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**(54) SEQUENCES FOR DIFFERENTIAL DIAGNOSTIC OF EHRLICHIA RUMINANTIUM AND USE
THEREOF**

SEQUENZEN ZUR DIFFERENTIELLEN DIAGNOSTIK VON EHRLICHIA RUMINANTIUM UND
VERWENDUNG DAVON

SEQUENCES DE DIAGNOSTIC DIFFÉRENTIEL D'EHRLICHIA RUMINANTIUM ET LEUR
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Description

[0001] *Rickettsia* are intracellular pathogenic bacteria responsible for various diseases on Humans and animals. *Rickettsia* are transmitted by arthropods, most frequently ticks, lice and mites, and cause major illnesses such as epidemic typhus or Rocky Mountain spotted fever. The genus *Ehrlichia* comprises several species pathogenic for humans and mammals such as *E. chaffeensis*, responsible for Human monocytic ehrlichiosis, *E. canis*, the causing agent of canine monocytic ehrlichiosis, or *E. phagocytophilia*, the agent of Human granulocytic ehrlichiosis.

[0002] Another species, *Ehrlichia ruminantium*, formerly known as *Cowdria ruminantium*, is the causing agent of heartwater or cowdriosis, an economically important disease of domestic ruminants. Heartwater can cause up to 80 % mortality in susceptible animals. *E. ruminantium* is transmitted by *Amblyomma* ticks and is present in Sub-Saharan Africa and surrounding islands, including Madagascar. Heartwater is also present in several Caribbean islands and is threatening the American mainland.

[0003] Serological diagnostic tests of heartwater using crude antigens from whole bacteria detect false positive reactions due to common antigenic determinants. ELISA-based and serological diagnostics have been developed using the Map 1 (WO 9914233; Sumption et al. Clin Diagn Lab Immunol. 10: 910-916, 2003) and the GroEL (WO 9914233) antigens. Other peptides for serological diagnostic have been described (US 2002004051, US 20020132789, WO 02/066652). Although they have dramatically improved specificity, they still display cross reaction with *E. canis* and *E. chaffeensis*. Furthermore, the life span of anti-Map 1 antibodies is rather short.

[0004] PCR-based diagnostic methods represent methods of choice for the sensitive and specific detection of *Ehrlichia* in clinically reactive or asymptomatic carrier ruminants, as well as in vectors. However, in the field, hosts and vectors can be co-infested by several parasites and the diversity of pathogen species is further complicated by the existence of extensive intra-species diversity. Improved methods are required to discriminate between strains of differing pathogenesis.

[0005] Vaccination against heartwater has long been based on "infection and treatment". Naïve animals are inoculated with blood containing virulent organisms, a procedure which carries a high risk of uncontrolled clinical reactions and the inadvertent spread of undesirable parasites and viruses. A first generation of cowdriosis inactivated vaccine based on cell-cultured derived elementary bodies was developed. Although representing a considerable improvement and the first heartwater vaccine acceptable for widespread use, the level of protection conferred is still not fully satisfactory. Indeed, all animals develop a clinical reaction at challenge despite vaccination. Furthermore, livestock also faces challenge by genetically and antigenically diverse strains.

[0006] Diversity of *E. ruminantium* is a key problem which has been recognized for a long time, but insufficient information is available for optimum vaccine formulation and specific diagnostic. The diversity of *E. ruminantium* was demonstrated at the antigenic level by cross-immunisation studies. Variable antigens were identified by ELISA and immunoblot using cross-absorbed immune sera. Genetic diversity was later demonstrated when sequencing the Map 1 gene which showed a high degree of sequence heterogeneity concentrated in three hypervariable regions. This DNA polymorphism was shown to correlate with antigenic polymorphism. Genomic polymorphism was also detected using RAPD and RFLP markers. The map 1 gene initially considered as a good marker for geographic diversity, was recently shown not to be geographically constrained. Furthermore, there is no evidence of evolution of *map1* under positive selection pressure. *Map1* was therefore reported as not being important for evasion of host immune response.

[0007] It is shown herein that between two strains of *Ehrlichia ruminantium*, i.e. strain Gardel and strain Welgevonden, the protection acquired through vaccination with one strain is without effect towards the other one. When an animal is protected by vaccination against the strain Gardel it remains susceptible to lethal infection with the strain Welgevonden, i.e. there is no cross protection between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

[0008] Thus, it is important to provide means and diagnostic tools allowing not only to identify *E. ruminantium* but also to differentiate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

[0009] The invention provides genetic markers to differentiate between these two strains, and methods using said genetic markers. More specifically, the disclosure identifies large genetic deletions that are specific either to *E. ruminantium* strain Gardel or to *E. ruminantium* strain Welgevonden, and provide means to distinguish between these two strains by detecting the presence or absence of at least one of said deletions. These deletions may result in the loss of a whole gene, or only of a part of it.

[0010] According to a first embodiment, the invention provides gene sequences present in only one of the two differing strains of *Ehrlichia ruminantium*. These sequences represent regions of high strain-specificity for development of diagnostic tools. They will be defined herein as "orphan genes", or "unique genes". They correspond to CDS which have no counterpart in the other strain.

[0011] According to a second embodiment, the present disclosure provides couples of genes which are present in both strains but whose sequences differ between the two strains, due to one or several mutations, including in particular deletions that represent a good target for strain-specific detection. These couples of genes will be defined herein as "allelic couples". The longest member of an allelic couple, that appears, on the basis of sequence data, to encode a

potentially functional protein will be defined herein as the "native gene", or the "native allele". The truncated member of the allelic couple, which appears, on the basis of sequence data, to encode a modified protein which is potentially non-functional, or functionally altered, will be defined herein as the "mutant gene", or the "mutant allele".

[0012] The CDS corresponding to the native gene in one strain may have, depending on the type of mutation, one or two counterparts in the other strain. More specifically, in case wherein the mutation induces a frameshift where the initial reading frame is changed due to deletion of bases and results in a shift of the frame, a second or additional CDS might be predicted by the annotation software package (i.e. GenoStar package - www.genostar.org) downstream from the site of mutation. This additional CDS is not a novel gene *per se* but merely the continuation of part of the original full length gene which was shortened by the mutation. If the part of the coding sequence located downstream from the mutation meets the prediction requirements regarding minimal size and presence of a start and a stop codon, it will be considered by the annotation software as a "novel" CDS. A specific number will therefore be attached to this additional CDS although it is only part of the initial full length gene and does not correspond to a biologically distinct gene. As a consequence, in some cases, the beginning of the mutant counterpart of the native gene will be found within a first CDS, while the end of the mutant counterpart of the native gene will be found within a second CDS.

[0013] The disclosure provides methods of detecting *Ehrlichia ruminantium* and, advantageously, of discriminating between *Ehrlichia ruminantium* strain Gardel and *Ehrlichia ruminantium* strain Welgevonden, using any of the orphan genes or allelic couples defined above, or any combination thereof.

[0014] Accordingly, a first object of the invention is a method for discriminating between *Ehrlichia ruminantium* strain Gardel and *Ehrlichia ruminantium* strain Welgevonden, wherein said method comprises the detection of the presence or the absence, in the bacteria to be tested, of the orphan gene ERGA_CDS_04980 (SEQ ID NO: 2).

[0015] According to a preferred embodiment of the method of the invention, it further comprises the detection of at least one orphan gene selected among:

ERGA_CDS_04340 (SEQ ID NO: 1)
 ERGA_CDS_05590 (SEQ ID NO: 3)
 ERGA_CDS_05600 (SEQ ID NO: 4)
 ERGA_CDS_07580 (SEQ ID NO: 5)
 ERWE_CDS_08330 (SEQ ID NO: 6),

[0016] ERGA_CDS_04340, ERGA_CDS_04980, ERGA_CDS_05590, ERGA_CDS_05600, and ERGA_CDS_07580 are found only in the genome of *E. ruminantium* strain Gardel.

[0017] ERWE_CDS_08330 is found only in the genome of *E. ruminantium* strain Welgevonden.

[0018] Advantageously, said preferred embodiment comprises the detection of at least one gene selected among ERGA_CDS_05590, and ERGA_CDS_07580.

[0019] The present invention also provides means for discriminating between *Ehrlichia ruminantium* strain Gardel and *Ehrlichia ruminantium* strain Welgevonden on the basis of the detection of a member of an allelic couple of genes, as defined above.

[0020] Thus according to a preferred embodiment, a method of the invention for discriminating between *Ehrlichia ruminantium* strain Gardel and *Ehrlichia ruminantium* strain Welgevonden further comprises the detection in the bacteria to be tested, of one of the members of at least one allelic couple of genes selected among:

- a couple consisting of ERGA_CDS_00120 (SEQ ID NO: 7) and ERWE_CDS_00120 (SEQ ID NO: 8);
- a couple consisting of ERGA_CDS_01350 (SEQ ID NO: 9) and ERWE_CDS_01390 (SEQ ID NO: 10);
- a couple consisting of ERGA_CDS_05740 (SEQ ID NO: 11) and ERWE_CDS_05830 (SEQ ID NO: 12);
- a couple consisting of ERGA_CDS_04500 (SEQ ID NO: 13) and ERWE_CDS_04590 (SEQ ID NO: 14) + ERWE_CDS_04600 (SEQ ID NO: 15)
- a couple consisting of ERGA_CDS_05350 (SEQ ID NO: 16) and ERWE_CDS_05460 (SEQ ID NO: 17) + ERWE_CDS_05470 (SEQ ID NO: 18)
- a couple consisting of ERGA_CDS_07330 (SEQ ID NO: 19) and ERWE_CDS_07410 (SEQ ID NO: 20).

[0021] ERGA_CDS_00120, ERGA_CDS_01350, ERGA_CDS_05740, ERGA_CDS_04500 and ERGA_CDS_05350, are alleles herein defined as native alleles, that are found in strain Gardel. ERWE_CDS_07410 is an allele herein defined as a native allele, that is found in strain Welgevonden.

[0022] The method may comprise the detection of a member of a single allelic couple among those listed above, or the detection of a member of each allelic couple in a combination of 2, 3, 4, 5, or 6 of those listed above.

[0023] In the allelic couples disclosed above, the mutant allele differs from the native allele by the presence of a deletion resulting in the loss of part of the transcribed region corresponding to the central part of the native coding sequence. This deletion generates a truncation, that can be accompanied by a frameshift.

[0024] Three main regions can therefore be considered. These regions are presented in Figure 1. The first region, named Zone 1, is the 5' region of the gene up to the beginning of the deletion in the mutant gene. Zone 1 is a conserved region of high similarity between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. An oligonucleotide designed to match this region will recognize both strains. Zone 2, corresponds to the region of deletion in the mutant gene and therefore only the native allele bears a sequence in this region and can be recognized by an oligonucleotide designed to match this region. Zone 3 is the second conserved region of high similarity between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. In this region also, an oligonucleotide designed to match Zone 3 will recognize both strains. In the mutant allele, Zone 1 adjoins Zone 3. An oligonucleotide designed to match the junction, i.e the regions of Zone 1 and Zone 3 immediately flanking the deletion, will recognize only the mutant strain.

[0025] The disclosure also discloses tools for detecting the presence or the absence of the orphan genes listed above, as well as tools for detecting the allelic couples of genes listed above, and differentiating their members.

[0026] These tools include in particular isolated polynucleotides defined by the sequences SEQ ID NO: 1 to 20 disclosed above or their complements, as well as fragments of at least 15 consecutive bp, preferably at least 18 consecutive bp, thereof. They also include polynucleotides that hybridize selectively, under stringent hybridization conditions, with one or two of the polynucleotides defined by the sequences SEQ ID NO: 1 to 20 described above, or with the complement thereof, without hybridizing to other sequences within the genome of *Ehrlichia ruminantium*. Among these, the polynucleotide defined by the sequence SEQ ID NO: 2 or its complement, as well as fragments of at least 15 consecutive bp, preferably at least 18 consecutive bp, thereof, and polynucleotides that hybridize selectively, under stringent hybridization conditions, with the polynucleotide SEQ ID NO: 2 described above, or with the complement thereof, are part of the object of the invention.

[0027] A polynucleotide that hybridize selectively with a given target sequence, is herein defined as a polynucleotide which does not hybridize, under the same hybridization conditions, with other sequences within the genome of *Ehrlichia ruminantium*.

[0028] Stringent hybridization conditions are defined as conditions that allow hybridization of only highly homologous sequences (i.e sequences having at least 90% and preferably at least 95 to 100% identity). It is known in the art that nucleic-acid hybridization is affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of mismatched bases between the hybridizing nucleic acids. Generally, stringent conditions for a given sequence can be obtained by performing hybridization at a temperature of about 10 to 20°C lower than the melting point (T_m) for the hybrid formed by said sequence and its exact complement, and at least one wash at a temperature of about 1 to 10°C lower, preferably at a temperature of about 1 to 5°C lower than the T_m for the hybrid formed by said sequence and its exact complement.

[0029] The above-mentioned polynucleotides can be divided in 3 sub-categories:

- polynucleotides specific to one of the orphan genes described above: said polynucleotides are fragments of anyone of the sequences SEQ ID NO: 1 to 6 or of its complement, as well as polynucleotides able to hybridize selectively, under stringent conditions, with anyone of the sequences SEQ ID NO: 1 to 6 or with its complement;
- polynucleotides common to both members of one of the allelic couples defined above: said polynucleotides are fragments, shared by both members of a given allelic couple, of anyone of the sequences SEQ ID NO: 7 to 20 or of its complement, or polynucleotides able to hybridize selectively, under stringent hybridization conditions, with a region shared by both members of a given allelic couple, of anyone of the sequences SEQ ID NO: 7 to 20 or of its complement. These polynucleotides are useful to detect *E. ruminantium*, and may also be used, as illustrated below, in methods for discriminating between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.
- polynucleotides specific to one of the members of one of the allelic couples disclosed above: said polynucleotides are fragments of anyone of the sequences SEQ ID NO: 7 to 20 or of its complement, that are present in only one of the members of a given allelic couple, or polynucleotides able to hybridize selectively, under stringent hybridization conditions, with a region of anyone of the sequences SEQ ID NO: 7 to 20 or of its complement, that is present in only one of the members of a given allelic couple. For a given allelic couple, these polynucleotides include those consisting of Zone 2 (or fragments thereof) as well as those spanning the junction between Zone 1 and Zone 3. In the case of a polynucleotide spanning the junction between Zone 1 and Zone 3, it will preferably be chosen in such a way that about half of its sequence is derived from Zone 1 and about half of its sequence is derived from Zone 3.

