7,1)	0.94	(0.73,1)	0.88	(0.42,1)
	EBD1+LCN2	0.75	(0.48,0.93)	0.89	(0.65,0.99)	0.64	(0.13,0.91)
	EBD1+LFN	0.69	(0.41,0.89)	1.00	(0.81,1)	0.69	(0.23,0.89)
3	EBD1+SLPI+LYZ	0.94	(0.7,1)	0.94	(0.73,1)	0.88	(0.42,1)

Sn, sensitivity; Sp, specificity; Y, Youden's index (Y=Sp+Sn-1)
CI, confidence interval
In the grey row are the values for the individual EBD1 gene assay (i.e. step 1). All other rows are the results of the assay of the respective gene analysed when considered in a series interpretation with EBD1 (i.e. a positive test outcome was defined as only when all tests were positive), with one other test (step 2) or two other test (step 3).
Series gene assays with a Youden's index ≥ 83% (as reached by individual EBD1 gene assay) are highlighted in bold and underwent further analyzes.

EXAMPLE 6

Pathogen Recognition Receptors and Tissue Inhibitors of Metallopeptidases

[0183] Gene expression of the pathogen recognition receptors TLR2, TLR4 and NLRC5, and TIMP1 was comparable in resistant and susceptible mares (Figure 1). There is a significant difference between susceptible and resistant mares.

EXAMPLE 7

Chemokines

[0184] Of the analyzed chemokines, only CCL2 had significantly higher mRNA levels in susceptible mares compared to resistant mares. CCL2 gene expression levels were 6.6 times higher in susceptible mares (Figure 2), after adjusting for cycle stage, bacteria and age of the mares.

EXAMPLE 8

Antimicrobial Peptides

[0185] Gene expression levels were significantly higher in susceptible mares for the antimicrobial peptides equine -defensin 1, lysozyme and SLPI, after adjusting for cycle stage and age of the mares. Cycle stage had an impact on EBD1 and P19 mRNA levels (Tables 9a and b). In an embodiment, sPLA2, LCN2 and LFN are also expressed with a significant difference between susceptible and resistant mares.

[0186] EBD1 gene expression was 501 times higher in susceptible mares. Lysozyme mRNA levels were 22 times higher in susceptible mares and comparable between cycle stages. Similarly, SLPI mRNA levels were 136 times higher in susceptible mares and cycle stage also did not influence these expression levels.

[0187] Uterocalin P19 mRNA levels were 10 times higher in samples taken during dioestrus in comparison to samples taken during oestrus, while susceptibility did not

influence gene expression for this gene.

[0188] Comparable gene expression levels were detected for uteroferrin in resistant and susceptible mares irrespective of cycle stage (Figure 3).

Correlations and ROC curve analyzes

[0189] Pathogen recognition receptor TLR 4 and chemokines CCL2 mRNA levels were significantly higher in susceptible mares in comparison to the control group. Detailed outputs from all ROC curve analyzes are presented in Tables 12 to 39. Youden's indices at the optimal cutpoints for TLR4 and CCL2 were 0.18 and 0.44, respectively. Thus, neither assay was further evaluated.

[0190] In the AMP gene group, EBD1, lysozyme, SLPI, sPLA2, LCN2 and lactoferrin mRNA levels were identified as differing significantly between the control group and susceptible mares, irrespective of cycle stage, age or presence of bacteria. Youden's index at the optimal cutpoint for sPLA2 was 0.57, so it was not evaluated further. The optimal cutpoints, and the sensitivity, specificity and Youden's indices for EBD1, lysozyme, SLPI, LCN and lactoferrin gene assays with Youden's indices >60% are presented in Table 3.

[0191] Following a series interpretation of combinations of all assays with Y >60%, EBD1 gene expression assay in combination with SLPI or Lysozyme reached similar or improved results when compared to the individual EBD1 gene expression assay (Table 4).

[0192] Combining EBD1, SLPI and lysozyme assays at their individually optimal cut-off points, using series interpretation, yielded a diagnostic sensitivity of 0.94 (95% CI: 0.7, 1.0) and a specificity of 0.94 (95% CI: 0.73, 1.0), similar to combining EBD1 or SLPI with LYZ alone (Table 4).

[0193] Gene expression of the EBDI and SLPI genes was found to be very highly correlated, while expression of the lysozyme gene was found to be highly correlated with EBDI and SLPI (Figure 5). Correlations between gene expression levels of each of these

three genes and age showed no to low correlation (R^2 between 0.16 and 0.39). When mRNA levels were separated by group there was no correlation with age, except a low negative correlation between lysozyme expression and age in resistant mares (Figure 6).

Table 12

2×2 Diagnostic Contingency Table for TLR4

TEST	TRUE	
	pos	**neg**
pos	10	8
neg	6	10

Table 13

Diagnostic Parameters at Optimal Cut-off Threshold for TLR4

	est	LCL	UCL
se	0.63	0.35	0.85
sp	0.56	0.31	0.78
youden	0.18	-0.34	0.63
ppv	0.56	0.31	0.78
npv	0.63	0.35	0.85
plr	1.41	0.74	2.67
nlr	0.68	0.32	1.44

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 14

2×2 Diagnostic Contingency Table for CCL2

TEST	TRUE	
	pos	**neg**
pos	7	0
neg	9	18

Table 15

Diagnostic Parameters at Optimal Cut-off Threshold for CCL2

	est	LCL	UCL
se	0.44	0.20	0.70
sp	1.00	0.81	1.00
youden	0.44	0.01	0.70
ppv	1.00	0.59	1.00
npv	0.67	0.46	0.83
plr	Inf	NaN	Inf
nlr	0.56	0.37	0.87

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 16

2×2 Diagnostic Contingency Table for EBD1

TEST	**TRUE**	
	pos	**neg**
pos	16	3
neg	0	15

Table 17

Diagnostic Parameters at Optimal Cut-off Threshold for EBD1

	est	LCL	UCL
se	1.00	0.79	1.00
sp	0.83	0.59	0.96
youden	0.83	0.38	0.96
ppv	0.84	0.60	0.97
npv	1.00	0.78	1.00
plr	6.00	2.14	16.86
nlr	0.00	0.00	NaN

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 18

2×2 Diagnostic Contingency Table for LYZ

TEST	TRUE	
	pos	**neg**
pos	15	4
neg	1	14

Table 19

Diagnostic Parameters at Optimal Cut-off Threshold for LYZ

	est	LCL	UCL
se	0.94	0.70	1.00
sp	0.78	0.52	0.94
youden	0.72	0.22	0.93
ppv	0.79	0.54	0.94
npv	0.93	0.68	1.00
plr	4.22	1.76	10.10
nlr	0.08	0.01	0.54

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 20

2×2 Diagnostic Contingency Table for SLPI

TEST	TRUE	
	pos	**neg**
pos	16	4
neg	0	14

Table 21

Diagnostic Parameters at Optimal Cut-off Threshold for SLPI

	est	LCL	UCL
se	1.00	0.79	1.00
sp	0.78	0.52	0.94
youden	0.78	0.32	0.94
ppv	0.80	0.56	0.94
npv	1.00	0.77	1.00
plr	4.50	1.90	10.68
nlr	0.00	0.00	NaN

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 22

2×2 Diagnostic Contingency Table for sPLA2

TEST	**TRUE**	
	pos	**neg**
pos	10	1
neg	6	17

Table 23

Diagnostic Parameters at Optimal Cut-off Threshold for sPLA2

	est	LCL	UCL
se	0.63	0.35	0.85
sp	0.94	0.73	1.00
youden	0.57	0.08	0.85
ppv	0.91	0.59	1.00
npv	0.74	0.52	0.90
plr	11.25	1.61	78.46
nlr	0.40	0.21	0.75

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 24

2×2 Diagnostic Contingency Table for LCN2

TEST	TRUE	
	pos	neg
pos	12	2
neg	4	16

Table 25

Diagnostic Parameters at Optimal Cut-off Threshold for LCN2

	est	LCL	UCL
se	0.75	0.48	0.93
sp	0.89	0.65	0.99
youden	0.64	0.13	0.91
ppv	0.86	0.57	0.98
npv	0.80	0.56	0.94
plr	6.75	1.77	25.70
nlr	0.28	0.12	0.67

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 26

2×2 Diagnostic Contingency Table for LFN

TEST	TRUE	
	pos	neg
pos	11	1
neg	5	17

Table 27

Diagnostic Parameters at Optimal Cut-off Threshold for LFN

	est	LCL	UCL
se	0.69	0.41	0.89
sp	0.94	0.73	1.00
youden	0.63	0.14	0.89
ppv	0.92	0.62	1.00
npv	0.77	0.55	0.92
plr	12.38	1.79	85.53
nlr	0.33	0.16	0.69

