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(54) Title: RECOMBINANT STRAIN OF A PATHOGENIC MICROORGANISM, AND A DOUBLE VACCINE

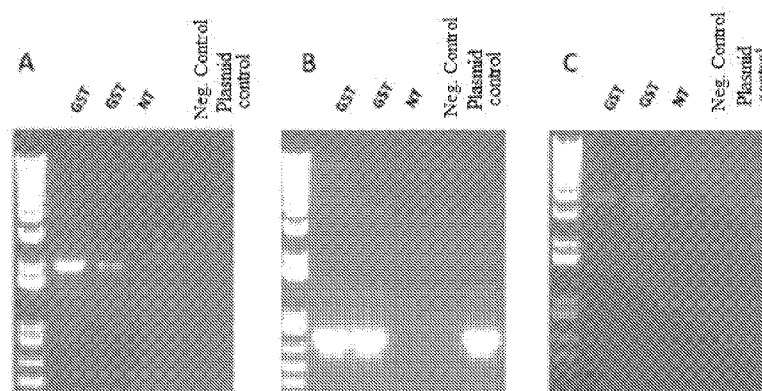


Fig. 1

(57) Abstract: The present invention proposes a recombinant strain of a pathogenic microorganism, comprised of an expression vector of an antigen derived from a parasite or disease vector, and a double vaccine comprising such a recombinant strain. In particular, the present invention consists of a double vaccine comprising a strain modified from *Babesia bovis* expressing glutathione S-transferase from ticks. The present invention is in the fields of biotechnology, veterinary medicine, and human medicine.



Recombinant strain of a pathogenic microorganism, and a double vaccineArea of technology of the invention:

(0001) The present invention relates to a recombinant strain of a pathogenic microorganism, and a double vaccine comprised of said recombinant strain. The present invention is in the area of technology of biotechnology, veterinary medicine, and human medicine.

Background of Invention:

(0002) The *Rhipicephalus microplus* tick is an important bovine ectoparasite because of the damage it causes to livestock production. The damage caused is due not only to the infestation of the animals by the arthropod, but also because this parasite is a vector of pathogenic microorganisms, such as *Babesia bigemina*, *Babesia bovis* and *Anaplasma spp.* This species of tick is the main parasitic problem for cattle husbandry in Brazil, in part of Uruguay, Argentina, and Australia. The need for substitutes for methods of control based on the use of chemical acaricides is increasingly urgent due to: (i) the emergence of tick populations which are resistant to available preparates; (ii) the time needed for development of new preparates is less than the time of emergence and resistance to them; and (iii) there is increasing consumer market demand for food produced by methods that do not leave traces of chemical residues originating in the production process.

(0003) For some time, there has been a consensus that the best alternative to replace chemical control is immunological control. Considering that it has been more than three decades since the market launch of two vaccines for this tick, they (the vaccines) have a degree of protection which is insufficient to replace the use of acaricides. This first immunoprotective protein has great variability among the populations of *R. microplus*, a fact which explains why these vaccines show very variable efficacy in various regions. In Australia and Cuba, they provide a certain degree of protection, and their use is indicated in association with chemical acaricides. In tick populations in South America they are ineffective. Consequently, the search for new antigens

for the vaccine against *R. microplus* and other tick species has continued. To date, several antigens have been identified, some of which are in the Biotechnology Center of the Federal University of Rio Grande do Sul. The degree of protection imparted by these antigens is very variable, and few have a degree of protection higher than 80%.

(0004) An important characteristic of commercial anti-tick vaccine antigens is that they are all hidden antigens. The concept of a hidden antigen was introduced when the Bm86 protein, the first antigen with immunoprotective activity against an ectoparasite, was identified; this is the antigen on which the commercial anti-tick vaccines are based. A hidden antigen is defined as an antigen of the parasite which under natural conditions does not come into contact with the immune system of the host, but when artificially inoculated with it the host mounts a response against it, and under natural conditions of infestation, antibodies reach the antigen. The Bm86 protein is a membrane protein of the *R. microplus* digestive tract epithelium with a role in the mechanism of exocytosis. Under natural conditions, this protein is inaccessible to the bovine immune system. However, once the host has developed antibodies by means of artificial inoculation with this protein, these antibodies, present in the digestive tract due to the blood supply, reach the protein. The demonstration that functional host antibodies circulate in the hemolymph of the tick opened the possibility that it might be promising to investigate antigens from other tissues, other than those from the digestive tract, for possible immunoprotective activity. It is assumed that the long time evolution of ticks together with their hosts has led to the current situation in which ticks are so well adapted to their hosts that they can avoid a response to their molecules that come into contact with the host. Hence the relevance of the hidden antigens in the search for an anti-tick vaccine.