[0030] These polynucleotides include in particular nucleic acid probes or PCR primers.

[0031] For use as PCR primers one will generally chose oligonucleotides of about 18 to 25 bp. A variety of procedures and softwares for designing appropriate primers for a target region are available. Thus, one skilled in the art can easily design, based on the information provided by the present invention, sets of PCR primers suitable to generate an amplification product specific to anyone of the orphan genes listed above, or to generate amplification products from both members of anyone of the allelic couples listed above, or to generate an amplification product from only one of the members of said allelic couple. By way of non-limitative example of oligonucleotide design software suitable for obtaining

PCR primers, one can mention the software Vector NTI Advance 9.0 (Informax).

[0032] For use as nucleic acid probes, one will prefer polynucleotides that comprise at least 30 bp, preferably at least 50 bp, and up to the whole length of the target sequence. Many softwares and procedures are available to the one skilled in the art, who can easily design, based on the information provided by the invention, suitable polynucleotides useful for efficiently discriminate *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden.

[0033] These polynucleotides can be DNA, RNA, or synthetic analogs, such as peptide nucleic acids, wherein the ribose phosphodiester backbone of the polynucleotide is replaced with a pseudo-peptide (polyamide) backbone.

[0034] They can be obtained by classic methods, well known to the one skilled in the art, such as chemical synthesis, restriction enzyme digestion, by PCR amplification, etc. They can also be labeled, by radioactive or cold labeling. Numerous protocols for polynucleotide synthesis or labeling are well known in the art (cf. for instance Sambrook and Russell, 2001 : Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

[0035] For the purpose of carrying out the invention, the polynucleotides can be used in many different ways, according to the various techniques for detection of a target nucleic acid sequence based on selective nucleic acid hybridization which are available in the art (cf. for instance Sambrook and Russell, 2001, mentioned above).

[0036] The methods of the invention can be performed either on whole bacteria previously lysed, or on nucleic acid (genomic DNA, cDNA or mRNA) isolated from said bacteria.

[0037] By way of example, polynucleotides can be used to detect *E. ruminantium*, and advantageously, to differentiate between *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden, in Southern hybridization, blot hybridization, Northern hybridization, colony hybridization on bacterial colonies, PCR amplification etc. They can be used individually in separate reactions, or they can be combined by 2 or more for use in a same reaction mixture. In this case, they will be labelled in order to be distinguished from each other, and/or they will be spatially separated by immobilization on different spots of a solid phase matrix.

[0038] According to a particularly preferred embodiment, these polynucleotides are used as immobilized probes in DNA arrays. For this use, polynucleotides of at least 30 bp, preferably of at least 50 bp will be preferred. Appropriate polynucleotides may be designed from the target sequences provided by the invention, by methods known in themselves, for instance using OligoArray 2.1 (Rouillard et al.. Nucleic Acids Research. 31 : 3057-3062, 2003).

[0039] Non-limitative examples of polynucleotides that can be used as nucleic acid probes for detecting the orphan genes defined above are: oligo-ERGA-4340, oligo-ERGA-4980, oligo-ERGA-5590, oligo-ERGA-5600, oligo-ERGA-7580, and oligo-ERWE-8330, that respectively hybridize selectively with the orphan genes ERGA_CDS_04340, ERGA_CDS_04980, ERGA_CDS_05590, ERGA_CDS_05600, ERGA_CDS_07580 and ERWE_CDS_08330.

[0040] Other non-limitative examples of polynucleotides that can be used as nucleic acid probes for detecting the same orphan genes are the amplicons PCR-oligo-ERGA-4340, PCR-oligo-ERGA-4980, PCR-oligo-ERGA-5590, PCR-oligo-ERGA-5600, PCR-oligo-ERGA-7580, and PCR-oligo-ERWE-8330 that respectively hybridize selectively with the orphan genes ERGA_CDS_04340, ERGA_CDS_04980, ERGA_CDS_05590, ERGA_CDS_05600, ERGA_CDS_07580 and ERWE_CDS_08330.

[0041] Also disclosed are nucleic acid probes useful for detecting *E. ruminantium* through the detection of any of the members of one or several allelic couple(s) of genes defined above. These probes can be directed to the Zone 1 region (P-Z-1 probe), or to the Zone 3 region (P-Z-3 probe), of the targeted allelic couple. A combination of a P-Z-1 probe and of a P-Z-3 probe can also be used.

[0042] Also disclosed are nucleic acid probes useful for differentiating between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden through the discrimination between the members of one or several allelic couple(s) of genes defined above. These probes are specific for the junction between Zone 1 and Zone 3, or preferably, for the Zone 2 region (P-Z-2 probe) of the targeted allelic couple. They are more particularly useful for discrimination between members of allelic couples where the size of the deletion in zone 2 is sufficiently important to allow its easy detection by DNA hybridization.

[0043] These allelic couples include:

- the couple consisting of ERGA_CDS_01350 and ERWE_CDS_01390;
- the couple consisting of ERGA_CDS_04500 and ERWE_CDS_04590+ ERWE_CDS_04600;
- the couple consisting of ERGA_CDS_05350 and ERWE_CDS_05460+ ERWE_CDS_05470.

[0044] The application also discloses triplets of nucleic acid probes useful for discrimination between members of allelic couples, comprising a probe specific of the Zone 1 region (P-Z-1 probe), a probe specific of the Zone 2 region (P-Z-2 probe), and a probe specific of the Zone 3 region (P-Z-3 probe).

[0045] Non limitative examples of said triplets of probes, designated MutERWE multiplexes, comprise the following oligonucleotides:

- MutERWE-1390N1, MutERWE-1390N2, and MutERWE-1390N3;

- MutERWE-4590N1, MutERWE-4590N2, and MutERWE-4600N3;
- MutERWE-5460N1, MutERWE-5460N2, and MutERWE-5460N3.

[0046] Advantageously, these nucleic acid probes are used in DNA arrays allowing to test simultaneously several genes of *E. ruminantium*, wherein said genes include at least one of the orphan genes defined above, and/or at least one member of any of the allelic couples defined above.

[0047] The disclosure also encompasses said DNA arrays.

[0048] DNA arrays are characterized in that they comprise at least one polynucleotide of the invention of at least 30 bp, preferably at least 50 bp, specific to orphan gene ERGA_CDS_04980 (SEQ ID NO: 2), and further at least one polynucleotide selected among :

- a polynucleotide specific to anyone of the other orphan genes defined above;
- a polynucleotide common to both members of anyone of the allelic couples defined above (i.e targeted to the Zone 1 or the Zone 3 region of said allelic couple);
- a polynucleotide specific to one of the members of anyone of the allelic couples disclosed above (i.e targeted either to the Zone 2 region or to the junction between Zone 1 and Zone 3 of said allelic couple).

[0049] Advantageously, DNA arrays comprise a combination of 2 or more of said polynucleotides.

[0050] Thus, DNA arrays of the invention allow to test in an *E. ruminantium* strain to be analyzed, various combinations of genes that can include from one to all of the orphan genes and/or from one to all of allelic couples defined above.

[0051] Non-limitative examples of DNA arrays of the invention are:

- i) a DNA array comprising the polynucleotide PCR-oligo-ERGA-4980, and at least one polynucleotide selected among the following: PCR-oligo-ERGA-4340, PCR-oligo-ERGA-5590, PCR-oligo-ERGA-5600, PCR-oligo-ERGA-7580, and PCR-oligo-ERWE-8330, or any combination of 2, 3, 4, or 5 of these polynucleotides;
- ii) a DNA array comprising the polynucleotide oligo-ERGA-4980, and at least one polynucleotide selected among the following: oligo-ERGA-4340, oligo-ERGA-5590, oligo-ERGA-5600, oligo-ERGA-7580, and oligo-ERWE-8330, or any combination of 2, 3, 4, or 5 of these polynucleotides.
- iii) a DNA array i) or ii) further comprising at least one polynucleotide selected among the following: MutERWE-1390N2, MutERWE-4590N2, and MutERWE-5460N2;
- iv) a DNA array i) or ii) further comprising at least one polynucleotide selected among the following: MutERWE-1390N1, MutERWE-1390N3, MutERWE-4590N1, MutERWE-4600N3, MutERWE-5460N1 and MutERWE-5460N3;
- v) a DNA array i) or ii) further comprising any combination of at least one of the polynucleotides listed in iii) with at least one of the polynucleotides listed in iv);

[0052] A large range of methods, protocols and techniques exist to develop and probe DNA arrays and read and analyse data. The person expert in the art has easy access to a large literature on the DNA array technology and can easily implement any kind of DNA array approach to identify *E. ruminantium*, and/or discriminate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden using information provided in the invention. One can find further information and start a broader literature review if needed from the following references: Lipshutz et al., Nat Genet. 21: 20-24, 1999 ; Kiechle and Holland-Staley, Arch. Pathol. Lab. Med. 127: 1089-1097, 2003 ; Rast et al., Dev Biol. 228: 270-286, 2000 ; Manduchi et al., Bioinformatics 16: 685-698, 2000 ; Mäder et al., J. Bacteriol. 184: 4288-4295, 2002 Bowtell, Nat. Genet. 21: 25-32, 1999 ; WO 2004/061111.

[0053] The present application also discloses combinations of polynucleotides that can be used as sets of PCR primers.

[0054] Non limitative examples of sets of primers that can be used to discriminate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden by detecting the presence or the absence of the orphan genes described above are: P-ERGA-4340-A/P-ERGA-4340-B; P-ERGA-4980-A/P-ERGA-4980-B; P-ERGA-5590-A/P-ERGA-5590-B; P-ERGA-5600-A/P-ERGA-5600-B; P-ERGA-7580-A/P-ERGA-7580-B; P-ERWE-8330-A/P-ERWE-8330-B.

[0055] Other examples of sets of primers are those which allow depending on the way they are used, either to detect *E. ruminantium*, or to discriminate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

[0056] This type of sets of primers includes combinations of polynucleotides common to both members of anyone of the allelic couples defined above under sequences SEQ ID NO: 7 to 20. Typically, said combinations comprise:

- at least one polynucleotide specific of the region upstream from the mutation (i.e Zone 1); and/or
- at least one polynucleotide specific of the region downstream from the mutation (i.e Zone 3).

[0057] These sets of PCR primers allow obtaining an amplification product from both members of the allelic couple. In this case, the discrimination between the native and the mutant allele will be performed on the basis of the difference

of size and/or of sequence between the amplification products, by way of example through their RFLP patterns after appropriate enzymatic digestion.

[0058] Non-limitative examples of such sets of primers are P-WEGA-120-S/P-WEGA-120-AS; P-WEGA-1350-S/P-WEGA-1350-AS; P-WEGA-4500-S/P-WEGA-4500-AS; P-WEGA-5350-S/P-WEGA-5350-AS; P-WEGA-5740-S/P-WEGA-5740-AS; P-WEGA-7410-S/P-WEGA-7410-AS.

[0059] Based on the information provided in the invention, a person skilled in the art can easily design other primers, detect other sequences and select different sets of restriction enzymes. A large range of RFLP strategies and techniques exist, some of them being for instance associated to PCR, to labeled probes, to Southern-blot analysis or to DNA sequencing, and a person expert in the art can easily implement a whole range of approaches to detect *E. ruminantium* or to discriminate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden using information from the invention. Similarly, a broad range of techniques exist to obtain, separate and analyze restriction fragments for RFLP analysis. Data provided in example 9 are compatible with any kind of technique for separation and analysis which are well described in the literature and known to a person expert in the art.

[0060] Another type of sets of primers are those which allow to discriminate between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden, by detecting the presence or the absence of an amplification product.

[0061] This type of sets of primers includes polynucleotides common to both members of anyone of the allelic couples defined above under sequences SEQ ID NO: 7 to 20, and polynucleotides specific to one of the members of said allelic couple.

[0062] Typically, a combination of this second type comprises:

- at least one polynucleotide specific to the mutated region (i.e Zone 2, or the junction between Zone 1 and Zone 3), and thus specific to one of the members of the allelic couple ; and
- at least one polynucleotide common to both members of the allelic couple, and specific of the region upstream from the mutation (i.e the Zone 1); and/or
- at least one polynucleotide common to both members of the allelic couple, and specific of the region downstream from the mutation (i.e the Zone 3).

[0063] Examples of such combinations include for instance:

- pairs of primers, where the sense primer recognizes the Zone 1 region (P-Z-1 primer) and the antisense primer recognises the Zone 2 region (P-Z-2-AS primer), or the sense primer recognises the Zone 2 region (P-Z-2-S primer) and the antisense primer recognises the Zone 3 region (P-Z-3-AS primer).
- pairs of primers where the sense primer recognizes the Zone 1 region (P-Z-1 primer) and the antisense primer recognises the junction between Zone 1 and Zone 3 (P-Z-1/3-AS primer), or the sense primer recognises the junction between Zone 1 and Zone 3 (P-Z-1/3-S primer) and the antisense primer recognises the Zone 3 region (P-Z-3-AS primer).

[0064] Such combinations are useful for instance to discriminate *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden, by carrying out a simple PCR in parallel on both strains, on the basis of the absence or presence of a PCR product.

- triplets of primers, wherein a sense primer recognizes the Zone 1 region (P-Z-1 primer), a first antisense primer recognises the Zone 2 region (P-Z-2-AS primer), or junction between Zone 1 and Zone 3 (P-Z-1/3-AS primer) and a second antisense primer recognises the Zone 3 region (P-Z-3 region). Such a combination allows discrimination between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden by multiplex PCR performed in parallel in the 2 strains.