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 28

2×2 Diagnostic Contingency Table for EBD1 and SLPI in series

TEST	**TRUE**	
	pos	**neg**
pos	16	3
neg	0	15

Table 29

Diagnostic Parameters at Optimal Cut-off Threshold for EBD1 and SLPI in series

	est	LCL	UCL
se	1.00	0.79	1.00
sp	0.83	0.59	0.96
youden	0.83	0.38	0.96
ppv	0.84	0.60	0.97
npv	1.00	0.78	1.00
plr	6.00	2.14	16.86
nlr	0.00	0.00	NaN

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 30

2×2 Diagnostic Contingency Table for EBD1 and LYZ in series

TEST	TRUE	
	pos	neg
pos	15	1
neg	1	17

Table 31

Diagnostic Parameters at Optimal Cut-off Threshold for EBD1 and LYZ in series

	est	LCL	UCL
se	0.94	0.70	1.00
sp	0.94	0.73	1.00
youden	0.88	0.42	1.00
ppv	0.94	0.70	1.00
npv	0.94	0.73	1.00
plr	16.88	2.50	113.84
nlr	0.07	0.01	0.44

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 32

2×2 Diagnostic Contingency Table for EBD1 and LCN2 in series

TEST	TRUE	
	pos	neg
pos	12	2
neg	4	16

Table 33

Diagnostic Parameters at Optimal Cut-off Threshold for EBD1 and LCN2 in series

	est	LCL	UCL
se	0.75	0.48	0.93
sp	0.89	0.65	0.99
youden	0.64	0.13	0.91
ppv	0.86	0.57	0.98
npv	0.80	0.56	0.94
plr	6.75	1.77	25.70
nlr	0.28	0.12	0.67

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 34

***2×2 Diagnostic Contingency Table** for EBD1 and LFN in series*

TEST	**TRUE**	
	pos	**neg**
pos	11	0
neg	5	18

Table 35

***Diagnostic Parameters at Optimal Cut-off Threshold** for EBD1 and LFN in series*

	est	LCL	UCL
se	0.69	0.41	0.89
sp	1.00	0.81	1.00
youden	0.69	0.23	0.89
ppv	1.00	0.72	1.00
npv	0.78	0.56	0.93
plr	Inf	NaN	Inf
nlr	0.31	0.15	0.65

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 36

2×2 Diagnostic Contingency Table for SLPI and LYZ in series

TEST	TRUE	
	pos	**neg**
pos	15	1
neg	1	17

Table 37

Diagnostic Parameters at Optimal Cut-off Threshold for SLPI and LYZ in series

	est	**LCL**	**UCL**
se	0.94	0.70	1.00
sp	0.94	0.73	1.00
youden	0.88	0.42	1.00
ppv	0.94	0.70	1.00
npv	0.94	0.73	1.00
plr	16.88	2.50	113.84
nlr	0.07	0.01	0.44

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

Table 38

2×2 Diagnostic Contingency Table for EBD1, SLPI and LYZ in series

TEST	TRUE	
	pos	**neg**
pos	15	1
neg	1	17

Table 39

Diagnostic Parameters at Optimal Cut-off Threshold for EBD1, SLPI and LYZ in series

	est	LCL	UCL
se	0.94	0.70	1.00
sp	0.94	0.73	1.00
youden	0.88	0.42	1.00
ppv	0.94	0.70	1.00
npv	0.94	0.73	1.00
plr	16.88	2.50	113.84
nlr	0.07	0.01	0.44

est, estimate; LCI, lower confidence interval; UCI, upper confidence interval; se, sensitivity; sp, specificity; ppv, positive predictive value; npv, negative predictive value; plr, positive likelihood ratio; nlr, negative likelihood ratio

[0194] Pathogen recognition receptor TLR 4 and chemokines CCL2 mRNA levels were significantly higher in susceptible mares in comparison to resistant mares. Youden's indices at the optimal cut points for the TLR4 and CCL2were 0.18 and 0.44, respectively, thus, none of these assays was further evaluated.

[0195] In the AMP gene group, EBD1, lysozyme, SLPI, sPLA2, LCN2 and LFN mRNA levels were identified as differing significantly between resistant and susceptible mares, irrespective of cycle stage, age and presence of bacteria.

[0196] The optimal cut point for the EBD1 gene expression assay was estimated to be 2.322, yielding a sensitivity of 1.0 (95% CI: 0.79, 1.0), a specificity of 0.83 (95% CI: 0.59,0.96) and an area under the ROC curve of 0.951. For the lysozyme gene expression assay, the optimal cut point was 3.468, yielding a sensitivity of 0.94 (95% CI: 0.7, 1.0), a specificity of 0.78 (95% CI: 0.52, 0.94) and an area under the ROC curve of 0.910. The optimal cut point for the SLPI gene expression assay was 2.841, yielding a sensitivity of 1.0 (95% CI:0.79 1.0), a specificity of 0.78 (95% CI: 0.52, 0.94) and an area under the ROC curve of 0.931 (Figure 4). Youden's indices for these three antimicrobial peptides were between 0.72 and 0.83, so further analyzes were performed on these genes.

[0197] Following series interpretation of the assays, the EBD1 and lysozyme gene expression assays yielded a combined sensitivity of 0.94 (95% CI: 0.7, 1.0) and a

specificity of 0.94 (95% CI: 0.73, 1.0), similar to the EBD1 gene expression assay on its own. Combining the EBD1 and SLPI gene expression assays yielded an estimated sensitivity of 1.0 (95% CI: 0.79, 1.0) and a specificity of 0.83 (95% CI: 0.59, 0.96). Combining all three assays at their individually optimal cut-off points, using series interpretation, yielded a diagnostic sensitivity of 0.94 (95% CI: 0.7, 1.0) and a specificity of 0.94 (95% CI: 0.73, 1.0).

[0198] Gene expression of the EBD1 and SLPI genes was found to be very highly correlated, while expression of the lysozyme gene was found to be highly correlated with EBD1 and SLPI (Figure 5). Correlations between gene expression levels of each of these three genes and age showed no to low correlation (R^2 between 0.16 and 0.39). When mRNA levels were separated by group there was no correlation with age (Figure 6), except a low negative correlation between lysozyme expression and age in resistant mares.

EXAMPLE 9

Development of diagnostic and therapeutic protocol for persistent endometritis

[0199] The present invention identifies diagnostic markers to predict susceptibility to persistent endometritis in mares based on expression levels of genes associated with the innate immune response. By analyzing the gene expression profiles, EBD1, lysozyme and SLPI are identified as key markers for diagnosing the disease. This is based on PMIE being a model for persistent endometritis.

[0200] The genes for CCL2, as well as the AMP EBD1, lysozyme, SLPI, sPLA2, LCN2 and LFN were consistently expressed at higher levels in susceptible mares, regardless of cycle stage. This increase was detected prior to breeding or any other introduction of potentially inflammatory agents. Further analyzes were performed on EBD1, lysozyme and SLPI due to the high diagnostic sensitivity and specificity of their gene expression assays when interpreted individually.

[0201] The defensin EBD1 has been detected in samples from several equine organs.

Examples 1 to 4 show EBD1 to be significantly up-regulated in response to the introduction of *E. coli* into the equine uterus, indicating an antimicrobial function. However, its exact mechanism of action has not been described and can only be inferred from other related defensins, which induce pore formation in the bacterial cell wall and activate the host immune system (Linde *et al.* (2008)). The higher levels of mRNA encoding this gene in susceptible mares compared to resistant mares indicate an association with chronic inflammation, even when no bacteria are cultured and no inflammation is histologically detected. This may indicate a more sensitive test for infertility than the routinely used combination of histopathological examination and microbiological culture.

[0202] The bactericidal function of lysozyme was first described over 90 years ago and is well characterized. This enzyme hydrolyzes the peptidoglycan bonds unique to bacteria, causing a loss of cell wall integrity and thus cell lysis (Callewaert and Michiels (2010) *Journal of Biosciences 35(1)*:127-160). Up-regulated lysozyme expression in response to intrauterine infusion of *Streptococcus zooepidemicus* (Pycock and Allen (1990) *Equine Veterinary Journal 22(6)*:422-425) and up-regulated lysozyme gene expression in response to *E. coli* (Examples 1 to 4) have been detected in the equine uterus. The consistently higher gene expression in susceptible mares also indicated that lysozyme may play a role in chronic, sometimes subclinical, endometritis leading to reduced fertility in affected horses.

[0203] The SLPI amino acid sequence is homologous to that of equine neutrophil antimicrobial peptide 2 (eNAP2) [Tomme *et al.* (1998) *Thorax 53(2)*:114-116], and both peptides have been shown to have antimicrobial function as a result of inhibition of bacterial enzymes (Couto *et al.* (1993) *Infection and Immunity 61(7)*:2991-2994; Hiemstra *et al.* (1996)). In addition, an *in vitro* study found that SLPI suppresses the activation of the pro-inflammatory NF- B pathway by LPS in murine macrophages (Jin *et al.* (1997) *Cell 88(3)*:417-426), indicating a potential anti-inflammatory role. SLPI gene expression has been detected during experimentally induced endometritis (Examples 1 to 4). Gene expression levels in susceptible mares were found to be higher than in resistant mares in

this study, indicating upregulation during chronic endometritis. While its function may be primarily antimicrobial, such high levels of expression may also reflect the uterus's attempt to limit persistent inflammation.