(0005) While on the one hand the use of hidden antigens has opened up the prospect of obtaining a tick vaccine, on the other hand it has the drawback that the post-immunization time becomes a limiting factor for protection, since reinfestations are not capable of acting as a booster vaccine. Effective protection would only be possible with frequent administration of the vaccine, which, due to the cost and the conditions of management of the animals, limits or even renders impracticable the use of vaccines.

(0006) Whereas the immunity of cattle to ticks due to natural infestations is very small and is manifested by a small decrease in the number of ticks over successive infestations, immunity against the hemoparasites *Babesia spp.* and *Anaplasma spp.* (both of which cause tick fever in cattle) is long-lasting as long as the cattle receive constant boosting that is provided by successive infestations by ticks. Continual infections by these organisms maintain an enzootic balance. Under prevailing conditions of bovine stock breeding in Brazil, where practically all tick populations are infected by these pathogens, continuous reinfection of cattle is responsible for maintaining immunity against *Babesia spp.* and *Anaplasma spp.* On the other hand, cattle from tick-free areas, or cattle that are kept totally free of ticks for some time, develop babesiosis and anaplasmosis if they are eventually infested with ticks, since the populations of ticks in almost all these regions are infected with *Babesia spp.* and *Anaplasma spp.* Mortality is high if these cattle are not treated with babesicidal drugs and antibiotics. Therefore, the use of highly effective tick vaccines can actually introduce -this problem into regions infested with *R. microplus*. In this case it is necessary to protect the cattle using the methodology of premunition (basically inoculating the cattle with the agents and controlling the levels of parasitemia by means of drugs) with the virulent microorganisms or vaccination with the attenuated microorganisms. It is envisaged that a very efficacious tick vaccine could lead to loss of enzootic stability and, consequently, an increase in cases of tick fever disease.

Summary of the invention:

(0007) Accordingly, the object of the present invention is to solve the known problems in the state of the art by means of a recombinant strain of a pathogenic microorganism comprising an expression vector of an antigen from a parasite or vector of diseases, and by means of a vaccine comprising this recombinant strain, acting to prevent diseases and to control parasites or disease vectors.

(0008) In a first aspect of the object of the invention, a recombinant strain of a pathogenic microorganism is provided which comprises an expression vector of an antigen derived from a parasite or vector of diseases.

(0009) In a second aspect of the object of the invention, a dual vaccine is provided which comprises an attenuated recombinant strain of a pathogenic microorganism comprising an expression vector of an antigen derived from a parasite or vector of diseases.

(0010) Further, the inventive concept common to all claimed protection contexts is the recombinant strain of a pathogenic microorganism comprising an expression vector of an antigen derived from a parasite or vector of diseases.

(0011) These and other objects of the invention will be readily appreciated by persons skilled in the art and by companies having an interest in the art, and will be described in sufficient detail to carry out the invention, in the following description.

Brief description of the Figures:

(0012) In order to better define and clarify the contents of the present patent application, the following figures are presented:

(0013) Fig. 1 shows a gel chromatogram for evaluating the integration of exogenous DNA into the protozoan DNA by the PCR technique. Genomic DNA obtained from parasites subjected to transfection with the plasmid containing the GST-HI (GST) sequence as well as un-transfected (NT) parasites were used as template for the PCR. As controls, a reaction without presence of DNA (negative control) and a control containing only the plasmid used in the GST transfection (plasmid control) were used. The groups of primers used were: A -- GST primer x genomic babesia primer, B -- GST primers, and C -- GFP primer x genomic babesia primer.

(0014) Fig. 2 shows the result (film) of a Southern blot of the mixed and clonal GST lines. Genomic DNA obtained from parasites of the clonal line (Clonal GST) or mixed line (Mixed GST), as well as a control transfected with a plasmid not related to this work (Non-Related -- NR) and a control not subjected to transfection were probed with probes for the region of the elongation factor. The difference in size between the Clonal, Mixed, and Un-Transfected GST groups shows that insertion into the region of interest in the protozoan genome actually occurred.

(0015) Fig. 3 shows the result (film) of GST expression in the lines of interest. The Western blot was made with an extract of proteins obtained from the cultivation of the Mixed GST line or Clonal GST line (mixed GST) or clonal line (GST Clonal) of parasites transfected with a plasmid not related to this work (Non-Related -- NR), or of parasites not subjected to transfection (Un-Transfected -- NT). Such samples were probed with rabbit serum immunized with GST (the GST) and with preimmune rabbit serum as a control. We can verify that there is actually GST expression in the first two groups of the membrane probed with GST, and that the other recognized points are also visualized in the material probed with preimmune serum, confirming that the interaction does not involve GST, but is a non-specific interaction.