[0065] Using such combinations of three primers in the same PCR reaction yields differential patterns for *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

[0066] Non-limitative examples of such pairs or triplets of primers, and of their use for discriminating *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden by simple PCR and multiplex PCR are:

- P-Z-1-ERGA-120; P-Z-2-ERGA-120-S; P-Z-2-ERGA-120-AS; P-Z-3-ERGA-120;
- P-Z-1-ERGA-1350; P-Z-2-ERGA-1350-S; P-Z-2-ERGA-1350-AS; P-Z-3-ERGA-1350;
- P-Z-1-ERGA-4500; P-Z-2-ERGA-4500-S; P-Z-2-ERGA-4500-AS; P-Z-3-ERGA-4500;
- P-Z-1-ERGA-5350; P-Z-2-ERGA-5350-S; P-Z-2-ERGA-5350-AS; P-Z-3-ERGA-5350;
- P-Z-1-ERGA-5740; P-Z-2-ERGA-5740-S; P-Z-2-ERGA-5740-AS; P-Z-3-ERGA-5740;
- P-Z-1-ERWE-7410; P-Z-2-ERWE-7410-S; P-Z-2-ERWE-7410-ASP-Z-3-ERWE-7410.

[0067] For all these primers, an amplification product is obtained only from the native member of the allelic couple when a P-Z-2-S primer or a P-Z-2-AS primer is used.

- P-Z-1-ERGA-4500;P-Z-1/3-ERGA-4500-S; P-Z-1/3-ERGA-4500-AS;P-Z-3-ERGA-4500;

[0068] For these primers, an amplification product is obtained only from the mutant member of the allelic couple when a P-Z-1/3-S primer or a P-Z-1/3-AS primer is used.

[0069] These combinations are meant to exemplify the approach and do not limit the invention. A person skilled in the art can easily define other primers combinations and/or other primer orientations which will lead to the detection of *E. ruminantium* or to a clear discrimination between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden.

[0070] The disclosure also comprises diagnostic kits for detecting *E. ruminantium* or for discriminating between strain Gardel and strain Welgevonden of *E. ruminantium*, wherein said kits comprise at least a nucleic acid probe and/or at least a set of primers for the detection of ERGA_CDS_04980 (SEQ ID NO:2).

[0071] The methods and tools provided by the invention are suitable for use at various stages of the life cycle of *E. ruminantium*, more specifically but not limited to the domestic-ruminants infectious stage, vector-interaction stage or reservoir animals-interaction stage. Preferred utilisations of the methods and tools of the invention include but are not limited to, the detection of *Ehrlichia ruminantium* in a given territory, the strain specific identification of *Ehrlichia ruminantium* in a given territory, the discrimination between strains of *Ehrlichia ruminantium* in a given territory or between different geographical regions, the analysis of strain movements within a region or between geographically distinct regions, the differential presence of strains of *Ehrlichia ruminantium* according to vector species and/or populations or the early detection and risk assessment in regions where potential vectors are present but where the disease has not been recorded yet.

[0072] For a better discrimination between *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden, one can advantageously combine the detection of at least one orphan gene or of any combination of orphan genes among those listed herein, with the detection of at least one allelic couple or any combination of allelic couples among those listed herein.

[0073] The orphan genes and allelic couples disclosed in the invention are also candidates of choice for the further development of vaccines. Orphan genes and mutant genes are the only genes differing between the strains Gardel and Welgevonden of *E. ruminantium*. Since these two strains do not generate cross protection through vaccination, protective proteins involved must be different in each strain. For instance a protective protein yielding protection against *E. ruminantium* strain Gardel is most likely to be absent or altered in *E. ruminantium* strain Welgevonden, since an animal vaccinated and protected against *E. ruminantium* strain Gardel is not protected and dies when infected with *E. ruminantium* strain Welgevonden. The most obvious group of absent or altered proteins between both strains are those encoded by the orphan or mutant genes. Accordingly, vaccines comprising these polypeptides will not only protect against heartwater, or cowdriosis, and *E. ruminantium*, but will permit to efficiently protect against non cross-protective strains and prevent the risk of deadly cross-infections.

[0074] Specifically exemplified herein is the identification of orphan genes and allelic couples from either or both the strains Gardel and Welgevonden from *Ehrlichia ruminantium*, and the use of PCR primers and nucleic acid probes derived from these genes for the development of DNA arrays, as well as the use of PCR primers derived from these genes for single-pair and multiplex PCR. The use of these primers and probes for differentiating *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden is also exemplified herein.

[0075] The genes and CDS described in these examples are designated according to the annotation of the genome sequences of strains Gardel and Welgevonden, which was performed using the GenoAnnot tool of the GenoStar package (www.genostar.org).

EXAMPLE 1. LACK OF VACCINAL CROSS-PROTECTION BETWEEN *E. RUMINANTIUM* STRAIN GARDEL AND *E. RUMINANTIUM* STRAIN WELGEVONDEN.

[0076] The strain Gardel of *E. ruminantium* was isolated in Guadeloupe island in 1982 from a goat injected with an homogenate of a female individual of *A. variegatum* collected on cows (Uilenberg et al., Rev. Elev. Méd. Vét. Trop. 34 : 34-42, 1985). The strain Welgevonden of *E. ruminantium* was isolated in South Africa in 1985 from mice injected with individually homogenised infected field-collected *A. hebraeum* ticks (Du Plessis, 1985). The strain Welgevonden was multiplied in mice for 8 passages (Du Plessis, 1985). *E. ruminantium* was multiplied on bovine umbilical endothelial cell (BUEC) grown in Glasgow-MEM medium complemented with foetal calf serum, tryptose-phosphate broth and antibiotics (Bezuidenhout et al., J. Vet. Res. 52: 113-120, 1985) at 37°C, 5% CO₂ with a weekly reinfection (Martinez et al., Vet. Parasitol. 67: 175-184, 1990). Unlike the Gardel strain, the strain Welgevonden is highly infective to both rodents (mice) and ruminants through intravenous injection.

[0077] Vaccination assays were conducted on Creole goats originating from Les Saintes islands, a heartwater-free

region of the Caribbean. Pre-bleed sera of all the animals were negative for anti-*Ehrlichia ruminantium* antibodies as determined by an indirect map-1b ELISA (van Vliet et al., J. Clin. Microbiol. 33: 2405-2410, 1995). After attenuated and virulent challenges, rectal temperature of each animal was daily monitored. *E. ruminantium* strain Gardel was attenuated after more than one hundred successive passages on goat endothelial cells. Virulent Gardel preparation (passage 34 and 42) derived from *in vitro* culture. The supernatant was collected when 70-80% of the cells were lysed by the bacteria for injection to goats. For immunization by infection with ticks and antibiotic treatment, *Amblyomma variegatum* larvae were fed on Gardel *E. ruminantium* infected animal. After moulting, infected nymphs, were engorged on a naive goat which was treated on the third day following hyperthermia with oxytetracycline at a dose of 20 mg/kg of body weight. For immunization with sublethal doses, *in vitro* *E. ruminantium* strain Gardel preparations (passage 14) were titrated by tissue culture lethal dose 50 (TCLD50) as described previously (Martinez et al., Vet Parasitol. 67: 175-184, 1996). Two-fold serial dilutions of inoculum were prepared to obtain sublethal *E. ruminantium* doses from 10 to 0.625 TCLD50. 5 groups of 5 goats were inoculated i.v. with these doses. Goats which survived following hyperthermia without antibiotic treatment were selected for the experiment.

[0078] Goats were vaccinated with *E. ruminantium* strain Gardel using different kinds of vaccines. The first kind of vaccine tested is an attenuated vaccine. In this case, the aggressiveness of the strain was reduced by successive passages on cell culture. In this case, strains are no longer aggressive after 200 serial passages. Strains having undergone less than 100 passages are virulent strains. Attenuated vaccine relies on a strain which is still alive. Another kind of vaccine, inactivated vaccine, was assessed. In this case, the bacteria were killed with sodium azide. Another means of vaccination investigated is infection with pathogenic strains followed by treatments with antibiotics. Animals were infected either with a virulent culture supernatant or by contact with infected ticks. When hyperthermia (fever) appears, the animals are then treated with tetracycline. Another way of vaccinating the animals was to inject sublethal doses of a virulent population and wait for the animal to recover without treatment with antibiotics. Experiments were conducted with animals vaccinated with all the various means of vaccination described above for further homologous or heterologous challenge with a virulent strain.

[0079] Vaccination and homologous and heterologous challenge experiments are summarized in Table 1 below.

TABLE 1

Goat NO	Vaccination		Homologous challenge <i>E. ruminantium</i> strain Gardel Hyperthermia	Heterologous challenge <i>E. ruminantium</i> strain Welgevonden Hyperthermia Death	
	E. ruminantium strain Gardel	Vaccine type Hyperthermia		Yes (12 D.A.I.)	Yes (15 D.A.I.)
9412	Attenuated	No	No	Yes (12 D.A.I.)	Yes (15 D.A.I.)
9642	Attenuated	No	No	Yes (13 D.A.I.)	Yes (15 D.A.I.)
9627	Sublethal dose	Yes	No	Yes (13 D.A.I.)	Yes (15 D.A.I.)
9506	Infected ticks AB	Yes 10 D.A.I.	No	Yes (12 D.A.I.)	Yes (15 D.A.I.)
0037	Control Yes 5 D.A.I. (A.B. to avoid death)			-	-
0038	Control Yes 6 D.A.I. (A.B. to avoid death)			-	-
9707	Control			Yes (12 D.A.I.)	Yes (15 D.A.I.)
AB : Treatment with antibiotics D.A.I : Day After Infection					

Experiments were conducted as follows:

55 Vaccination with attenuated vaccine (Goat NO 9642)

[0080] 4/11/96 Injection of *E. ruminantium* strain Gardel attenuated after 224 passages.

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No clinical reaction was observed indicating that the strain injected was not virulent. 20/03/97 Homologous challenge with a virulent population of *E. ruminantium* strain Gardel (34 passages) - 2 ml of culture supernatant were injected intravenously.

No clinical reaction was observed, indicating that the animal was protected against *E. ruminantium* strain Gardel.

5 28/04/98 Heterologous challenge with a virulent population of *E. ruminantium* strain Welgevonden (8 passages) - 800 µl of culture supernatant were injected intravenously.

Hyperthermia appeared 12 days post infection and the animal was treated with antibiotic after 15 days to avoid death. This indicates that the animal was not protected against *E. ruminantium* strain Welgevonden.

10 Vaccination with attenuated vaccine and serial challenge (Goat NO 9412)

[0081] 28/02/94 Injection of *E. ruminantium* strain Gardel attenuated after 136 passages.

No clinical reaction was observed indicating that the strain injected was not virulent.

15 7/06/94 Homologous challenge with an a virulent population of *E. ruminantium* strain Gardel (42 passages) - 2 ml of culture supernatant were injected intravenously.

No clinical reaction was observed, indicating that the animal was protected against *E. ruminantium* strain Gardel.

20/03/97 Homologous challenge with a virulent population of *E. ruminantium* strain Gardel (34 passages) - 2 ml of culture supernatant were injected intravenously

No clinical reaction was observed, indicating that the animal remained protected against *E. ruminantium* strain Gardel after 3 years.

25 28/04/98 Heterologous challenge with a virulent population of *E. ruminantium* strain Welgevonden (8 passages) - 800 µl of culture supernatant were injected intravenously. Hyperthermia appeared 12 days post infection. The animal was not treated with antibiotic and death occurred 15 days after infection with *E. ruminantium* strain Welgevonden. This indicates that the animal was not protected against *E. ruminantium* strain Welgevonden.

25 Vaccination with sublethal doses (Goat NO 9642)

[0082] 4/10/96 Infection with a sublethal dose of *E. ruminantium* strain Gardel (0.625 TCID₅₀ of *E. ruminantium* strain Gardel after 14 passages).

30 Hyperthermia was observed but the animal survived and recovered without treatment with antibiotics.

20/03/97 Homologous challenge with a virulent population of *E. ruminantium* strain Gardel (34 passages) - 2 ml of culture supernatant were injected intravenously.

No clinical reaction was observed, indicating that the animal was protected against *E. ruminantium* strain Gardel.

28/04/98 Heterologous challenge with a virulent population of *E. ruminantium* strain Welgevonden (8 passages) - 800 µl of culture supernatant were injected intravenously.

35 Hyperthermia appeared 13 days post infection and the animal was treated with antibiotic after 15 days to avoid death. This indicates that the animal was not protected against *E. ruminantium* strain Welgevonden.

40 Vaccination with infected ticks (Goat NO 9506)

[0083] 20/06/96 Infection with ticks infected with *E. ruminantium* strain Gardel.

Hyperthermia was observed 10 days post infection and the animal was treated with antibiotics to avoid death. The animal recovered and survived.

20/03/97 Homologous challenge with a virulent population of *E. ruminantium* strain Gardel (34 passages) - 2 ml of culture supernatant were injected intravenously.

No clinical reaction was observed, indicating that the animal was protected against *E. ruminantium* strain Gardel.

28/04/98 Heterologous challenge with a virulent population of *E. ruminantium* strain Welgevonden (8 passages) - 800 µl of culture supernatant were injected intravenously.

45 Hyperthermia appeared 12 days post infection and the animal was treated with antibiotic after 15 days to avoid death.

50 This indicates that the animal was not protected against *E. ruminantium* strain Welgevonden.

Control for susceptibility to *E. ruminantium* strain Gardel (Goats NO 0037 and 038)

[0084] 28/04.98 Infection of naive goats (not vaccinated) with a virulent population of *E. ruminantium* strain Gardel (32 passages) - 500 µl of culture supernatant were injected intravenously.

55 Hyperthermia appeared 5 and 6 days post infection. Animals were treated with antibiotic to avoid death. This indicates that the animals were susceptible to *E. ruminantium* strain Gardel.