[0204] Levels of expression of genes for EBD1, lysozyme and SLPI were highly correlated (Figure 5). Levels of expression of these three genes did not differ between samples from which bacteria could be cultured and other samples from susceptible mares from which bacteria could not be cultured.

[0205] This is particularly important in light of the differences in the age of mares between the groups compared in this study. There was no evidence of an association between age and expression of EBD1, lysozyme or SLPI, once samples were stratified according to their susceptibility to PMIE. This indicates that the increased expression of these genes is due to susceptibility and not old age alone, which advocates the potential of their gene expression assays as diagnostic tools (Figure 6).

[0206] For the 40 samples analyzed, diagnostic sensitivity of between 94% and 100% was achieved based on ROC curve-derived optimal cut-offs for expression of EBD1, lysozyme or SLPI, interpreted individually, with specificity ranging from 78% to 83%. The best overall diagnostic predictor for PMIE involved combining assays of EBD1 and lysozyme gene expression, which yielded sensitivity and specificity of 94%. Gene expression of EBD1, SLPI and lysozyme enable the prediction of susceptibility of mares to PMIE and therefore persistent endometritis. A decision can then be made to avoid breeding, insemination or embryo transfer, to manage the breeding very intensely or to first treat the animal to reduce EBD1, lysozyme and/or SLPI.

[0207] In conclusion, this study has yielded valuable insights into both the pathophysiological mechanisms associated with persistent endometritis and the diagnostic potential of assays measuring levels expression some of analyzed genes, in particular those for EBD1, lysozyme and SLPI. Aspects disclosed herein has been published in part after the priority date in Marth *et al.* (2017) *Reproduction, Fertility and Development*

https://doi.org/10.1071/RD17157, Marth *et al.* (2016a) *Reprod. Fertil. Dev. 28*:1810-1824, Marth *et* al. (2016b) *Vet. Res. 47*:110 which are incorporated herein by reference.

[0208] Those skilled in the art will appreciate that the disclosure described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure contemplates all such variations and modifications. The disclosure also enables all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features or compositions or compounds.

[0209] All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference in their entirety as if physically present in this specification.

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CLAIMS:

1. A method for determining the likelihood of achieving a clinical pregnancy in a female equine animal, said method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of a biomarker selected from the list consisting of equine β-defensin1 (EBD1), lysozyme and secretory leukoprotein inhibitor (SLPI) wherein an elevated level of any one of these biomarkers relative to a control is indicative of subjects prone to develop persistent endometritis and an increased likelihood of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and an increased likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer.

2. A method for determining the likelihood of a female equine animal establishing endometritis including persistent mating-induced endometritis, said method comprising obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of a biomarker selected from the list consisting of equine β-defensin1 (EBD1), lysozyme and secretory leukoprotein inhibitor (SLPI) wherein an elevated level of any one of these biomarkers relative to a control is indicative of subjects prone to develop persistent endometritis.

3. The method of Claim 1 or 2 wherein the equine animal is selected from the group consisting of a horse, a Przewalski horse, zebra and an ass.

4. The method of Claim 3 wherein the equine animal is a horse.

5. The method of Claim 4 wherein the level of sensitivity for detecting persistent endometritis is greater than approximately 80% with a specificity of about 70%.

6. The method of any one of Claims 1 to 4 wherein the biomarker assayed is EBD1.

7. The method of any one of Claims 1 to 4 wherein the levels of lysozyme and/or

SLPI are determined.

8. The method of Claim 7 wherein the levels of lysozyme and/or SLPI are determined in combination with EBD1.

9. The method of any one of Claims 1 to 8 wherein a level of EBD1 and/or lysozyme and/or SLPI within a statistically validated range not associated with persistent endometritis is indicative of a duration of endometritis not likely to prevent achieving a clinical pregnancy.

10. The method of any one of Claims 1 to 9 further comprising determining the level of another biomarker selected from the group consisting of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and P19.

11. The method of any one of Claims 1 to 10 wherein the level of the biomarker is determined by the level of mRNA expressed or corresponding cDNA encoding the biomarker.

12. The method of any one of Claims 1 to 10 wherein the level of the biomarker is determined by the amount of biomarker protein in the sample.

13. The method of any one of Claims 1 to 12 wherein natural breeding or artificial insemination or embryo transfer occurs in the presence of low levels of one or more of the biomarkers.

14. The method of any one of Claims 1 to 12 wherein natural breeding or artificial insemination or embryo transfer does not occur in the presence of high levels of one or more of the biomarkers.

15. The method of any one of Claims 1 to 12 wherein if a biomarker is found elevated, an antagonist is administered of that biomarker to reduce its activity or levels prior to

natural breeding or artificial insemination or embryo transfer.

16. The method of Claim 15 wherein the antagonist is a biomarker-specific antibody.

17. The method of Claim 15 wherein the antagonist is a chemical molecule or proteinaceous inhibitor of the biomarker or soluble ligand to the biomarker.

18. Use of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI in the manufacture of an assay to detect persistent endometritis in female equine animals.

19. Use of Claim 18 wherein the equine animal is selected from the group consisting of a horse, a Przewalski horse, zebra and an ass.

20. Use of Claim 19 wherein the equine animal is a horse.

21. Use of Claim 20 wherein the level of sensitivity for detecting persistent endometritis is greater than approximately 80% with a specificity of greater than 70%.

22. Use of any one of Claims 17 to 21 wherein the biomarker assayed is EBD1.

23. Use of any one of Claims 17 to 21 wherein the levels of lysozyme and/or SLPI are determined.

24. Use of Claim 23 wherein the levels of lysozyme and/or SLPI are determined in combination with EBD1.

25. Use of any one of Claims 17 to 24 wherein a level of EBD1 and/or lysozyme and/or SLPI within a statistically validated range not associated with persistent endometritis is indicative of a level of endometritis not likely to prevent achieving a clinical pregnancy.

26. Use of any one of Claims 17 to 25 further comprising determining the level of another biomarker selected from the group consisting of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and P19.

27. Use of any one of Claims 17 to 26 wherein the level of the biomarker is determined by the level of mRNA expressed or corresponding cDNA encoding the biomarker.

28. Use of any one of Claims 17 to 26 wherein the level of the biomarker is determined by the amount of biomarker protein in the sample.

29. Use of any one of Claims 17 to 28 wherein natural breeding or artificial insemination or embryo transfer is to occur in the presence of low levels of one or more of the biomarkers.

30. Use of any one of Claims 17 to 28 wherein natural breeding or artificial insemination or embryo transfer is not to occur in the presence of high levels of one or more of the biomarkers.

31. Use of antagonist of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI in the manufacture of a medicament to facilitate a successful pregnancy in female equine animals.

32. Use of Claim 31 wherein the equine animal is selected from the list consisting of a horse, a Przewalski horse, zebra and an ass.

33. Use of Claim 32 wherein the equine animal is a horse.

34. Use of Claim 32 wherein the level of sensitivity for detecting persistent endometritis is greater than approximately 80% with a specificity of greater than 70%.

35. Use of any one of Claims 31 to 34 wherein the biomarker assayed is EBD1.

36. Use of any one of Claims 30 to 34 wherein the levels of lysozyme and/or SLPI are determined.

37. Use of Claim 36 wherein the levels of lysozyme and/or SLPI are determined in combination with EBD1.

38. Use of any one of Claims 30 to 37 wherein a level of EBD1 and/or lysozyme and/or SLPI within a statistically validated range not associated with persistent endometritis is indicative of a duration of endometritis not likely to prevent achieving a clinical pregnancy.

39. Use of any one of Claims 30 to 38 further determining the level of another biomarker selected from the group consisting of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and P19.

40. Use of any one of Claims 30 to 38 wherein the level of the biomarker is determined by the level of mRNA expressed or corresponding cDNA encoding the biomarker.

41. Use of any one of Claims 30 to 40 wherein the level of the biomarker is determined by the amount of biomarker protein in the sample.

42. Use of any one of Claims 30 to 41 wherein natural breeding or artificial insemination or embryo transfer is to occur in the presence of low levels of one or more of the biomarkers.

43. Use of any one of Claims 30 to 41 wherein natural breeding or artificial insemination or embryo transfer is not to occur in the presence of high levels of one or more of the biomarkers.

44. A mechanical device which enables detecting the elevation of a biomarker selected from the group consisting of EBD1, lysozyme and SLPI in a uterine biopsy, cytobrush or lavage sample from a female equine animal, said device comprises a sample end and comprising an immobilized ligand of the biomarker to be measured or an immobilized nucleic acid completely to part of the mRNA or cDNA encoding the biomarker, the device comprising reading means to determine if the biomarker is bound to its ligand wherein an elevated amount of the biomarker compared to a statistically validated level or control is indicative that the female equine animal is less receptive for a successful pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and an increased likelihood of a successful pregnancy following natural breeding, artificial insemination or embryo transfer.