Control for susceptibility to *E. ruminantium* strain Welgevonden (Goat NO 9707)

[0085] 28/04/98 Infection of a naïve goat (not vaccinated) with a virulent population of *E. ruminantium* strain Welgevonden (8 passages) - 800 µl of culture supernatant were injected intravenously.

5 Hyperthermia appeared 12 days post infection. The animal was not treated with antibiotic and death occurred 15 days after infection. This indicates that the animals were susceptible to *E. ruminantium* strain Welgevonden.

EXAMPLE 2. GENERAL FEATURES AND SEQUENCE REFERENCE

10 [0086] For each strain, purified DNA was broken by sonication to generate fragments of differing sizes. After filling up the ends with Klenow polymerase, DNA fragments ranging from 0.5 kb to 4 kb were separated in a 0.8% agarose gel and collected after gelase (Epicentre) digestion of a cut agarose band. Blunt-end DNA fragments were inserted into pBluescript II KS (Stratagene) digested with EcoRV and dephosphorylated. Ligation was performed with the Fast-Link DNA Ligation kit (Epicentre) and competent DH10B *E. coli* were transformed prior to colony isolation on LB-agar+ Ampicillin + Xgal +IPTG. About 15000 clones were isolated for each strain of *E. ruminantium*. Plasmidic DNA from recombinant *E. coli* strains was extracted according to the alkaline lysis method and inserts were sequenced on both strands using universal forward and reverse M13 primers and the ET DYEnamic terminator kit (Amersham). Sequences were obtained with ABI 373 et ABI 377 automated sequencers (Applied Biosystems)- Data were analysed and contigs were assembled using Phred-Phrap et Consed software packages (<http://www.genome.washington.edu>). Gaps were filled in through primer-directed sequencing using custom made primers. A total of about 20000 raw sequence runs were generated and analysed for each *E. ruminantium* strain to generate a full length consensus sequence with a coverage of 6x to 7x.

20 [0087] The genome of *E. ruminantium* strains Gardel and Welgevonden is arranged as a circular chromosome of 1499920 bp and 1512977 bp, respectively. The respective G+C contents for the strains Gardel and Welgevonden is 27.51 % and 27.48 %. The genome of *E. ruminantium* strain Gardel comprises 948 coding sequences of an average size of 1018 bp which represent a total coding surface of 63 % of the whole genome. The genome of *E. ruminantium* strain Welgevonden bears 957 genes of the same average size of 1018 bp. The genome surface of this strain devoted to coding sequences is 62 %. Both genomes comprise 36 transfer RNAs (tRNA) and 3 ribosomal RNAs (rRNA).

30 **EXAMPLE 3. IDENTIFICATION OF ORPHAN GENES IN THE GARDEL AND WELGEVONDEN STRAINS OF *E. RUMINANTIUM***

35 [0088] The differential analysis of the whole genomes of *E. ruminantium* strains Gardel and Welgevonden showed the presence of coding sequences which are present in only one of the strains and not in the other (orphan gene sequences). Some of the CDS which are unique to *E. ruminantium* strain Gardel and found only in the genome of this strain are presented in Table 2 (Seq ID NO 1 to Seq ID NO 5). One of the CDS which is unique to *E. ruminantium* strain Welgevonden and found only in the genome of this strain is also presented in Table 2 (Seq ID NO 6). Since these sequences are unique to one or the other strain, they clearly represent targets for the differential detection of *E. ruminantium* strain Gardel versus *E. ruminantium* strain Welgevonden.

40 TABLE 2

CDS name	SeqID	Size (bp)	Annotated function	Strain
ERGA_CDS_04340	Seq ID NO 1	186	unknown	Gardel
ERGA_CDS_04980	Seq ID NO 2	270	unknown	Gardel
ERGA_CDS_05590	Seq ID NO 3	630	unknown	Gardel
ERGA_CDS_05600	Seq ID NO 4	828	unknown	Gardel
ERGA_CDS_07580	Seq ID NO 5	303	unknown	Gardel
ERWE_CDS_08330	Seq ID NO 6	225	unknown	Welgevonden

EXAMPLE 4. IDENTIFICATION OF MUTANT ALLELES IN THE GARDEL AND WELGEVONDEN STRAINS OF *E. RUMINANTIUM*

55 [0089] The differential analysis of the whole genomes of *E. ruminantium* strains Gardel and Welgevonden also showed the presence of coding sequences which are affected by one or several mutations in one of the two strains and for which a non-mutated, functionally active and normal allele is present in the genome of the other strain. These allelic couples of coding sequences are presented in Table 3.

TABLE 3

Mutant allele in	SEQ ID NO	Size (bp)	Nature of mutation	Native allele in:	SEQ ID NO	Size (bp)	Annotated Function
Gardel				Welgevonden			
ERGA CDS 07330	19	3522	Deletion	ERWE CDS 07410	20	4122	Unknown
Welgevonden				Gardel			
ERWE CDS 00120	8	1176	Deletion	ERGA CDS 00120	7	1266	Unknown
ERWE CDS 01390	10	2856	Deletion	ERGA CDS 01350	9	3252	Unknown
ERWE CDS 05830	12	1659	Deletion	ERGA CDS 05740	11	1836	Unknown
ERWE_CDS_04590 + ERWE CDS 04600	14 + 15	873 + 1740	Deletion + Frameshift	ERGA_CDS_04500	13	3570	Un known
ERWE_CDS_05460 + ERWE CDS 05470	17 + 18	2361 + 4473	Deletion + Frameshift	ERGA_CDS_05350	16	6903	Un known

EXAMPLE 5. DIFFERENTIAL DNA ARRAY DETECTION OF STRAINS OF *E. RUMINANTIUM* BASED ON RECOGNITION OF ORPHAN GENES WITH AMPLICONS

[0090] PCR primers were designed using Vector NTI Advance 9.0 (Informatix).

[0091] These oligonucleotides are used to produce amplicons by PCR using *E. ruminantium* strain Welgevonden or strain Gardel DNA as template. Oligonucleotides used in this example as PCR primers, and the resulting amplicons are listed in Table 4. The sequence of the PCR primers is indicated in Table 15A.

TABLE 4

Primer name	Orientation	Position relative to CDS	Size (mer)	CDS	Amplicon (size)
Strain Gardel					
P-ERGA-4340-A	Sense	1-21	21	ERGA_CDS_04340	PCR oligo ERGA 4340 (173 bp)
P-ERGA-4340-B	Antisense	153-173	21	ERGA_CDS_04340	PCR oligo ERGA 4340 (173 bp)
P-ERGA-4980-A	Sense	1-25	25	ERGA_CDS_4980	PCR oligo ERGA 4980 (218 bp)
P-ERGA-4980-B	Antisense	196-218	23	ERGA_CDS_4980	PCR oligo ERGA 4980 (218 bp)

(continued)

	Primer name	Orientation	Position relative to CDS	Size (mer)	CDS	Amplicon (size)
5	P-ERGA-5590-A	Sense	1-20	20	ERGA_CDS_05590	PCR oligo ERGA 5590 (509 bp)
	P-ERGA-5590-B	Antisense	490-509	20	ERGA_CDS_05590	PCR oligo ERGA 5590 (509 bp)
10	P-ERGA-5600-A	Sense	56-74	19	ERGA_CDS_05600	PCR oligo ERGA 5600 (643 bp)
	P-ERGA-5600-B	Antisense	677-698	22	ERGA_CDS_05600	PCR oligo ERGA 5600 (643 bp)
15	P-ERGA-7580-A	Sense	1-23	23	ERGA_CDS_07580	PCR oligo ERGA 7580 (239 bp)
	P-ERGA-7580-B	Antisense	221-239	19	ERGA_CDS_07580	PCR oligo ERGA 7580 (239 bp)
Strain Welgevonden						
20	P-ERWE-8330-A	Sense	14-38	25	ERWE_CDS_08330	PCR oligo ERWE 8330 (180 bp)
	P-ERWE-8330-B	Antisense	173-193	21	ERWE_CDS_08330	PCR oligo ERWE 8330 (180 bp)
Strain Welgevonden						
25	P-ERWE-8330-A	Sense	14-38	25	ERWE_CDS_08330	PCR oligo ERWE 8330 (180 bp)
	P-ERWE-8330-B	Antisense	173-193	21	ERWE_CDS_08330	PCR oligo ERWE 8330 (180 bp)
Strain Welgevonden						
30	P-ERWE-8330-A	Sense	14-38	25	ERWE_CDS_08330	PCR oligo ERWE 8330 (180 bp)
	P-ERWE-8330-B	Antisense	173-193	21	ERWE_CDS_08330	PCR oligo ERWE 8330 (180 bp)

Preparation of the amplicons

[0092] DNA is extracted from elementary bodies of *E. ruminantium* as described by Perez et al. (FEMS Microbiol. Lett. 154: 73-79, 1997). *E. ruminantium* strains are grown in BUEC cells as described in Example 1 above. Elementary bodies are purified from the culture supernatant by differential centrifugation and resuspended in 350 µl of PBS to which is added 150 µl of buffer containing 25 mM Tris-HCl (pH 8.0), 10 mM MgCl₂ and 125 µg of DNase in order to remove contaminating host cell DNA. After incubation for 90 min. at 37°C, the reaction is stopped by addition of 25 mM EDTA. Elementary bodies are washed three times in water and lysed by overnight incubation at 55°C in a solution of 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 25 mM EDTA, 1.5% SDS and 250 µg/ml of proteinase K. Bacterial DNA is extracted with phenol-chloroform, precipitated with cold ethanol and resuspended in sterile distilled water. Contamination with cell DNA is evaluated by slot blot hybridization using labelled bovine DNA as a probe and dilutions of bovine DNA (12.5 ng and 25 ng) as positive controls.

[0093] Amplicons are amplified from DNA extracted from *E. ruminantium* elementary bodies using the primers described in Table 4. A standard procedure is used to obtain the amplicons through PCR (Sambrook & Russe1, "Molecular Cloning :a laboratory manual", 3rd Edition, vol. 2, Chapter 8). PCR amplification of amplicons are obtained by mixing 250 ng of *E. ruminantium* DNA, 2.5 U of Taq DNA polymerase, 200 nM of each dNTP, 1 µM of each, sense and antisense, primer and 3 mM MgCl₂ in a final volume of 50 µl. Amplification is carried out under the following conditions: 5 min denaturation at 94°C, followed by 30 cycles of amplification with a 1-min denaturation, 45 sec of annealing at 45°C and 2 min extension at 72°C. An extra extension step of 10 min at 72°C is added after completion of the 30 cycles. PCR products, i.e. amplicons, are analysed by 1% agarose gel electrophoresis in Tris-borate-EDTA buffer.

[0094] Table 5 below indicates the size and position of the amplicons relative to the corresponding CDS.

TABLE 5

Amplicon			CDS	
Name	Size	Position relative to CDS	Name	Size
Strain Gardel				
PCR-oligo-ERGA-4340	173 bp	1-173	ERGA_CDS_04340	186 bp
PCR-oligo-ERGA-4980	218 bp	1-218	ERGA_CDS_04980	270 bp
PCR-oligo-ERGA-5590	509 bp	1-509	ERGA_CDS_05590	630 bp
PCR-oligo-ERGA-5600	643 bp	56-698	ERGA_CDS_05600	828 bp
PCR-oligo-ERGA-7580	239 bp	1-239	ERGA CDS 07580	303 bp
Strain Welgevonden				
PCR-oligo-ERWE-8330	180 bp	14-193	ERWE CDS 08330	225 bp

Preparation of DNA microarrays

[0095] Amplicons are spotted using the Amersham Biosciences Lucidea Array spotter on aminosilane-coated mirror glass slides (7 Star, Amersham Biosciences) following the procedure recommended by the supplier. Negative and positive control DNAs are also printed into 24 different sectors of each slide. After printing, the slides are stored at room temperature in a dessicator. Prior to hybridization, DNA is cross-linked to the slides by UV irradiation, washed twice with 0.2% SDS solution and rinsed twice with distilled water.

Preparation of labelled DNA from *E. ruminantium*

[0096] DNA is extracted from elementary bodies of *E. ruminantium* strain Gardel or strain Welgevonden as described above in this example. Purified DNA is fragmented by sonication as follows. A 500-µl DNA solution in TE buffer (10 mM Tris-HCl pH8.0, 0.3 mM EDTA) is sonicated for 3 cycles of 1 min each at amplitude 5. Samples are placed on ice while sonicated. Samples are incubated on ice for 1 min and centrifuged briefly to concentration the solution on the bottom of the tube after each cycle of sonication. The size of DNA fragments is checked by 1% agarose-gel electrophoresis under standard conditions. Preferred samples are ranging between 0.3 and 0.5 kb in size. Sonicated DNA from *E. ruminantium* strains Gardel and Welgevonden are labelled with different dyes. DNA from *E. ruminantium* Gardel is labelled with Cy3-dCTP (Amersham Biosciences) whereas *E. ruminantium* Welgevonden is labelled with Cy5-dCTP (Amersham Biosciences). In both cases DNA is labelled by random priming (BioPrime Array CGH Genomic labeling System from Invitrogen) following the procedure recommended by the supplier. 1 µg of sonicated DNA in 21 µl of sterile distilled water is mixed with 20 µl of the random primers solution and incubated at 95°C for 5 min and immediately cooled on ice. Still on ice, the following reagents are added: 5 µL of 10X dCTP nucleotide mix, 3 µl of Cy3-dCTP (or 3 µl of Cy5-dCTP depending on the strain), 1 µl of exo-klenow fragment enzyme. The solution is mixed and quickly spinned down prior to incubation at 37°C for 2 hours. Reaction is stopped by addition of 5µl of stop buffer. Probes are purified through purification columns as recommended by the supplier of the kit. 45 µl of TE buffer are added as well as 400 µl of purification buffer A. Solution is vortexed for 30 sec and loaded on a purification column within a collection tube. The column is centrifuged at 11000 g for 1 min at room temperature and the flow-through is discarded. 600 µl of purification buffer B are added to the column prior to centrifugation at 11000 g for 1 min at room temperature and the flow-through is discarded. The purification column is placed in another collection tube and 50 µl of sterile distilled water is added. The column is incubated 1 min at room temperature and centrifuged at 11000 g for 1 min at room temperature. The flow-through contains the purified probe. *E. coli* DNA prepared and labelled in the very same way is used as control. When using Cy3-labelled DNA from *E. ruminantium* Gardel, the *E. coli* control DNA is labelled with Cy5-dCTP, whereas when using Cy5-labelled DNA from *E. ruminantium* Welgevonden, the *E. coli* control DNA is labelled with Cy3-dCTP.

Hybridization of DNA to the microarrays

[0097] The labelled DNA preparations, samples and control, (0.9-1.2 µg per strain) are denatured at 95°C for 5 min. They are subsequently added to microarray hybridization buffer (Amersham Biosciences) and are applied to the microarrays in individual chambers of an automated slide processor (Amersham Biosciences). Hybridization is carried out at 42°C for PCR array (or 37 °C for oligos array) for 12 h. Hybridized slides are washed at 45°C successively with 1 X SSC, 0.2% SDS for 10 min, twice with 0.1 X SSC, 0.2% SDS for 10 min, with 0.1 X SSC for 1 min and with isopropanol before air drying. Microarrays are immediately scanned in both Cy3 and Cy5 channels with Amersham generation III array

scanner with a 10 µm resolution.

Results

- 5 [0098] DNA from strain Gardel hybridizes with all the spots of the micro-array bearing the amplicons PCR-oligo-ERGA-4340, PCR-oligo-ERGA-4980, PCR-oligo-ERGA-5590, PCR-oligo-ERGA-5600, PCR-oligo-ERGA-7580 and does not hybridizes with the spots bearing the amplicon PCR-oligo-ERWE-8330. On the other hand, DNA from strain Welgevonden hybridizes only with the spot bearing the amplicon PCR-oligo-ERWE-8330
- 10 [0099] These results show that *E. ruminantium* strain Gardel can be specifically discriminated from *E. ruminantium* strain Welgevonden using any combination from 1 to 6 of these amplicons.

EXAMPLE 6. DIFFERENTIAL DNA ARRAY DETECTION OF STRAINS OF *E. RUMINANTIUM* BASED ON RECOGNITION OF ORPHAN GENES WITH OLIGONUCLEOTIDES

- 15 [0100] This example is based on the use of oligonucleotide probes specific to the orphan genes to be detected. Oligonucleotides were designed using OligoArray 2.1 (Rouillard et al., Nucleic Acids Research. 31: 3057-3062, 2003). Table 6 below indicates the size of these oligonucleotides and their positions relative to the corresponding CDS.

TABLE 6

Oligonucleotide			CDS recognized by the oligonucleotide
Name	Size (bp)	Position relative to CDS sequence	
Oligo-ERGA-4340	50	137-186	ERGA_CDS_04340
Oligo-ERGA-4980	51	180-230	ERGA_CDS_04980
Oligo-ERGA-5590	50	561-610	ERGA_CDS_05590
Oligo-ERGA-5600	50	710-759	ERGA_CDS_05600
Oligo-ERGA-7580	50	194-243	ERGA_CDS_07580
Oligo-ERWE-8330	51	35-85	ERWE_CDS_08330

30 [0101] To prepare DNA arrays, the oligonucleotides are spotted on aminosilane-coated mirror glass slides (7 Star, Amersham Biosciences) following the procedure described for amplicons in Example 5 above. Labelled DNAs from *E. ruminantium* strain Gardel or *E. ruminantium* strain Welgevonden are prepared and hybridized to said arrays as disclosed in Example 5 above.

35 [0102] DNA from strain Gardel hybridizes with all the spots of the micro-array bearing the oligonucleotides Oligo-ERGA-4340, Oligo-ERGA-4980, Oligo-ERGA-5590, Oligo-ERGA-5600, Oligo-ERGA-7580 and does not hybridize with the spot bearing the oligonucleotide Oligo-ERWE-8330. On the other hand, DNA from strain Welgevonden hybridizes only with the spot bearing the oligonucleotide Oligo-ERWE-8330.

40 [0103] Thus *E. ruminantium* strain Gardel can be specifically discriminated from *E. ruminantium* strain Welgevonden using any combination from 1 to 6 of these oligonucleotides.

EXAMPLE 7. DIFFERENTIAL DNA ARRAY DETECTION OF STRAINS OF *E. RUMINANTIUM* BASED ON RECOGNITION OF MUTATED GENES WITH OLIGONUCLEOTIDES

45 [0104] The genes targeted in this example are truncated genes for which part of the transcribed region is lost in the central part of the initial coding sequence. Three main regions can therefore be considered. The first region, named Zone 1, is the 5' region of the gene up to the beginning of the deletion in the mutated gene. Zone 1 is a conserved region of high similarity between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. An oligonucleotide designed to match this region (N1) recognizes both strains. Zone 2, corresponds to the region of deletion in the mutant allele and therefore only the native full length allele bears a sequence in this region and can be recognized by an oligonucleotide (N2). Zone 3 is the second conserved region of high similarity between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. In this region also, an oligonucleotide designed to match Zone 3 (N3) recognizes both strains.

55 [0105] Oligonucleotides probes targeted to Zone 1, Zone 2, and Zone 3 of each of the allelic couples:

ERGA_CDS_01350/ERWE_CDS_01390,
ERGA_CDS_04500/(ERWE_GDS_04590 + ERWE_CDS_4600)

ERGA_CDS_05350/(ERWE_CDS_05460 + ERWE_CDS_5470)

were designed using OligoArray 2.1 (Rouillard *et al.*, cited above). Table 7 below indicates the size of these oligonucleotides and their positions relative to the corresponding CDS. The sequence of these oligonucleotides is indicated in Table 15A.

TABLE 7

Oligonucleotide name	Zone	Size (mer)	Position relative to CDS	Mutant allele	Position relative to CDS	Native allele
MutERWE-1390N1	1	50	507-556 - 2771-2820	ERWE_CDS_01390	507-556 2328-2377 3167-3216	ERGA_CDS_01350
MutERWE-1390N2	2	50		ERWE_CDS_01390		ERGA_CDS_01350
MutERWE-1390N3	3	50		ERWE_CDS_01390		CDS 01350
MutERWE-4590N1	1	50	455-504 - 962-1011	ERWE_CDS_04590	449-498 536-585 2777-2826	ERGA_CDS_04500
MutERWE-4590N2	2	50		ERWE_CDS_04590		ERGA_CDS_04500
MutERWE-4600N3	3	50		ERWE_CDS_04600		ERGA_CDS_04500
MutERWE-5460N1	1	50	2251-2300 - 3440-3489	ERWE_CDS_05460	6793-6842 5455-5504 3440-3489	ERGA_CDS_05350
MutERWE-5460N2	2	50		ERWE_CDS_05460		ERGA_CDS_05350
MutERWE-5470N3	3	50		ERWE_CDS_05470		ERGA_CDS_05350

[0106] These oligonucleotides can be used as oligonucleotide multiplexes for DNA array detection of *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. These oligonucleotide multiplexes are listed in Table 8 below.

TABLE 8

Mut oligonucleotide multiplexes	Oligonucleotides	Mutant allele	Native allele	Zone
MutERWE-1390	MutERWE 1390N1	ERWE_CDS_01390	ERGA_CDS_01350	1
	MutERWE 1390N2	ERWE_CDS_01390	ERGA_CDS_01350	2
	MutERWE 1390N3	ERWE CDS 01390	ERGA CDS 01350	3
MutERWE-4590/4600	MutERWE 4590N1	ERWE_CDS_04590	ERGA_CDS_04500	1
	MutERWE 4590N2	ERWE_CDS_04590	ERGA_CDS_04500	2
	MutERWE 4600N3	ERWE CDS 04600	ERGA CDS 04500	3
MutERW E-5460/5470	MutERWE 5460N1	ERWE_CDS_05460	ERGA_CDS_05350	1
	MutERWE 5460N2	ERWE_CDS_05460	ERGA_CDS_05350	2
	MutERWE 5470N3	ERWE CDS 05470	ERGA CDS 05350	3

[0107] To prepare DNA arrays, the oligonucleotides are spotted on aminosilane-coated mirror glass slides following the procedure described for amplicons in Example 5 above. Labelled DNAs from *E. ruminantium* strain Gardel or *E. ruminantium* strain Welgevonden are prepared and hybridized to said arrays as disclosed in Example 5 above.

[0108] A differential analysis on both strains is carried out by running a separate reaction for each oligonucleotide, N 1, N2 and N3 in each strain making a total of six separate reactions, each on a specific spot (six separate spots). All members of the series hybridize with DNA from the strain bearing the native full length allele yielding three positive responses. The N 1 and N3 oligonucleotides specific to the conserved Zone 1 and 3 hybridize with DNA from the strains bearing the mutation. The N2 oligonucleotide specific to the deleted region (Zone 2) does not hybridize onto the stain bearing the mutation and yields a negative response. A typical pattern for the strain bearing the mutation is a positive

response for oligonucleotide N1 and N3 and a negative response for oligonucleotide N2.

[0109] The Mut oligonucleotide series described in this example allow differential discrimination between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden through DNA arrays on three different genes with three different series of three oligonucleotides. This provides a high level of confidence in the specific identification of each strain.

5

EXAMPLE 8. DIFFERENTIAL PCR DETECTION OF STRAINS OF *E. RUMINANTIUM*

[0110] This example illustrates the discrimination between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden based on the differential PCR- amplification of native and mutant alleles of 6 different genes:

10

ERGA_CDS_00120/ERWE_CDS_00120
 ERGA_CDS_01350/ERWE_CDS_01390
 ERGA_CDS_05740/ERWE_CDS_05830
 ERGA_CDS_04500/(ERWE_CDS_04590 + ERWE_CDS_4600)
 ERGA_CDS_05350/(ERWE_CDS_05460 + ERWE_CDS_5470)
 ERGA_CDS_07330/ERWE_CDS_07410

15

[0111] Primers were designed using VectorNTI Advance 9.0 (Informax).

[0112] Table 9 below provides a list of these primers and indicates their positions relative to the corresponding CDS.

20

The sequence of these PCR primers is indicated in Table 15B.

TABLE 9.

Primer (orientation)	Zone	relative to CDS	Native allele	relative to CDS	Mutant allele
P-Z-1-ERGA-120 (sense)	1	5-29	ERGA_CDS_00120	5-29	ERWE_CDS_00120
P-Z-2-ERGA-120-S (sense)		442-466	ERGA_CDS_00120	-	ERWE_CDS_00120
P-Z-2-ERGA-120-AS (antisense)		442-466	ERGA_CDS_00120	-	ERWE_CDS_00120
P-Z-3-ERGA-120 (antisense)		1222-1246	ERGA_CDS_00120	1132-1156	ERWE_CDS_00120
P-Z-1-ERGA-1350 (sense)	2	240-264	ERGA_CDS_01350	240-264	ERWE_CDS_01390
P-Z-2-ERGA-1350-S (sense)		2344-2368	ERGA_CDS_01350	-	ERWE_CDS_01390
P-Z-2-ERGA-1350- AS (antisense)		2344-2368	ERGA_CDS_01350	-	ERWE_CDS_01390
P-Z-3-ERGA-1350 (antisense)		3007-3031	ERGA_CDS_01350	2611-2635	ERWE_CDS_01390
P-Z-1-ERGA-4500 (sense)	3	301-325	ERGA_CDS_04500	307-331	ERWE_CDS_04590
P-Z-2-ERGA-4500-S (sense)		548-572	ERGA_CDS_04500	-	ERWE_CDS_04590
P-Z-2-ERGA-4500- AS (antisense)		548-572	ERGA_CDS_04500	-	ERWE_CDS_04590
P-Z-3-ERGA-4500 (antisense)		829-853	ERGA_CDS_04500	775-799	ERWE_CDS_04590
P-Z-1-ERGA-5350 (sense)	4	4525-4549	ERGA_CDS_05350	223-247	ERWE_CDS_05460
P-Z-2-ERGA-5350-S (sense)		5466-5491	ERGA_CDS_05350	-	ERWE_CDS_05460

(continued)

Primer (orientation)	Zone	relative to CDS	Native allele	relative to CDS	Mutant allele
P-Z-2-ERGA-5350- AS (antisense)	2	5466-5491	ERGA_CDS_05350	-	ERWE_CDS_05460
P-Z-3-ERGA-5350 (antisense)		6071-6095	ERGA_CDS_05350	1532-1556	ERWE_CDS_05460
P-Z-1-ERGA-5740 (sense)	1	443-467	ERGA_CDS_05740	449-473	ERWE_CDS_05830
P-Z-2-ERGA-5740-S (sense)		1376-1400	ERGA_CDS_05740	-	ERWE_CDS_05830
P-Z-2-ERGA-5740- AS (antisense)	2	1376-1400	ERGA_CDS_05740	-	ERWE_CDS_05830
P-Z-3-ERGA-5740 (antisense)		1780-1804	ERGA_CDS_05740	1603-1627	ERWE_CDS_05830
P-Z-1-ERWE-7410 (sense)	1	151-175	ERWE_CDS_07410	151-175	ERGA_CDS_07330
P-Z-2- ERWE-7410- S (sense)		639-663	ERWE_CDS_07410	-	ERGA_CDS_07330
P-Z-2- ERWE-7410- AS (antisense)	2	639-663	ERWE_CDS_0741	-	ERGA_CDS_07330
P-Z-3- ERWE-120 (antisense)		1818-1843	ERWE_CDS_07410	1222-1246	ERGA_CDS_07330

[0113] P-Z-1 and P-Z-3 primers recognize the conserved regions Zone 1 and Zone 3, respectively, in both *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden. P-Z-2 primers are specifically binding to Zone 2 in the native full length allele and do not hybridise to the genome of the strain bearing the corresponding mutant allele.