45. The mechanical device of Claim 44 wherein the equine animal is selected from the list consisting of a horse, a Przewalski horse, zebra and an ass.

46. The mechanical device of Claim 45 wherein the equine animal is a horse.

47. The mechanical device of Claim 46 wherein the level of sensitivity for detecting persistent endometritis is greater than approximately 80% with a specificity of about 70%.

48. The mechanical device of any one of Claims 44 to 46 wherein the biomarker assayed is EBD1.

49. The mechanical device of any one of Claims 44 to 46 wherein the levels of lysozyme and/or SLPI are determined.

50. The mechanical device of Claim 49 wherein the levels of lysozyme and/or SLPI are determined in combination with EBD1.

51. The mechanical device of any one of Claims 43 to 50 further comprising determining the levels of another biomarker selected from the group consisting of TLR2,

TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and P19.

52. A method of treating a female equine animal to facilitate a successful pregnancy following natural breeding, artificial insemination or embryo transfer, said method comprising determining the likelihood of a successful pregnancy by obtaining a uterine biopsy, cytobrush or lavage sample, determining the level of a biomarker selected from the list consisting of EBD1, lysozyme and SLPI wherein an elevated level of any one of these biomarkers relative to a control is indicative of mares prone to develop persistent endometritis and an increased likelihood of not achieving a clinical pregnancy wherein a non-elevated level relative to a control is indicative of non-persistent endometritis and an increased likelihood of achieving a clinical pregnancy following natural breeding, artificial insemination or embryo transfer wherein if there is a likelihood of persistent endometritis at a level to prevent a successful pregnancy, a medicament is administered to the female equine animal to reduce the level of one or more of EBD1, lysozyme and/or SLPI for a time and condition sufficient for an embryo to successfully develop *in utero* and/or the female equine animal is subject to medical intervention.

53. The method of Claim 52 wherein the equine animal is selected from the group consisting of a horse, a Przewalski horse, zebra and an ass.

54. The method of Claim 53 wherein the equine animal is a horse.

55. The method of any one of Claims 52 to 54 wherein the biomarker assayed is EBD1.

56. The method of any one of Claims 52 to 54 wherein the levels of lysozyme and/or SLPI are determined.

57. The method of Claim 56 wherein the levels of lysozyme and/or SLPI are determined in combination with EBD1.

58. The method of any one of Claims 52 to 57 further determining the level of another biomarker selected from the group consisting of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and P19.

59. The method of Claim 52 wherein the medicament is an anti-inflammatory agent or anti-microbial agent.

60. An animal model for endometritis, the animal model comprising a female equine animal artificially subject to intrauterine infection with a microorganism for a time and under conditions sufficient to induce elevated expression of biomarkers of innate immunity, the biomarkers selected from the group consisting of TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL9, CXCL10, CXCL11, EBD1, lysozyme, SLIPI, LCN2, lactoferrin and uteroferrin as well as sPLA$_2$ and P19.

61. The animal model of Claim 60 wherein the equine animal is selected from a horse, a Przewalski horse, zebra and ass.

62. The animal model of Claim 61 wherein the equine animal is a female horse.

63. The animal model of any one of Claims 60 to 62 wherein the microorganism is *E. coli*.

64. The animal model of any one of Claims 60 to 62 wherein the microorganism is *Streptococcus equi*.

65. The animal model of Claim 64 wherein the *Streptococcus equi* is sub-species *zooepidemicus*.

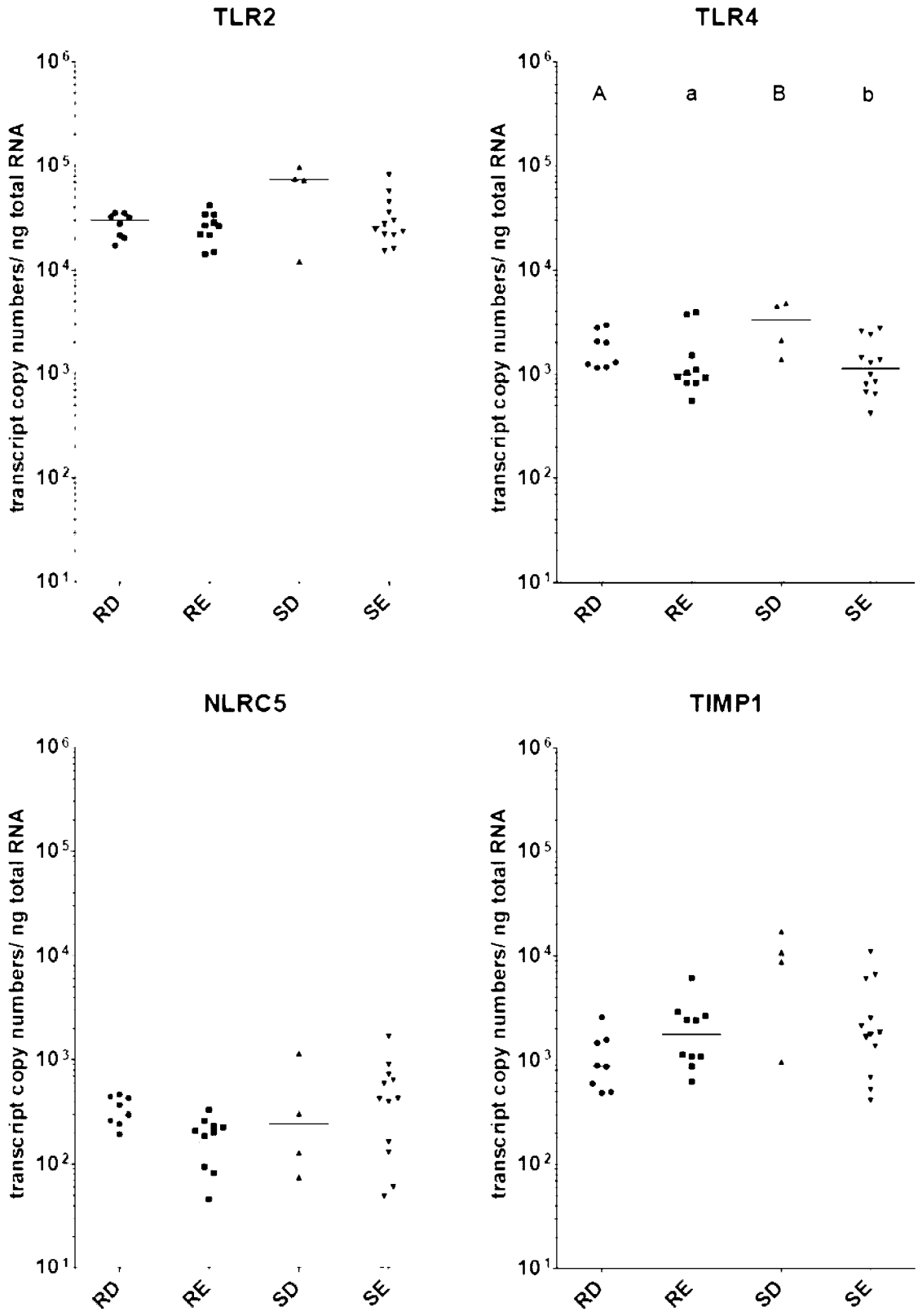
66. A business model comprising determining whether a non-human female animal is prone to the development of a condition of persistent endometritis by the determination of levels of EBD1, lysozyme and/or SLPI, individually or together and optionally with one or

more of TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin and/or P19 wherein a decision to purchase the animal, use the animal in a breeding program and/or to insure the animal is based on the level of risk that persistent endometritis may impair the successful initiation of a pregnancy.

67. The business model of Claim 66 wherein the equine animal is a horse.

68. An assay to determine the presence or elevation in EBD1, lysozyme and/or SLPI in a uterine biopsy, cytobrush or lavage from a subject wherein said assay comprises isolation of the sample and screening the sample for the presence of one or more of EBD1, lysozyme and/or SLPI.