[0114] In this example, P-Z-1 primers are sense primers whereas P-Z-3 primers are antisense primers. Two kinds of P-Z-2 primers are used, sense primer (labelled -S) and antisense primers (labelled -AS). They recognized the same region but on complementary strands and directed amplification in opposite orientations.

[0115] Two different ways of use of these primers are exemplified herein: simple PCR and multiplex PCR.

Simple PCR

[0116] DNA is extracted from *E. ruminantium* elementary bodies, as described in example 5 above. Simple direct PCR is performed using a standard procedure (Sambrook & Russel, Molecular Cloning "a laboratory manual", 3rd Edition, vol. 2, Chapter 8).

[0117] 250 ng of *E. ruminantium* DNA, 2.5 U of Taq DNA polymerase, 200 nM of each dNTP, 1 µM of each, sense and antisense, primer and 3 µM MgCl₂ are mixed in a final volume of 50 µl. Amplification is done under the following conditions: 5 min denaturation at 94°C, followed by 30 cycles of amplification with a 1-min denaturation, 45 sec of annealing at 45°C and 2 min extension at 72°C. An extra extension step of 10 min at 72°C is added after completion of the 30 cycles. PCR products are analysed by 1% agarose gel electrophoresis in Tris-borate-EDTA buffer.

[0118] The amplification patterns obtained using three different pairs of primers (P-Z-1 + P-Z-3, P-Z-1 + P-Z-2-AS and P-Z-3 + P-Z-2-S) are shown in Table 10.

TABLE 10.

Primer pairs	E. ruminantium strain Gardel (size)	E. ruminantium strain Welgevonden (size)
P-Z-1-ERGA-120 + P-Z-3-ERGA-120	Positive (1242 bp)	Positive (1152 bp)
P-Z-1-ERGA-120 + P-Z-2-ERGA-120-AS	Positive (462 bp)	Negative
P-Z-3-ERGA-120 + p-Z-2-ERGA-120-S	Positive (805 bp)	Negative

(continued)

	Primer pairs	E. ruminantium strain Gardel (size)	E. ruminantium strain Welgevonden (size)
5	P-Z-1-ERGA-1350 + P-Z-3-ERGA-1350	Positive (2792 bp)	Positive (2396 bp)
	P-Z-1-ERGA-1350 + P-Z-2-ERGA-1350-AS	Positive (2129 bp)	Negative
	P-Z-3-ERGA-1350 + P-Z-2-ERGA-1350-S	Positive (688 bp)	Negative
10	P-Z-1-ERGA-4500 + P-Z-3-ERGA-4500	Positive (553 bp)	Positive (493 bp)
	P-Z-1-ERGA-4500 + P-Z-2-ERGA-4500-AS	Positive (272 bp)	Negative
	P-Z-3-ERGA-4500 + P-Z-2-ERGA-4500-S	Positive (306 bp)	Negative
15	P-Z-1-ERGA-5350 + P-Z-3-ERGA-5350	Positive (1571 bp)	Positive (1334 bp)
	P-Z-1-ERGA-5350 + P-Z-2-ERGA-5350-AS	Positive (967 bp)	Negative
	P-Z-3-ERGA-5350 + P-Z-2-ERGA-5350-AS	Positive (630 bp)	Negative
20	P-Z-1-ERGA-5740 + P-Z-3-ERGA-5740	Positive (1362 bp)	Positive (1179 bp)
	P-Z-1-ERGA-5740 + P-Z-2-ERGA-5740-AS	Positive (958 bp)	Negative
	P-Z-3-ERGA-5740 + P-Z-2-ERGA-5740-S	Positive (429 bp)	Negative
25	P-Z-1-ERWE-7410 + P-Z-3-ERWE-7410	Positive (1096 bp)	Positive (1693 bp)
	P-Z-1-ERWE-7410 + P-Z-2-ERWE-7410-AS	Negative	Positive (513 bp)
	P-Z-3-ERWE-7410 + P-Z-2-ERWE-7410-S	Negative	Positive (1205 bp)

[0119] For each doublet of native and mutant alleles, *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden can be discriminated according to the expected size of the amplification products.

Multiplex PCR:

[0120] Differential detection through multiplex PCR is performed on DNA prepared from *E. ruminantium* elementary bodies as described in Example 5 above.

[0121] For each couple of allele, the multiplex PCR is carried out by mixing 250 ng of *E. ruminantium* DNA, 2.5 U of Taq DNA polymerase, 200 nM of each dNTP, 1 µM of P-Z-1 primer, 0.5 µM of P-Z-2 primer, 0.5 µM of P-Z-3 primer and 3 mM MgCl₂ in a final volume of 50 µl. Amplification is done under the following conditions: 5 min denaturation at 94°C, followed by 30 cycles of amplification with a 1-min denaturation, 45 sec of annealing at 45°C and 2 min extension at 72°C. An extra extension step of 10 min at 72°C is added after completion of the 30 cycles. PCR products, i.e. doublets, are analysed by 1% agarose gel electrophoresis in Tris-borate-EDTA buffer.

[0122] The use, for any of the targeted gene, of a P-Z-1 sense primer and two antisense primers, i.e. P-Z-2-AS and P-Z-3, generates two PCR products of differing size in the same reaction when the gene present in the strain is the native full length allele. These two PCR products correspond to an amplification driven by the P-Z-1/P-Z-2-AS pair and by the P-Z-1/P-Z-3 pair. When a mutant allele is present, the PCR reaction generates only one amplification product driven by the P-Z-1/P-Z-3 pair.

[0123] The amplification patterns obtained on *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden are shown in Table 11.

TABLE 11.

	Primer triplets	E. ruminantium strain Gardel (size)	E. ruminantium strain Welgevonden (size)
50	P-Z-1-ERGA-120 + P-Z-2-ERGA-120-AS + P-Z-3-ERGA-120	2 PCR products (1242 bp and 462 bp)	1 PCR product (1172 bp)
	P-Z-1-ERGA-1350 + P-Z-2-ERGA-1350-AS + P-Z-3-ERGA-1350	2 PCR products (2792 bp and 2129 bp)	1 PCR product (2396 bp)

(continued)

	Primer triplets	E. ruminantium strain Gardel (size)	E. ruminantium strain Welgevonden (size)
5	P-Z-1-ERGA-4500 + P-Z-2-ERGA-4500-AS + P-Z-3-ERGA4500	2 PCR products (553 bp and 272 bp)	1 PCR product (493 bp)
	P-Z-1-ERGA-5350 + P-Z-2-ERGA-5350-AS + P-Z-3-ERGA-5350	2 PCR products (1571 bp and 967 bp)	1 PCR product (1334 bp)
	P-Z-1-ERGA-5740 + P-Z-2-ERGA-5740-AS + P-Z-3-ERGA-5740	2 PCR products (1362 bp and 958 bp)	1 PCR product (1179 bp)
10	P-Z-1-ERWE-7410 + P-Z-2-ERWE-7410-AS + P-Z-3-ERWE-7410	1 PCR product (1096 bp)	2 PCR products (1693 bp and 513 bp)
15			

20 [0124] In the same way as for the simple PCR described above, *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden can be discriminated according to the size of the amplification products.

25 **EXAMPLE 9. DIFFERENTIAL DISCRIMINATION BETWEEN *E. RUMINANTIUM* STRAIN GARDEL AND *E. RUMINANTIUM* STRAIN WELGEVONDEN BASED ON RFLP AND SEQUENCE ANALYSIS-RELATED MEANS**

30 [0125] This example illustrates the discrimination between *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden by PCR amplification of the members of same couples of genes as in example 8, followed by RFLP analysis of the amplification products.

35 [0126] Primers targeted to sequences that are present in both *E. ruminantium* strain Gardel and *E. ruminantium* strain Welgevonden and giving amplification products of different sizes depending on the strain, were designed using VectorNTI Advance 9.0 (Informatix).

[0127] Table 12 below provides a list of these primers and indicates their positions relative to the corresponding CDS. The sequence of these PCR primers is indicated in Table 15B.

35 TABLE 12.

	Primers	Orientation	Position relative to CDS Strain Gardel	Position relative to CDS Strain Welgevonden
40	P-WEGA-120-S	Sense	1-25 (ERGA_CDS_00120)	1-25 (ERWE_CDS_00120)
	P-WEGA-120-AS	Antisense	1237-1261(ERGA_CDS_00120)	1147-1171 (ERWE_CDS_00120)
45	P-WEGA-1350-S	Sense	1430-1454 (ERGA_CDS_01350)	1430-1454 (ERWE_CDS_01390)
	P-WEGA-1350-AS	Antisense	2887-2911(ERGA_CDS_01350)	2491-2515 (ERWE_CDS_01390)
50	P-WEGA-4500-S	Sense	301-325 (ERGA_CDS_04500)	307-331 (ERWE_CDS_04590)
	P-WEGA-4500-AS	Antisense	3541-3565 (ERGA_CDS_04500)	1711-1735 (ERWE_CDS_04600)
55	P-WEGA-5350-S	Sense	3365-3389 (ERGA_CDS_05350)	3365-3389 (ERVVE_CDS_05470)
	P-WEGA-5350-AS	Antisense	6079-6103 (ERGA_CDS_05350)	1540-1564 (ERWE_CDS_05460)
60	P-WEGA-5740-S	Sense	241-268 (ERGA_CDS_05740)	244-271 (ERWE_CDS_05830)
	P-WEGA-5740-AS	Antisense	1779-1806 (ERGA_CDS_05740)	1602-1629 (ERWE_CDS_05830)
65	P-WEGA-7410-S	Sense	1-25 (ERGA_CDS_07330)	1-25 (ERWE_CDS_07410)
	P-WEGA-7410-AS	Antisense	1365-1389 (ERGA_CDS_07330)	1962-1986 (ERWE_CDS_07410)

55 **PCR amplification**

[0128] PCR amplification is carried out, for each couple of allele, by mixing 250 ng of *E. ruminantium* DNA, 2.5 U of

Taq DNA polymerase, 200 nM of each dNTP, 1 µM of each, sense (S) and antisense (AS) primer, and 3 mM MgCl₂ in a final volume of 50 µl. Amplification is done under the following conditions: 5 min denaturation at 94°C, followed by 30 cycles of amplification with a 1-min denaturation, 45 sec of annealing at 45°C and 2 min extension at 72°C. An extra extension step of 10 min at 72°C is added after completion of the 30 cycles. PCR products are checked by running an aliquot on 1% agarose gel electrophoresis in Tris-borate-EDTA buffer.

[0129] The P-WEGA-120-S/P-WEGA-120-AS pair directs the amplification of a 1261 bp PCR product from ERGA_CDS_00120 in *E. ruminantium* strain Gardel (i.e. RFLP-ERGA-120) and a 1171 bp PCR product from ERWE_CDS_00120 in *E. ruminantium* strain Welgevonden (i.e. RFLP-ERWE-120).

[0130] The P-WEGA-1350-S/P-WEGA-1350-AS pair yields a 1482 bp PCR product from ERGA_CDS_01350 in *E. ruminantium* strain Gardel (i.e. RFLP-ERGA-1350) and a 1086 bp PCR product from ERWE_CDS_1390 in *E. ruminantium* strain Welgevonden (i.e. RFLP-ERWE-1390).

[0131] The P-WEGA-4500-S/P-WEGA-4500-AS pair drives amplification of a 3265 bp PCR product from ERGA_CDS_04500 in *E. ruminantium* strain Gardel (RFLP-ERGA-4500) and a 2675 bp PCR product from ERWE_CDS-04590/ERWE_CDS_04600 in *E. ruminantium* strain Welgevonden (i.e. RFLP-ERWE-4590/4600).

[0132] The P-WEGA-5350-S/P-WEGA-5350-AS pair drives amplification of a 2739 bp PCR product from ERGA_CDS_05350 in *E. ruminantium* strain Gardel (RFLP-ERGA-05350) and a 2765 bp PCR product from ERWE_CDS_05460/ERWE_CDS_05470 in *E. ruminantium* strain Welgevonden (i.e. RFLP-ERWE-5460/5470).

[0133] The P-WEGA-5740-S/P-WEGA-5740-AS pair drives amplification of a 1566 bp PCR product from ERGA_CDS_05740 in *E. ruminantium* strain Gardel (RFLP-ERGA-05740) and a 1386 bp PCR product from ERWE_CDS_05830 in *E. ruminantium* strain Welgevonden (RFLP-ERWE-5830).

[0134] Similarly, P-WEGA-7410-S/P-WEGA-7410-AS pair drives amplification of a 1389 bp PCR product from ERGA_CDS_07330 (RFLP-ERGA-07330), and a 1986 bp PCR product from ERWE_CDS_07410 in *E. ruminantium* strain Welgevonden (RFLP-ERWE-7410).

[0135] These PCR products are listed in Table 13 below.

TABLE 13

PCR-product	Sense-primer	Antisense-primer	Gene-detected	Strain
RFLP-ERGA-120	P-WEGA-120-S	P-WEGA-120-AS	ERGA_CDS_00120	Gardel
RFLP-ERWE-120	P-WEGA-120-S	P-WEGA-120-AS	ERWE_CDS_00120	Welgevonden
RFLP-ERGA-1350	P-WEGA-1350-S	P-WEGA-1350-AS	ERGA_CDS_01350	Gardel
RFLP-ERWE-1390	P-WEGA-1350-S	P-WEGA-1350-AS	ERWE_CDS_01390	Welgevonden
RFLP-ERGA-4500	P-WEGA-4500-S	P-WEGA-4500-AS	ERGA_CDS_04500	Gardel
RFLP-ERWE-4590/4600	P-WEGA-4500-S	P-WEGA-4500-AS	ERWE_CDS_04590 ERWE_CDS_04600	Welgevonden
RFLP-ERGA-5350	P-WEGA-5350-S	P-WEGA-5350-AS	ERGA_CDS_05350	Gardel
RFLP-ERWE-5460/5470	P-WEGA-5350-S	P-WEGA-5350-AS	ERWE_CDS_05460 ERWE_CDS_05470	Welgevonden
RFLP-ERGA-5740	P-WEGA-5740-S	P-WEGA-5740-AS	ERGA_CDS_05740	Gardel
RFLP-ERWE-5830	P-WEGA-5740-S	P-WEGA-5740-AS	ERWE_CDS_05830	Welgevonden
RFLP-ERGA-7330	P-WEGA-7410-S	P-WEGA-7410-AS	ERGA_CDS_07330	Gardel
RFLP-ERWE-7410	P-WEGA-7410-S	P-WEGA-7410-AS	ERWE_CDS_07410	Welgevonden

RFLP analysis

[0136] The PCR products are used for further RFLP analysis with the following restriction endonucleases: *Alu**I*, *Dra**I*, *Eco**R**V*, *Hin**f**I*, *Rsa**I* and *Taq**I*.