69. The assay of Claim 68 further comprising assaying TLR2, TLR4, NLRC5, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 and/or P19.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TLR4
NLRC5
TIMP1
transcript copy numbers/ ng total RNA
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10^5
10^4
10^3
10^2
10^1
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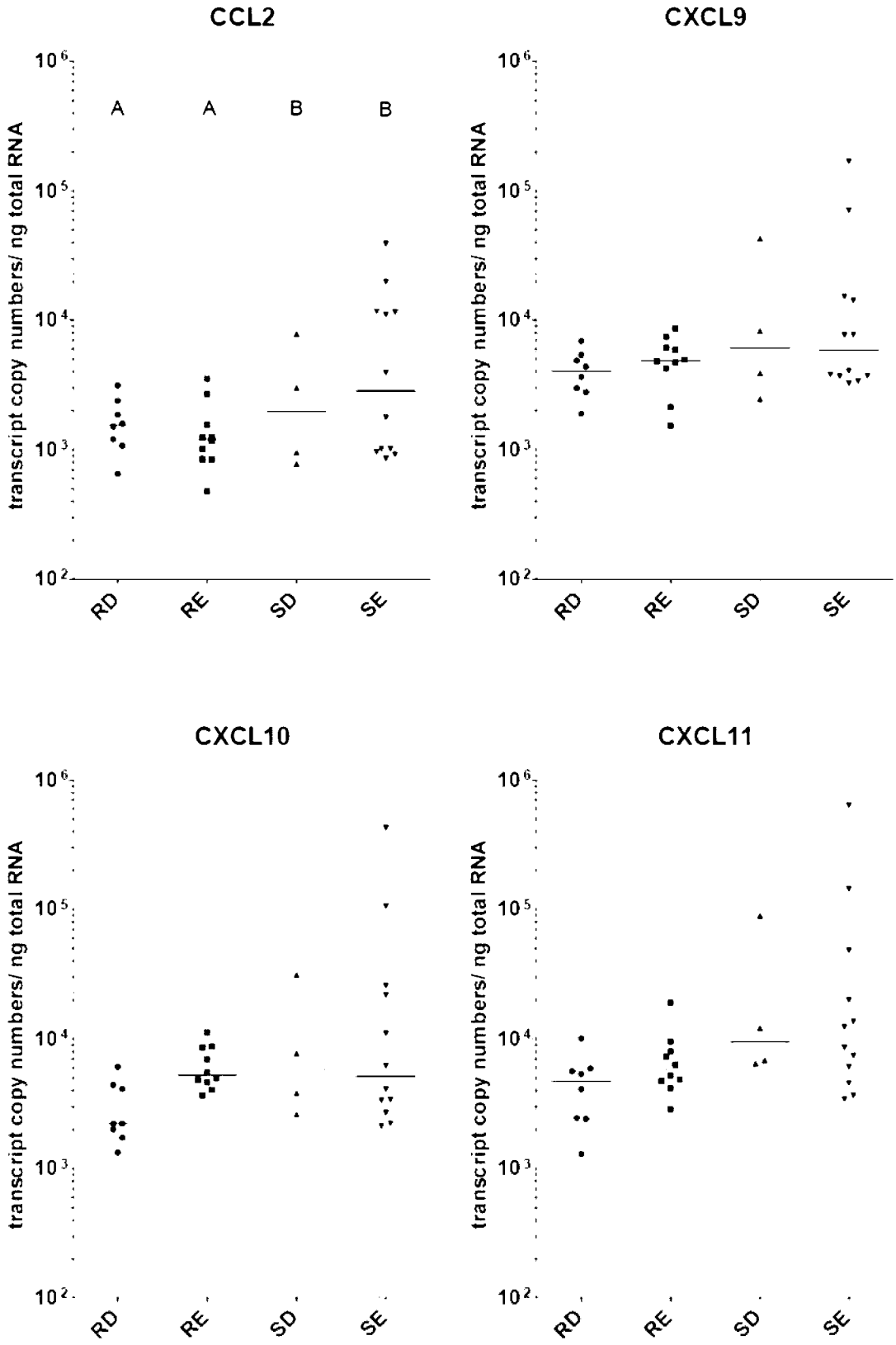
RE
SD
SE
A
a
B
b

Figure 1


CCL2
CXCL9
CXCL10
CXCL11
transcript copy numbers/ ng total RNA
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10^5
10^4
10^3
10^2
A
A
B
B
RD
RE
SD
SE