[0137] PCR products are digested in a final volume of 20 µl with the selected enzyme under the conditions, i.e. buffer, time and temperature, recommended by the supplier of the enzyme. Following digestion, the restriction fragments are separated on 2% agarose gel electrophoresis in Tris-borate-EDTA buffer.

[0138] The results are shown in Table 14 below.

TABLE 14

PCR product	Number of sites for selected restriction enzymes					
	Alul	DraI	EcoRV	HinfI	RsaI	TaqI
RFLP-ERGA-120	4	2	4	11	8	4
	4	2	1	9	9	1
RFLP-ERGA-1350	8	2	1	10	14	5
	5	4	1	1	10	1
RFLP-ERGA-4500	6	5	4	10	14	5
	5	9	1	8	8	6
RFLP-ERGA-5350	5	2	2	6	13	2
	2	2	2	8	17	2
RFLP-ERGA-5740	2	4	1	8	17	2
	3	3	2	6	12	4
RFLP-ERGA-7330	2	3	1	2	9	1
	2	3	1	2	17	1
RFLP-ERWE-120	4	2	1	9	9	1
	4	2	1	9	9	1
RFLP-ERWE-1390	5	4	1	1	10	1
	5	4	1	1	10	1
RFLP-ERWE-4590/4600	5	9	1	8	8	6
	5	9	1	8	8	6
RFLP-ERWE-5460/5470	2	2	2	8	17	2
	2	2	2	8	17	2
RFLP-ERWE-5830	3	3	2	6	12	4
	3	3	2	6	12	4
RFLP-ERWE-7410	2	3	1	2	17	1
	2	3	1	2	17	1

[0139] These results show that depending on the strain's DNA used as template for the PCR, all the PCR products yield a different number of bands for at least one of the tested restriction enzymes.

EXAMPLE 10. DISCRIMINATION BETWEEN *E. RUMINANTIUM* STRAIN GARDEL AND *E. RUMINANTIUM* STRAIN WELGEVONDEN BASED ON DNA HYBRIDIZATION

[0140] This example illustrates the use of the Mut oligonucleotide series described in Example 7 as labelled DNA probes to specifically discriminate *E. ruminantium* strain Gardel from *E. ruminantium* strain Welgevonden through DNA hybridization analysis.

[0141] The Mut oligonucleotides used as DNA probes are labelled with α -32P dCTP by random priming using the Rediprime II DNA labeling system (Amersham Bioscience) following the procedure described by the supplier. DNA concentration is adjusted so that 45 μ l of DNA solution in TE buffer (10 mM Tris-HCl, pH 8.0) contains 25 ng of DNA. DNA is denatured by incubation at 100°C for 5 min, followed by immediate incubation in ice-cold water for 5 min. The tube is briefly centrifuged to bring the contents to the bottom of the tube. Denatured DNA is transferred to the reaction tube provided in the kit and mixed to 5 μ l of Redivue [32P] dCTP. Reaction is incubated at 37°C for 10 min and stopped by addition of 5 μ l of 0.2 M EDTA. The labelled probe is heat denatured by incubation at 100°C for 5 min followed by incubation for 5 min in ice-cold water prior to hybridization with *E. ruminantium* DNA.

[0142] Total DNA from strain Gardel or from strain Welgevonden is obtained as described in Example 5.

[0143] Heat denatured DNA is spotted on a nylon membrane (Hybond N+, Amersham) using a slot blot manifold (Hoeffer Scientific Instruments) following the procedure recommended by the supplier of the membrane (Amersham). DNA-DNA hybridization is conducted as described in the Hybond N+ user manual. Prehybridization is conducted overnight in an hybridization tube (meant for use in hybridization ovens) and incubated at 68°C with 20 ml of prehybridization buffer (Denhart 5x, SSPE 2x, SDS .5x, SSPE-Dextran 4x, denatured herring-sperm DNA). After removal of the prehybridization buffer, the membrane is incubated overnight at 68°C in 10ml of hybridization buffer (Denhart 5x, SSPE 2x, SDS .5x, SSPE-Dextran 4x, denatured herring-sperm DNA, 30 ng/ml of labelled DNA probe). Following hybridization, the membrane is washed successively for 30 min in 2x SSC, 0.5 % SDS at room temperature and in 0.1x SSC, 0.5 % SDS at 68°C.

[0144] Hybridized probes are revealed by autoradiography.

[0145] MutERWE 1390N1, MutERWE 1390N3, MutERWE 4590N1, MutERWE 4600N3, MutERWE 5460N1, and MutERWE 5470N3, hybridize with DNA from both strains Gardel and Welgevonden, while MutERWE 1390N2, MutERWE 4590N2, MutERWE 5460N2 only hybridize with DNA from strain Gardel.

TABLE 15A

TABLE 15B

Oligonucleotide or Primer name	Orientation	Sequence (from 5' to 3')	SEQ ID NO
P-Z-1-ERGA-120	sense	gtattgataattatgatggtgaaac	48
P-Z-2-ERGA-120-S	sense	gcacatgatatacgaaatcgaggtc	49
P-Z-2-ERGA-120-AS	antisense	gaactgcgttgcgtatcatgtgc	50
P-Z-3-ERGA-120	antisense	ggttacaaggacaatgatgagtg	51
P-Z-1-ERGA-1350	sense	tccaccagagatgttattgtaaag	52
P-Z-2-ERGA-1350-S	sense	cactatgtaaacgcgtgtAACATTGG	53
P-Z-2-ERGA-1350-AS	antisense	ccaaatgttacatgcgttacatagt	54
P-Z-3-ERGA-1350	antisense	caacagaacttcgtattaaaagc	55
P-Z-1-ERGA-4500	sense	gttaagtgtgaaatgttattgtttag	56
P-Z-2-ERGA-4500-S	sense	cgtctgatctaataatgcgtctga	57
P-Z-2-ERGA-4500-AS	antisense	tcagacgcattttagatcagacg	58
P-Z-1/3-ERGA-4500-S	sense	cttagtaaggaaagaaaaacttaagc	59
P-Z-1/3-ERGA-4500-AS	antisense	gtcttaaggttttcttccttacttag	60
P-Z-3-ERGA-4500	antisense	cactttctgttaattcaaaagttaga	61
P-Z-1-ERGA-5350	sense	gaattaattgtatgtaatgcagaag	62
P-Z-2-ERGA-5350-S	sense	ggtaggagaatttgaagttagcttgcag	63
P-Z-2-ERGA-5350-AS	antisense	cttcgtacttcaaattctccattacc	64

(continued)

Oligonucleotide or Primer name	Orientation	Sequence (from 5' to 3')	SEQ ID NO
P-Z-3-ERGA-5350	antisense	cttgttagattcttctgtgtac	65
P-Z-1-ERGA-5740	sense	gtaggccaaaaagtataggtaa	66
P-Z-2-ERGA-5740-S	sense	ttagaccaaaaacatttgcattc	67
P-Z-2-ERGA-5740-AS	antisense	ctagatgcaaatgttttgtctaa	68
P-Z-3-ERGA-5740	antisense	caacaaaatacatcatctcaagg	69
P-Z-1-ERWE-7410	sense	agggttactttatgttagtcagg	70
P-Z-2-ERWE-7410-S	sense	gagaagggtgttactgatacagcg	71
P-Z-2-ERWE-7410-AS	antisense	cgcgttatcgttaacatcccttc	72
P-Z-3-ERWE-7410	antisense	cctcttcgtatacaggattaccatt	73
P-WEGA-120-S	sense	atgggttattgtataattatgtatgg	74
P-W EGA-120-AS	antisense	caaataatgtttatccatggttacaagg	75
P-WEGA-1350-S	sense	gcgttgttataactgttttcaggtaa	76
P-W EGA-1350-AS	antisense	catgagatgttatcttgtactcac	77
P-W EGA-4500-S	sense	gttaagtgtgaaatgttattgtttag	78
P-WEGA-4500-AS	antisense	ctaaatcttactttgagattttatg	79
P-W EGA-5350-S	sense	atttatcagcgactgttattcttag	80
P-W EGA-5350-AS	antisense	ctgtacactgttagattcttc	81
P-W EGA-5740-S	sense	cgtaataatctttacaaaatgtgacac	82
P-WEGA-5740-AS	antisense	ttcaacaaaatacatcatctcaaggta	83
P-WEGA-7410-S	sense	atgaatgagataatcctatacacag	84
P-WEGA-7410-AS	antisense	agtcacatcatattgtactgtcaca	85

SEQUENCE LISTING

[0146]

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Centre National de la Recherche Scientifique (CNRS) FRUTOS, Roger
FERRAZ, Conception
DEMAILLE, Jacques
MARTINEZ, Dominique

<120> SEQUENCES FOR DIFFERENTIAL DIAGNOSTIC OF EHRlichia RUMINANTIUM AND USE THEREOF

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Claims

1. A method for discriminating between *Ehrlichia ruminantium* strain Gardel and *Ehrlichia ruminantium* strain Welgev-

onden, wherein said method comprises the detection of the presence or the absence, in the bacteria to be tested, of the orphan gene ERGA_CDS_04980, having the sequence SEQ ID NO: 2.

2. A method of claim 1, which further comprises the detection of the presence or the absence, in the bacteria to be tested, of at least one orphan gene selected among:

- ERGA_CDS_04340 having the sequence SEQ ID NO: 1;
- ERGA_CDS_05590 having the sequence SEQ ID NO: 3;
- ERGA_CDS_05600 having the sequence SEQ ID NO: 4;
- ERGA_CDS_07580 having the sequence SEQ ID NO: 5;
- ERWE_CDS_08330 having the sequence SEQ ID NO: 6.

3. The method of any of claims 1 or 2, which further comprises the detection in the bacteria to be tested, of one of the members of at least one allelic couple of genes selected among :

- a couple consisting of ERGA_CDS_00120 having the sequence SEQ ID NO: 7 and ERWE_CDS_00120 having the sequence SEQ ID NO: 8;
- a couple consisting of ERGA_CDS_01350 having the sequence SEQ ID NO: 9 and ERWE_CDS_01390 having the sequence SEQ ID NO: 10;
- a couple consisting of ERGA_CDS_05740 having the sequence SEQ ID NO: 11 and ERWE_CDS_05830 having the sequence SEQ ID NO: 12;
- a couple consisting of ERGA_CDS_04500 having the sequence SEQ_ID_NO: 13 and ERWE_CDS_04590 having the sequence SEQ ID NO: 14 and ERWE_CDS_04600 having the sequence SEQ ID NO: 15
- a couple consisting of ERGA_CDS_05350 having the sequence SEQ ID NO: 16 and ERWE_CDS_05460 having the sequence SEQ ID NO: 17 and ERWE_CDS_05470 having the sequence SEQ ID NO: 18
- a couple consisting of ERGA_CDS_07330 having the sequence SEQ ID NO: 19 and ERWE_CDS_07410 having the sequence SEQ ID NO: 20.

4. An isolated polynucleotide selected among:

- a) a polynucleotide of SEQ ID NO: 2 or the complement thereof;
- b) a fragment of at least 15 consecutive base pairs of a polynucleotide a);
- c) a polynucleotide of at least 15 bp that hybridizes selectively, under stringent hybridization conditions, with a polynucleotide a).

5. A DNA array comprising a polynucleotide of claim 4, and at least one polynucleotide selected among:

- a) a polynucleotide of SEQ ID NO: 3, a polynucleotide of SEQ ID NO: 5, a polynucleotide of SEQ ID NO: 4, a polynucleotide of SEQ ID NO: 6, and a polynucleotide of SEQ ID NO: 1, or their complements;
- b) a fragment of at least 30 consecutive base pairs of a polynucleotide a);
- c) a polynucleotide of at least 30 bp that hybridizes selectively, under stringent hybridization conditions, with a polynucleotide a).

6. A DNA array of claim 5 further comprising at least one polynucleotide selected among:

- a) the portion of SEQ ID NO: 9 that is deleted in SEQ ID NO: 10;
- b) the portion of SEQ ID NO: 13, that is deleted in SEQ ID NO: 14 and SEQ ID NO: 15;
- c) the portion of SEQ ID NO: 16 that is deleted in SEQ ID NO: 17 and SEQ ID NO: 18;
- d) a fragment of at least 30 consecutive base pairs of a polynucleotide a), b), or c);
- e) a polynucleotide of at least 30 bp that hybridizes selectively, under stringent hybridization conditions, with a polynucleotide a), b, or c.

7. A DNA array of claim 6, further comprising at least one polynucleotide selected among:

- a) a portion of SEQ ID NO: 9 that is conserved in SEQ ID NO: 10;
- b) a portion of SEQ ID NO: 13, that is conserved in SEQ ID NO: 14 and SEQ ID NO: 15;
- c) a portion of SEQ ID NO: 16 that is conserved in SEQ ID NO: 17 and SEQ ID NO: 18;
- d) a fragment of at least 30 consecutive base pairs of a polynucleotide a), b), or c);

e) a polynucleotide of at least 30 bp that hybridizes selectively, under stringent hybridization conditions, with a polynucleotide a), b, or c.