Figure 2

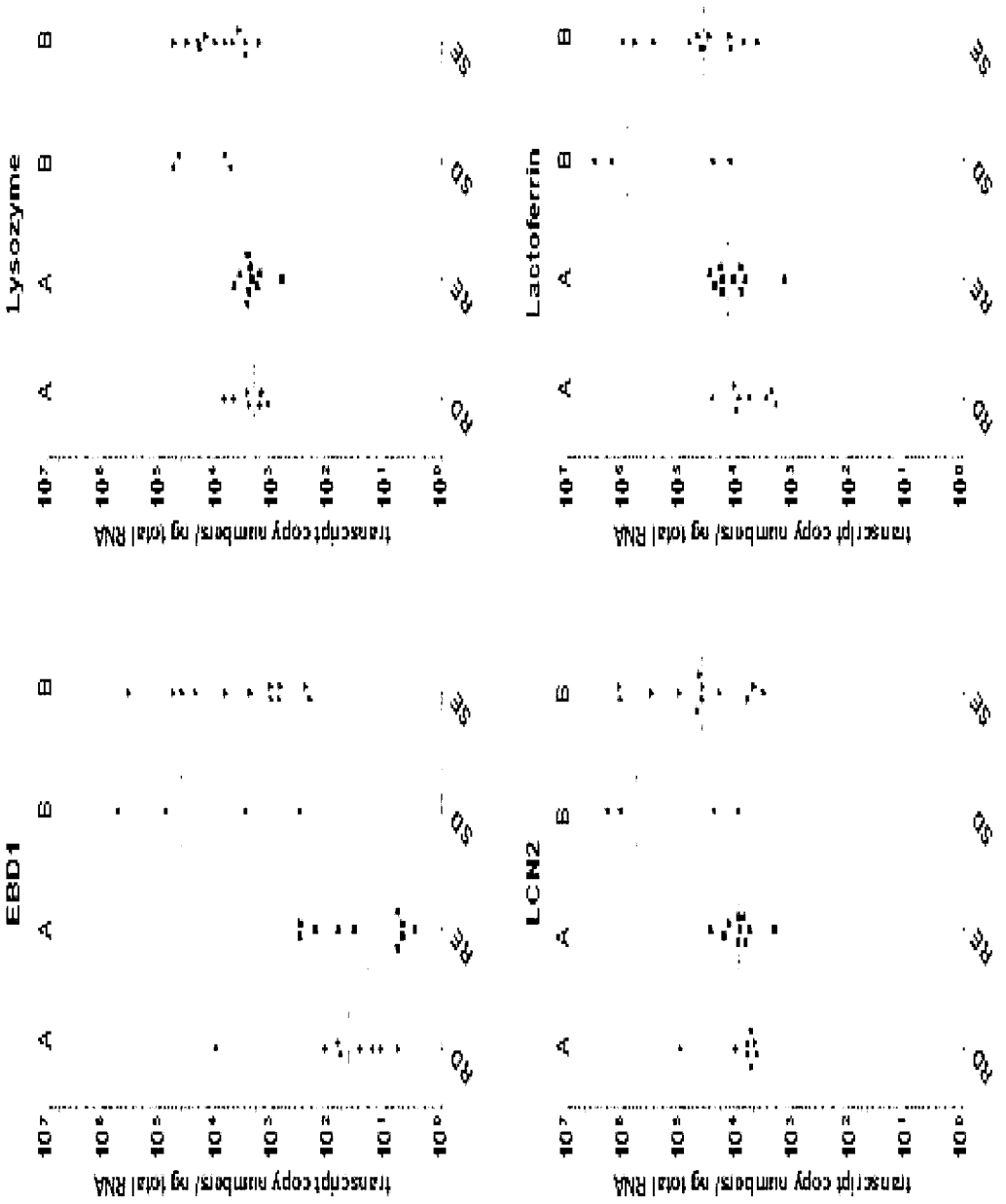


Figure 3A

H:\ar-Interwoven\NRPortbl\DCC\AAR\15953293_1.docx-3/11/2017

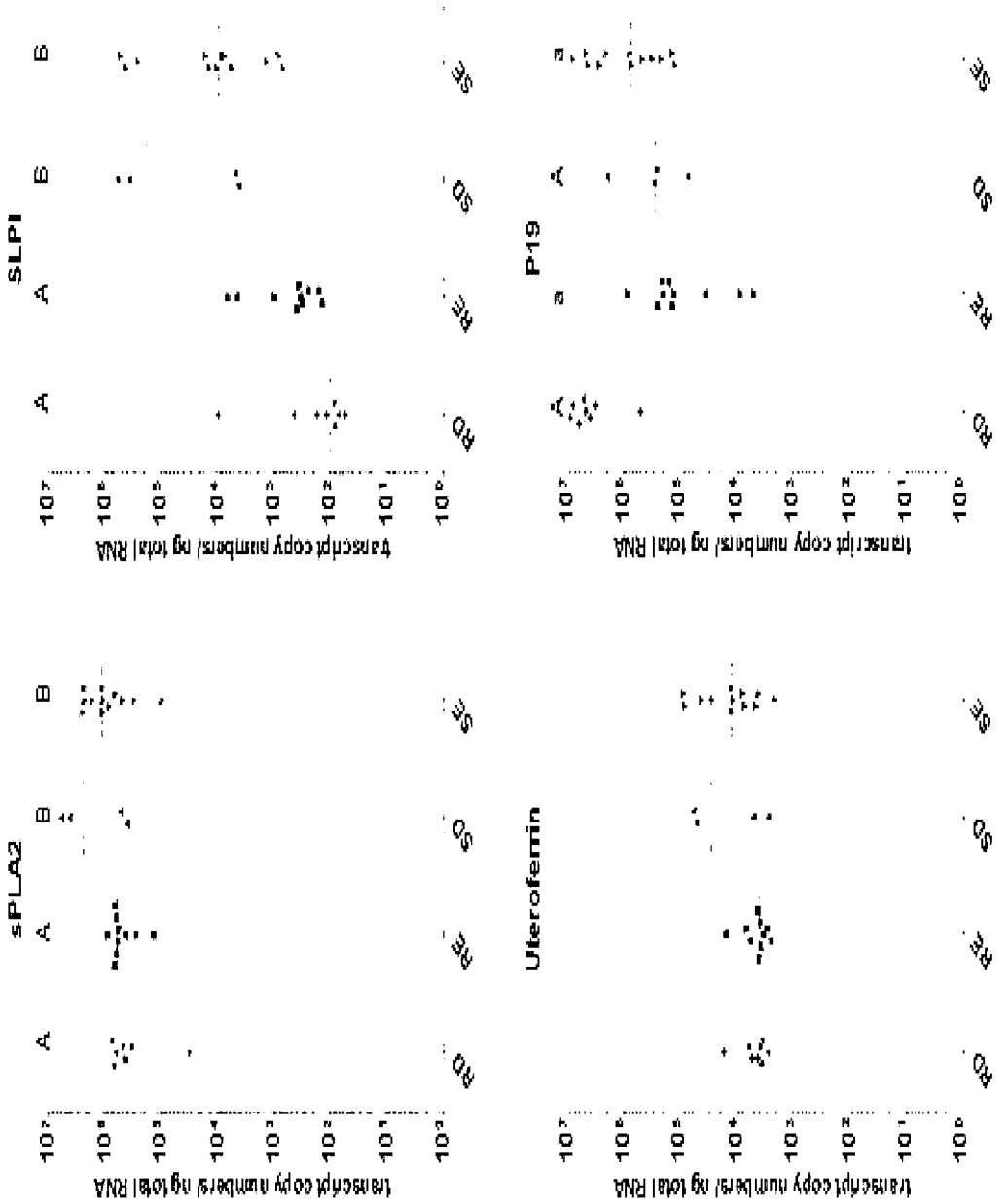


Figure 3B

H:\ar-Interwoven\NRPortbl\DCC\AAR\15953293_1.docx-3/11/2017

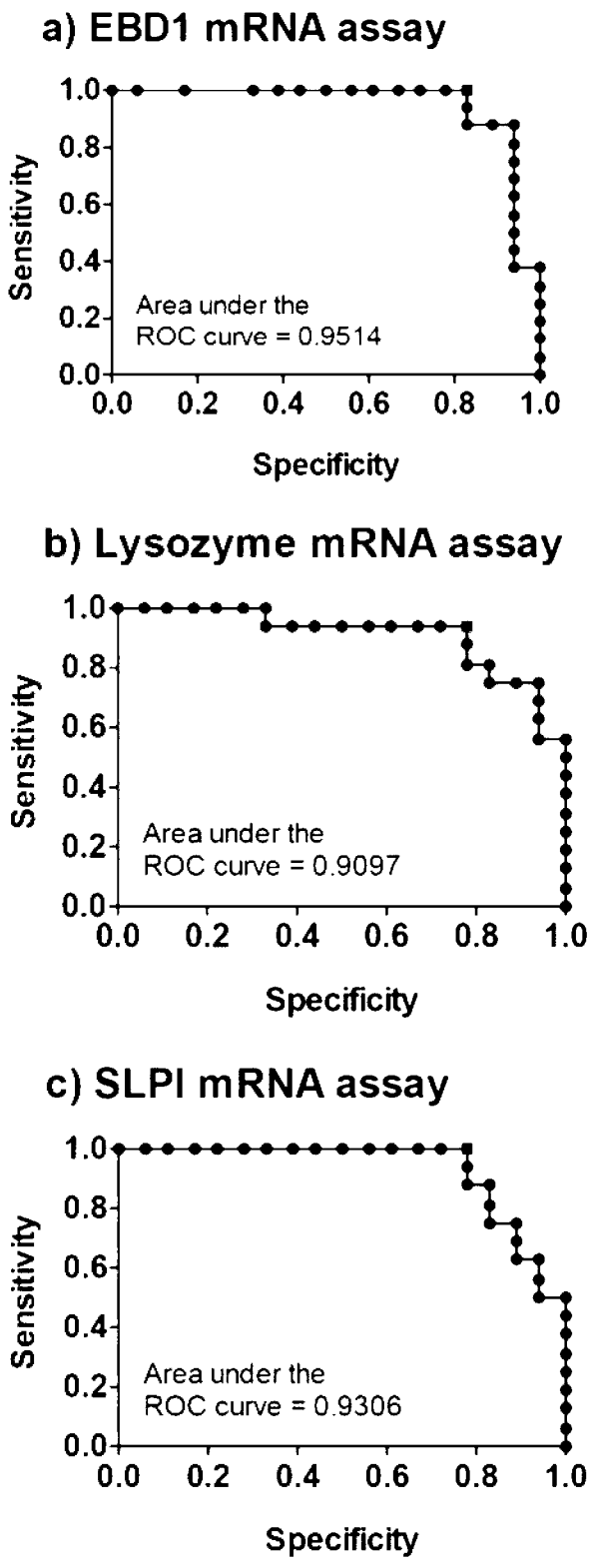
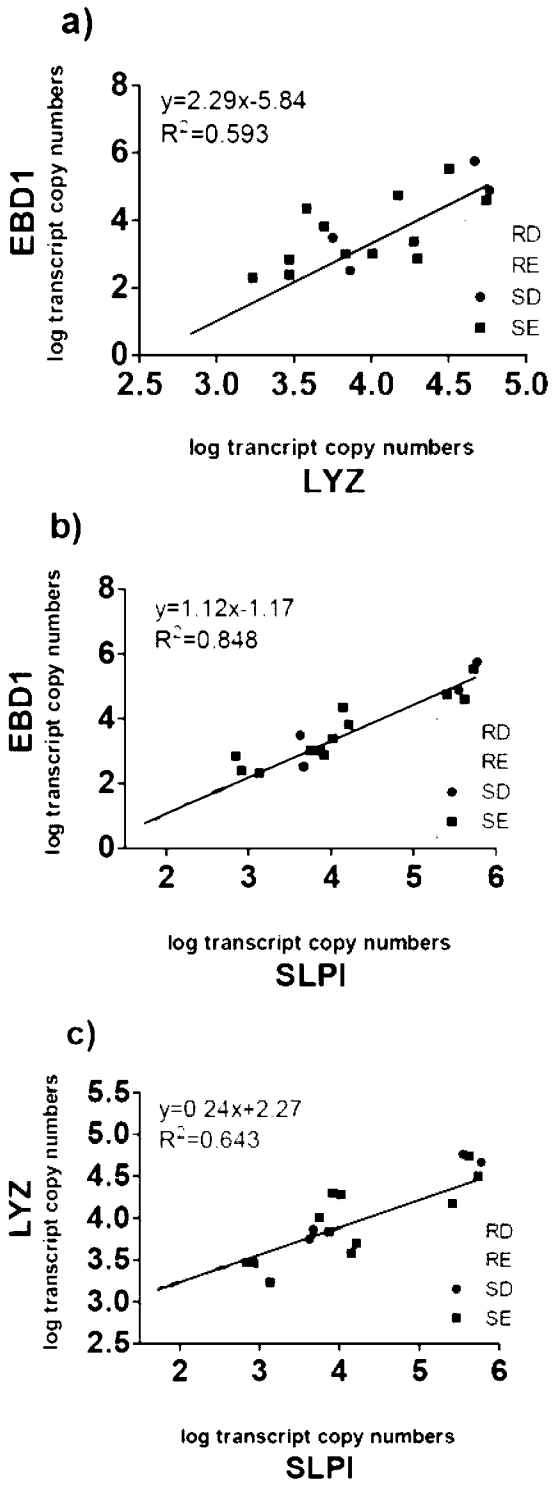

a) EBD1 mRNA assay
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
Area under the
ROC curve = 0.9514
0.0 0.2 0.4 0.6 0.8 1.0
Specificity
b) Lysozyme mRNA assay
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
Area under the
ROC curve = 0.9097
0.0 0.2 0.4 0.6 0.8 1.0
Specificity
c) SLPI mRNA assay
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
Area under the
ROC curve = 0.9306
0.0 0.2 0.4 0.6 0.8 1.0
Specificity

Figure 4


a)
y=2.29x-5.84
R²=0.593
EBD1
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RD
RE
SD
SE
log trancript copy numbers
LYZ
b)
y=1.12x-1.17
R²=0.848
EBD1
log transcript copy numbers
RD
RE
SD
SE
log transcript copy numbers
SLPI
c)
y=0.24x+2.27
R²=0.643
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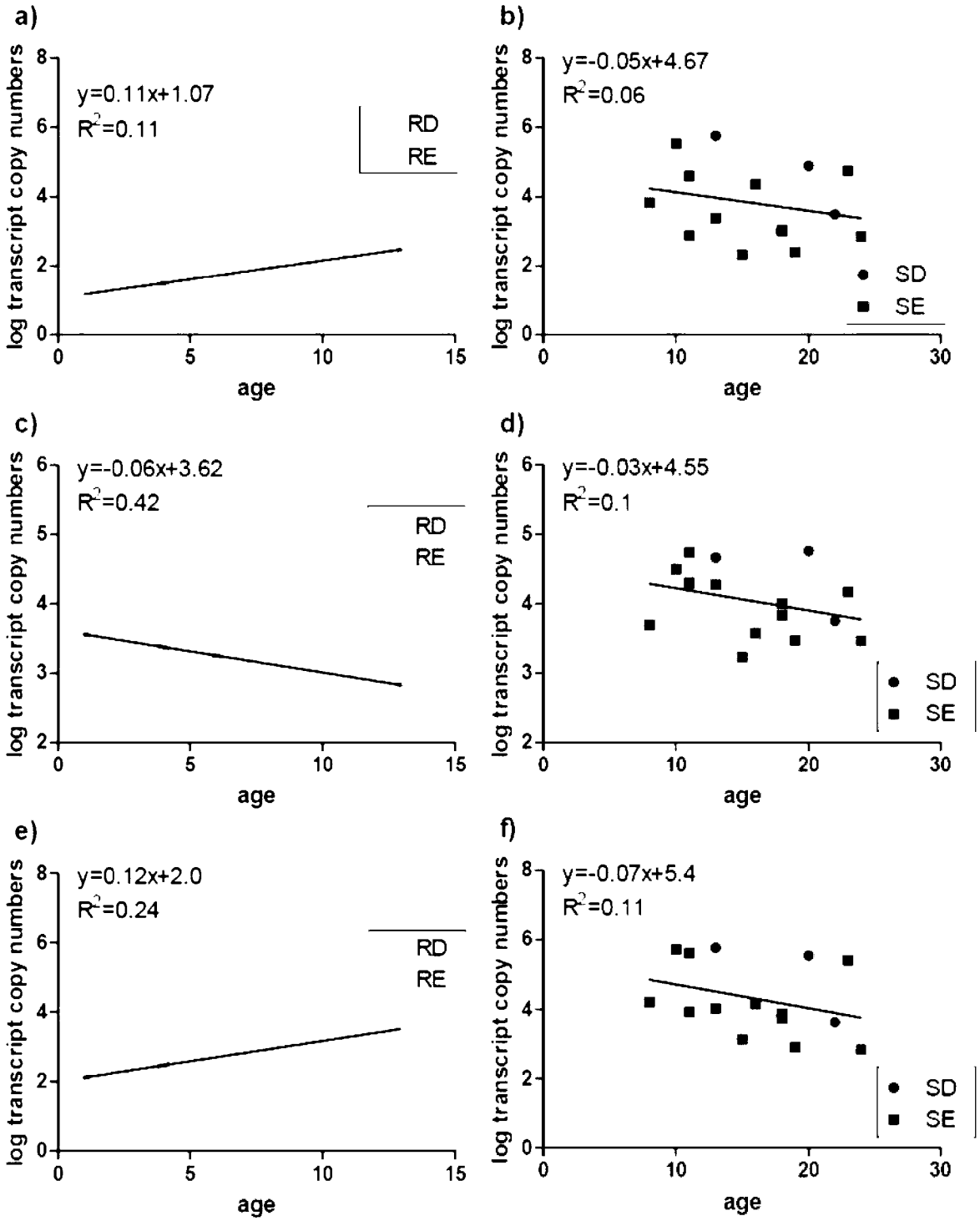
SE
log transcript copy numbers
SLPI

Figure 5


a)
y=0.11x+1.07
R2=0.11
RD
RE
log transcript copy numbers
age
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R2=0.06
SD
SE
log transcript copy numbers
age
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y=-0.06x+3.62
R2=0.42
RD
RE
log transcript copy numbers
age
d)
y=-0.03x+4.55
R2=0.1
SD
SE
log transcript copy numbers
age
e)
y=0.12x+2.0
R2=0.24
RD
RE
log transcript copy numbers
age
f)
y=-0.07x+5.4
R2=0.11
SD
SE
log transcript copy numbers
age

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2017/051178

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68 (2018.01) G01N 33/48 (2006.01) A01K 67/02 (2006.01) A61D 19/00 (2006.01)

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)

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)

EPOQUE (PATENW) and STN (BIOSIS, CAPLUS, EMBASE, MEDLINE): C12Q1/68, G01N33/48; A01K67/02; A61D19/00 and keywords used - equine, horse, equus, streptococcus, zooepidemicus, e. coli, endometritis, pmie, uterine inflammatory response, contagious equine metritis, equine ß-defensin 1, EBD1, lysozyme, secretory leukoprotein inhibitor, SLPI, induce, increase, elevate, biomarker, diagnosis, detect, identify, assay and like terms.

Google Scholar, Espacenet, AusPat, PubMed, Google Patents - inventor names and applicant.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 2018	16 January 2018

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INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. PCT/AU2017/051178
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X L	MARTH C.D. et al., 'Deep sequencing of the uterine immune response to bacteria during the equine oestrous cycle', BMC Genomics. (2015), vol. 16, article 934 (online journal) <DOI 10.1186/s12864-015-2139-3 >. Abstract; Materials and methods, pages 3-5; page 12; Figures 2 and 5; pages 14-15: Antimicrobial peptides and proteins; and conclusion. Lack of unity	1-30, 60-69
X L	ARLAS T.R. et al., 'Proteomics of endometrial fluid after dexamethasone treatment in mares susceptible to endometritis', Theriogenology. (2015), vol. 84, pages 617-623. Abstract; Materials and methods, pages 618-620; Figures 2-4; and Table 1. Lack of unity	60-65
X L	CHRISTOFFERSEN M. et al., 'Inflammatory responses to induced infectious endometritis in mares resistant or susceptible to persistent endometritis', BMC Veterinary Research. (2012), vol. 8, article 41 (online journal) < http://www.biomedcentral.com/1746-6148/8/41 >. Abstract; Methods pages 3-4; pages 7-8; Figure 2; and conclusion. Lack of unity	60-65
X L	CHRISTOFFERSEN M. et al., 'Effect of immunomodulatory therapy on the endometrial inflammatory response to induced infectious endometritis in susceptible mares', Theriogenology. (2012), vol. 78, pages 991-1004. Abstract; Materials and Methods, pages 992-993; and page 997. Lack of unity	60-65
X L	WOLF C.A. et al., 'Effect of corticotherapy on proteomics of endometrial fluid from mares susceptible to persistent postbreeding endometritis', Theriogenology. (2012), vol. 77, pages 1351-1359. Abstract; and Materials and methods, 2.5 Experimental Infection (Infected), pages 1352-1353; Tables 2-3; and conclusion. Lack of unity	60-65
X	WO 2010/069335 A2 (KOBENHAVNS UNIVERSITET) 24 June 2010 Example 1	60-65
X	WO 2014/118764 A2 (EPONA BIOTECH LTD) 07 August 2014 Abstract; lines 40-43, page 9; lines 4-7, page 10; claims 19, 38-44, 47; and Figures 1-3, 13, 14, 16.	44-47
X	REILAS T. et al., 'Relationship between embryo recovery rate and uterine lavage fluid composition in postpartum mares', Reproduction Nutrition Development. (2000), vol. 40, pages 383-391. Abstract; Materials and Methods, pages 385-387; and Tables I and II.	68
X	NIKOLAITCHOUK N. et al., 'The lower genital tract microbiota in relation to cytokine-, SLPI- and endotoxin levels: application of checkerboard DNA-DNA hybridization (CDH)', APMIS. (2008), vol. 116, issue 4, pages 263-277. Abstract; Materials and Methods, pages 264-267; and Table 9.	68
X	WO 2016/065415 A1 (THE UNIVERSITY OR QUEENSLAND) 06 May 2016 Abstract; and claims.	44-47, 49

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2017/051178**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015/0226735 A1 (UNIVERSAL BIOSENSORS PTY LTD) 13 August 2015 Abstract; and claims	44-47, 49
X	HALL D.A. et al., 'A 256 pixel magnetoresistive biosensor microarray in 0.18μm CMOS', IEEE Journal of Solid-State Circuits. (2013), vol. 48, no. 5, pages 1290-1301. Abstract.	44-47, 49
A	WO 2002/072624 A2 (CAMBRIDGE UNIVERSITY TECHNICAL SERVICES LIMITED) 19 September 2002	
P,X	MARTH C.D. et al., 'Innate immune genes in persistent mating-induced endometritis in horses', Reproduction, Fertility and Development. (August, 2017). (online journal) <URL: https://doi.org/10.1071/RD17157>. Abstract; Materials and methods; Results; Table 2; Table 3; and Table 4.	1-69
P,X	MARTH C.D. et al., 'Oestrous cycle-dependent equine uterine immune response to induced infectious endometritis', Veterinary Research. (November, 2016), vol. 47, article 110 (online journal) < DOI 10.1186/s13567-016-0398-x>. Abstract; Materials and methods; Table 1; Table 4; Table 5; and Results.	1-69