5 **Patentansprüche**

1. Verfahren zur Unterscheidung zwischen *Ehrlichia-ruminantium-Stamm* Gardel und *Ehrlichia-ruminantium-Stamm* Welgevonden, wobei das Verfahren die Detektion des Vorliegens oder der Abwesenheit des Orphan-Gens ERGA_CDS_04980 mit der Sequenz SEQ ID NO: 2 in den zu testenden Bakterien umfasst.

10 2. Verfahren gemäß Anspruch 1, das außerdem die Detektion des Vorliegens oder der Abwesenheit mindestens eines Orphan-Gens, ausgewählt aus:

- 15 - ERGA_CDS_04340 mit der Sequenz SEQ ID NO: 1;
 - ERGA_CDS_05590 mit der Sequenz SEQ ID NO: 3;
 - ERGA_CDS_05600 mit der Sequenz SEQ ID NO: 4;
 - ERGA_CDS_07580 mit der Sequenz SEQ ID NO: 5;
 - ERGA_CDS_08330 mit der Sequenz SEQ ID NO: 6

20 in den zu testenden Bakterien umfasst.

3. Verfahren gemäß einem der Ansprüche 1 oder 2, welches die Detektion von einem der Mitglieder aus wenigstens einem allelischen Paar von Genen, ausgewählt aus:

- 25 - einem Paar, bestehend aus ERGA_CDS_00120 mit der Sequenz SEQ ID NO: 7 und ERWE_CDS_00120 mit der Sequenz SEQ ID NO: 8;
 - einem Paar, bestehend aus ERGA_CDS_01350 mit der Sequenz SEQ 10 NO: 9 und ERWE_CDS_01390 mit der Sequenz SEQ ID NO: 10;
 - einem Paar, bestehend aus ERGA_CDS_05740 mit der Sequenz SEQ ID NO: 11 und ERWE_CDS_05830 mit der Sequenz SEQ ID NO: 12;
 - einem Paar, bestehend aus ERGA_CDS_04500 mit der Sequenz SEQ ID NO: 13 und ERWE_CDS_04590 mit der Sequenz SEQ ID NO: 14, und ERWE_CDS_04600 mit der Sequenz SEQ ID NO: 15;
 - einem Paar, bestehend aus ERGA_CDS_05350 mit der Sequenz SEQ ID NO: 16 und ERWE_CDS_05460 mit der Sequenz SEQ ID NO: 17, und ERWE_CDS_05470 mit der Sequenz SEQ ID NO: 18;
 30 - einem Paar, bestehend aus ERGA_CDS_07330 mit der Sequenz SEQ ID NO: 19 und ERWE_CDS_07410 mit der Sequenz SEQ ID NO: 20,

35 in den zu testenden Bakterien umfasst.

40 4. Isoliertes Polynucleotid, ausgewählt aus:

- a) einem Polynucleotid von SEQ ID NO: 2 oder dem Komplement davon;
 b) einem Fragment aus wenigstens 15 fortlaufenden Basenpaaren von einem Nucleotid a);
 c) einem Polynucleotid aus wenigstens 15 bp, das selektiv, unter stringenten Hybridisierungsbedingungen, mit einem Polynucleotid a) hybridisiert.

45 5. DNA-Array, umfassend ein Polynucleotid gemäß Anspruch 4 und wenigstens ein Polynucleotid, ausgewählt aus:

- 50 a) einem Polynucleotid von SEQ ID NO: 3, einem Polynucleotid von SEQ ID NO: 5, einem Polynucleotid von SEQ ID NO: 4, einem Polynucleotid von SEQ ID NO: 6 und einem Polynucleotid von SEQ ID NO: 1 oder deren Komplementen;
 b) einem Fragment aus wenigstens 30 fortlaufenden Basenpaaren von einem Polynucleotid a);
 c) einem Polynucleotid aus wenigstens 30 bp, das selektiv, unter stringenten Hybridisierungsbedingungen, mit einem Polynucleotid a) hybridisiert.

55 6. DNA-Array gemäß Anspruch 5, außerdem umfassend wenigstens ein Polynucleotid, ausgewählt aus:

- a) dem Teil von SEQ ID NO: 9, der in SEQ ID NO: 10 deletiert ist;

- 5 b) dem Teil von SEQ ID NO: 13, der in SEQ ID NO: 14 und SEQ ID NO: 15 deletiert ist;
 c) dem Teil von SEQ ID NO: 6, der in SEQ ID NO: 17 und SEQ ID NO: 18 deletiert ist;
 d) einem Fragment aus wenigstens 30 fortlaufenden Basenpaaren von einem Polynucleotid a), b) oder c);
 e) einem Polynucleotid aus wenigstens 30 bp, das selektiv, unter stringenten Hybridisierungsbedingungen, mit
 einem Polynucleotid a), b) oder c) hybridisiert.

7. DNA-Array gemäß Anspruch 6, außerdem umfassend wenigstens ein Polynucleotid, ausgewählt aus:

- 10 a) einem Teil von SEQ ID NO: 9, der in SEQ ID NO: 10 konserviert ist;
 b) einem Teil von SEQ ID NO: 13, der in SEQ ID NO: 14 und SEQ ID NO: 15 konserviert ist;
 c) einem Teil von SEQ ID NO: 16, der in SEQ ID NO: 17 und SEQ ID NO: 18 konserviert ist;
 d) einem Fragment aus wenigstens 30 fortlaufenden Basenpaaren von einem Polynucleotid a), b) oder c);
 e) einem Polynucleotid aus wenigstens 30 bp, das selektiv, unter stringenten Hybridisierungsbedingungen, mit
 einem Polynucleotid a), b) oder c) hybridisiert.

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Revendications

1. Procédé de discrimination entre la souche Gardel de *Ehrlichia ruminantium* et la souche Welgevonden de *Ehrlichia ruminantium*, où ledit procédé comprend la détection de la présence ou de l'absence, dans les bactéries à tester, du gène orphelin ERGA_CDS_04980, ayant la séquence SEQ ID NO : 2.

2. Procédé selon la revendication 1, qui comprend en outre la détection de la présence ou de l'absence, dans les bactéries à tester, d'au moins un gène orphelin choisi parme :

- 25 ERGA_CDS_04340 ayant la séquence SEQ ID NO : 1 ;
 ERGA_CDS_05590 ayant la séquence SEQ ID NO : 3 ;
 ERGA_CDS_05600 ayant la séquence SEQ ID NO : 4 ;
 ERGA_CDS_07580 ayant la séquence SEQ ID NO : 5 ;
 30 ERWE_CDS_08330 ayant la séquence SEQ ID NO : 6.

3. Procédé selon l'une quelconque des revendications 1 ou 2, qui comprend en outre la détection dans les bactéries à tester, d'un des membres d'au moins un couple allélique de gènes choisi parme :

- 35 un couple constitué de ERGA_CDS_00120 ayant la séquence SEQ ID NO : 7 et de ERWE_CDS_00120 ayant la séquence SEQ ID NO : 8 ;
 un couple constitué de ERGA_CDS_01350 ayant la séquence SEQ ID NO : 9 et de ERWE_CDS_01390 ayant la séquence SEQ ID NO : 10 ;
 40 un couple constitué de ERGA_CDS_05740 ayant la séquence SEQ ID NO : 11 et de ERWE_CDS_05830 ayant la séquence SEQ ID NO : 12 ;
 un couple constitué de ERGA_CDS_04500 ayant la séquence SEQ ID NO : 13 et de ERWE_CDS_04590 ayant la séquence SEQ ID NO : 14 et de ERWE_CDS_04600 ayant la séquence SEQ ID NO : 15 ;
 45 un couple constitué de ERGA_CDS_05350 ayant la séquence SEQ ID NO : 16 et de ERWE_CDS_05460 ayant la séquence SEQ ID NO : 17 et de ERWE_CDS_05470 ayant la séquence SEQ ID NO : 18 ;
 un couple constitué de ERGA_CDS_07330 ayant la séquence SEQ ID NO : 19 et de ERWE_CDS_07410 ayant la séquence SEQ ID NO : 20.

4. Polynucléotide isolé choisi parme

- 50 a) un polynucléotide de SEQ ID NO : 2 ou le complémentaire de celui-ci ;
 b) un fragment d'au moins 15 paires de bases consécutives d'un polynucléotide a) ;
 c) un polynucléotide d'au moins 15 pb qui s'hybride sélectivement, dans des conditions d'hybridation stringentes, à un polynucléotide a).

55 5. Puce d'ADN comprenant un polynucléotide selon la revendication 4 et au moins un polynucléotide choisi parme :

- a) un polynucléotide de SEQ ID NO : 3, un polynucléotide de SEQ ID NO : 5, un polynucléotide de SEQ ID NO : 4, un polynucléotide de SEQ ID NO : 6 et un polynucléotide de SEQ ID NO : 1, ou leurs complémentaires ;

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- b) un fragment d'au moins 30 paires de bases consécutives d'un polynucléotide a) ;
c) un polynucléotide d'au moins 30 pb qui s'hybride sélectivement, dans des conditions d'hybridation stringentes, à un polynucléotide a).

5 **6.** Puce d'ADN selon la revendication 5, comprenant en outre au moins un polynucléotide choisi parmi :

- a) la partie de SEQ ID NO : 9 qui est déletée dans SEQ ID NO : 10 ;
b) la partie de SEQ ID NO : 13 qui est déletée dans SEQ ID NO : 14 et SEQ ID NO : 15 ;
c) la partie de SEQ ID NO : 16 qui est déletée dans SEQ ID NO : 17 et SEQ ID NO : 18 ;
10 d) un fragment d'au moins 30 paires de bases consécutives d'un polynucléotide a), b) ou c) ;
e) un polynucléotide d'au moins 30 pb qui s'hybride sélectivement, dans des conditions d'hybridation stringentes, à un polynucléotide a), b) ou c).

15 **7.** Puce d'ADN selon la revendication 6, comprenant en outre au moins un polynucléotide choisi parmi :

- a) une partie de SEQ ID NO : 9 qui est conservée dans SEQ ID NO : 10 ;
b) une partie de SEQ ID NO : 13 qui est conservée dans SEQ ID NO : 14 et SEQ ID NO : 15 ;
c) une partie de SEQ ID NO : 16 qui est conservée dans SEQ ID NO : 17 et SEQ ID NO : 18 ;
d) un fragment d'au moins 30 paires de bases consécutives d'un polynucléotide a), b) ou c) ;
20 e) un polynucléotide d'au moins 30 pb qui s'hybride sélectivement, dans des conditions d'hybridation stringentes, à un polynucléotide a), b) ou c).

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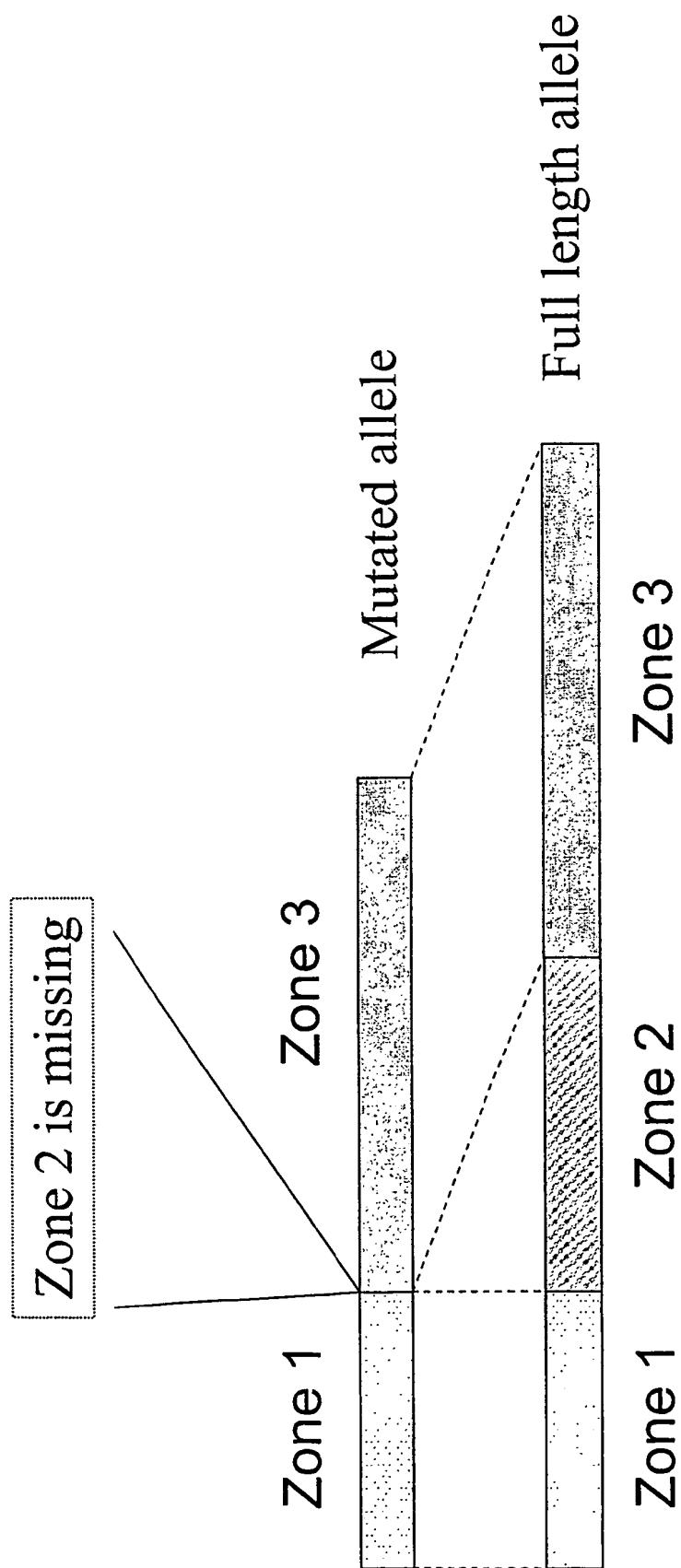


Figure 1

REFERENCES CITED IN THE DESCRIPTION

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