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

   No sequence searching was required when completing the International Search Report and International Search Opinion.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2017/051178

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☐ Claims Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box

Continuation of: **Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

Independent Claims

The inventive concept appears to reside in the use of biomarkers equine β-defensin 1 (EBD1), lysozyme or secretory leukoprotein inhibitor (SLPI) to indicate the likelihood of developing persistent endometritis. Claims 1 and 2 are directed towards diagnostic methods by measuring the level of biomarker EBD1, lysozyme or SLPI in a uterine biopsy, cytobrush or lavage sample, wherein an elevated level compared to a control is indicative of the development of persistent endometritis. Other independent claims are directed to the use of EBD1, lysozyme or SLPI in the manufacture of an assay to detect persistent endometritis; the use of EBD1, lysozyme or SLPI antagonists in the manufacture of a medicament; a mechanical device to detect elevated levels of EBD1, lysozyme or SLPI biomarkers; a method of treatment using EBD1, lysozyme or SLPI antagonists; a business model based on the presence of elevated biomarker levels of EBD1, lysozyme or SLPI; and an assay to detect EBD1, lysozyme or SLPI biomarkers. Also claimed is an animal model wherein a female equine is artificially infected with a microorganism to cause intrauterine infection to induce elevated levels of at least one biomarker selected from TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL9, CXCL10, CXCL11, EBD1, lysozyme, SLPI, LCN2, lactoferrin, uteroferrin, sPLA2 or P19.

The only feature common to all of the claimed inventions and which provides a technical relationship among them is the presence or use of biomarkers associated with endometritis in equines.

However this cannot be viewed as a special technical feature as biomarkers for equine endometritis have been identified in the prior art (see below), and furthermore the three biomarkers exemplified (EBD1, lysozyme and SLPI) do not satisfy the criteria set out for Markush practice for a group of chemical compounds.

Prior Art Examples

D1: MARTH C.D. et al., 'Deep sequencing of the uterine immune response to bacteria during the equine oestrous cycle', BMC Genomics. (2015), vol. 16, article 934, (online journal), <DOI 10.1186/s12864-015-2139-3> - discloses that expression of EBD1, lysozyme and SLPI are significantly up-regulated in endometrial biopsies after subjects have been inoculated with *E. coli* and identifies them as potential treatment targets (see page 12; Figure 2c; and pages 14-15: *Antimicrobial peptides and proteins*).

D2: ARLAS T.R. et al., 'Proteomics of endometrial fluid after dexamethasone treatment in mares susceptible to endometritis', Theriogenology. (2015), vol. 84, pages 617-623 - analyses protein profiles (albumin; apolipoprotein A1; transthyretin; and actin) of uterine fluid samples from equines whilst investigating dexamethasone treatment (see abstract; Materials and methods, pages 618-620; Figures 2-4; and Table 1).

D3: CHRISTOFFERSEN M. et al., 'Inflammatory responses to induced infectious endometritis in mares resistance or susceptible to persistent endometritis', BMC Veterinary Research. (2012), vol. 8, article 41, (online journal), <http://www.biomedcentral.com/1746-6148/8/41> - investigates a number of inflammatory markers, and in particular IL-1β and IL-1ra were significantly higher in mares with severe uterine infection (see abstract; Methods; pages 7-8: Endometrial gene expression related to uterine infection; Figure 2; and conclusion).

D4: CHRISTOFFERSEN M. et al., 'Effect of immunomodulatory therapy on the endometrial inflammatory response to induced infectious endometritis in susceptible mares', Theriogenology. (2012), vol. 78, pages 991-1004 - investigates mRNA expression in a number of inflammatory markers, and found that IL-10 expression increased in mares with severe uterine infection induced by *E. coli* and glucocorticoid treatment (see abstract; Materials and Methods, 2.2 *E. coli* inoculation and collection of urine samples; Table 1; and Figure 3). Furthermore endometrial gene expression of IL-1ß, IL-6 and SAA was significantly increased after *E. coli* inoculation and no glucocorticoid treatment (see paragraph 4.1: *Effect of immunomodulatory therapy on endometrial gene expression of cytokines and SAA*, page 997).

D5: WOLF C.A. et al., 'Effect of corticotherapy on proteomics of endometrial fluid from mares susceptible to persistent postbreeding endometritis', Theriogenology. (2012), vol. 77, pages 1351-1359 - investigates the use of glucococorticoid therapy in

Supplemental Box

equines infected with *S. equi* subspecies *zooepidemicus* through protein analysis (α_1-antitrypsin; transthyretin; actin; immunoglobulin G; haptoglobulin (Hp); apolipoprotein A-1 (ApoA-1) and transferrin) and concluded that uterine infection is characterised by increased levels of Hp and ApoA-1 (see Abstract; and Materials and methods, pages 1352-1354; Tables 2-3; and concluding paragraph).

The above documents disclose special technical feature being biomarkers associated with endometritis in equines. It is noted that not all independent claims are limited to the use of biomarkers to investigate persistent endometritis and associated embodiments, and therefore the special technical feature is considered to be the broader concept of endometritis, rather than persistent endometritis. According to paragraph [0039] of the description endometritis refers to an inflammatory condition which includes persistent and non-persistent endometritis. Also noted is that only D1 discloses the exemplified biomarkers of the current claims - EBD1, SLPI and/or lysozyme.

Markush Practice

Under Markush practice for a group of chemical compounds (in this instance biomarkers EBD1, lysozyme and SLPI), a technical interrelationship as defined in Rule 13.2, is considered met when the following criteria are fulfilled:

"(A) all alternatives have a common property or activity, and (B)(1) a common structure is present, that is, a significant structural element is shared by all of the alternatives, or (B)(2) in cases where the common structure cannot be the unifying criteria, all alternatives belong to a recognized class of chemical compounds in the art to which the invention pertains."

PCT Search and Examination Guidelines, paragraph 10.17

The special technical feature of the claims resides in the detection of the presence biomarkers EBD1, lysozyme or SLPI. The identified biomarkers would be regarded as having the same or corresponding technical feature if they had a common property or activity, and shared a significant structural element that is essential to the common property or formed part of a recognised class of compounds in the art.

While each of the biomarkers may share the common property of being elevated in endometritis, there is no shared significant structural element, nor any recognised class of chemical compounds to which all the alternatives belong.

Therefore, there is no common special technical feature, and therefore the inventions are not unified.

Number of Inventions

The number of inventions are broadly defined as follows:

Group 1: is defined by claims 1-5 (partial), 6 (in full), 8-21 (partial), 22 (in full), 24-34 (partial), 35 (in full), 37-47 (partial), 48 (in full), 50-54 (partial), 55 (in full) and 56-69 (partial) wherein biomarker **EBD1** is specific to this group of claims.

Group 2: is defined by claims 1-5 (partial), 7-21 (partial), 23-34 (partial), 36-47 (partial), 49-54 (partial) and 56-69 (partial) wherein biomarker **lysozyme** is specific to this group of claims.

Group 3: is defined by claims 1-5 (partial), 7-21 (partial), 23-34 (partial), 36-47 (partial), 49-54 (partial) and 56-69 (partial) wherein biomarker **SPLI** is specific to this group of claims.

Groups 4-16: are defined by claims 60-65 (partial). These claims are directed equine animal model for endometritis with elevated expression of biomarkers. Each biomarker is considered a separate invention as the claim requires a minimum of any one of TLR2, TLR4, NLRC5, TIMP1, CCL2, CXCL9, CXCL10, CXCL11, LCN2, lactoferrin, uteroferrin, sPLA2 or P19.

For the fee already paid, the ISA will search and examine the invention defined by Groups 1, 2 and 3 where the biomarker is EBD1, lysozyme or SLPI. It is considered that search and examination for the inventions defined in these three groups require negligible additional search and examination effort, and can be searched for the one search fee.

Note that claims 60-65 relate to an animal model for endometritis, wherein a microorganism is introduced into a female equine animal to cause an intrauterine infection. As currently drafted, the elevated expression levels of the exemplified biomarkers may regarded as an inherent feature, and may include situations which occur in nature. As such, it is not possible to determine the entire scope of the claim, and these claims will only be searched in relation to the specific biomarkers EBD1, lysozyme or SLPI as set out in the Groups 1, 2 and 3 above.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2017/051178**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
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		US 2012058087 A1	08 Mar 2012
		US 8633007 B2	21 Jan 2014
WO 2014/118764 A2	07 August 2014	WO 2014118764 A2	07 Aug 2014
		AU 2014210744 A1	17 Sep 2015
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		EP 2951584 A2	09 Dec 2015
		US 2016153878 A1	02 Jun 2016
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		EP 3213073 A1	06 Sep 2017
		US 2017315114 A1	02 Nov 2017
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

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Patent Document/s Cited in Search Report		Patent Family Member/s	
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		SG 11201501853S A	29 Apr 2015
		WO 2014045122 A1	27 Mar 2014
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		AU 2002249342 A1	24 Sep 2002

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.