(0016) Fig. 4 shows plots of the measurements of the hematocrit and the rectal temperature in experimentally infected animals. Key: GST = animal infected with the babesia clonal line expressing GSTHI. GFP = control, namely an animal infected with the control clonal line that is unable to express GSTHI.

(0017) Fig. 5 shows the result (film) of the Western blot assay performed with the GSTHI protein, probed with serum from experimentally infected animals.

Detailed description of the invention:

(0018) Within this context, the present invention proposes a dual vaccine using an attenuated strain of a pathogenic microorganism transformed to express the protein of a parasite or a vector of diseases, in which case the protein is expected to behave as an antigen. Thus, the vaccine comprising said strain is dual, because it acts against the pathogenic microorganism and also against the parasite or disease vector. According to one embodiment, maintenance of enzootic stability of *Babesia bovis* is expected to occur, which is expected to cause the bovine population to be continually stimulated by hidden or non-hidden tick antigens, which will keep the tick population at a low level.

(0019) In a first aspect, the present invention proposes a recombinant strain of a pathogenic microorganism comprising an expression vector of an antigen derived from a parasite or vector of diseases.

(0020) In a second aspect, the present invention proposes a dual vaccine comprising an attenuated recombinant strain of a pathogenic microorganism comprising an expression vector of an antigen derived from a parasite or vector of diseases.

(0021) According to an embodiment, said antigen is derived from a parasite or vector of diseases.

(0022) According to another embodiment, said antigen is a protein fused to a signal peptide.

(0023) According to yet another embodiment, said antigen is glutathione S-transferase fused to an MSA-1 signal peptide.

(0024) According to still another embodiment, said glutathione S-transferase is from the species *Haemaphysalis longicornis*.

(0025) According to still another embodiment, said pathogenic microorganisms is *Babesia bovis*.

(0026) According to another embodiment, the said parasite or disease vector is a tick.

(0027) According to yet another embodiment, said vaccine comprises adjuvants, preservatives, antibiotics, stabilizers, or a combination of these.

(0028) According to still another embodiment, said vaccine is a booster vaccine.

(0029) According to an embodiment, said vaccine is a live vaccine.

(0030) According to another embodiment, said vaccine further comprises other antigens and/or pathogenic microorganisms.

(0031) Transfection techniques allow the incorporation of a DNA not belonging to the organism, and expression of heterologous proteins. This type of methodology is commonly used to study mechanisms of gene regulation and characterization of genes, but can also be used for other purposes such as improvement of the transfected organism, as well as to fulfill experimental or biotechnological needs, as is the case in the development of vaccines. The transfection process can be divided into two processes [(sub-processes)]: transient transfection and stable transfection. In the first case, the outer DNA sequence is not inserted into the DNA of the recipient organism, many of them being of short duration. In the second case, DNA is inserted into the recipient organism.

(0032) According to an embodiment, stable transfection is used, since the transfected babesia needs to be able to propagate while maintaining the desired character. To effectively carry out the stable transfection process, we need a plasmid which contains all the components necessary for such a condition, namely: promoter, termination signal, selection agent, reporter gene, and region for integration into the genome. Previous studies of the region responsible for elongation factor expression provided important information that culminated in the selection of the bidirectional promoter responsible for the production of such a protein, and also selection of the insertion site. It is important to emphasize that the site where the integration takes place will suffer an interruption in its coding sequence, which may result in "knock out" of the gene. Therefore, the site of insertion must be well studied, so that even the interruption does not prevent the development of the parasite.

(0033) The ability to insert exogenous DNA into an organism is an essential activity in molecular biology and genetic engineering. The process of insertion of genetic information is viable based on various transformation techniques which are frequently dependent on the organism to be transformed. The principal techniques used for transformation and transfection will be described below.

(0034) In bacteria, the most common technique is the use of circular plasmids that will not integrate into the genome of the bacterium but will coexist with the circular DNA of this prokaryote. The most widely used technique for delivery of exogenous DNA is via chemical transformation, in which the cell is rendered competent by washing with different solutions which will be responsible for increasing the membrane fluidity of the bacterium and facilitating DNA insertion. The competent cell is incubated with the plasmid, and both undergo a thermal shock step, where membrane depolarization occurs, allowing plasmid DNA to enter. Another technique for destabilizing the membrane and allowing insertion of the plasmid DNA involves the use of an electric pulse, whereby the interaction of the generated electric field and the lipid layer of the cell allows permeabilization of the membrane, thus increasing the transport of the nucleic acids through it. Although it is very commonly used with bacteria, electroporation can be used in other cell types as well, such as, e.g., unicellular protozoa.

(0035) Bioballistics involves the use of small particles coated with DNA, which are accelerated at high speeds. When applied in tissues, they are able to penetrate the cells without promoting cellular lysis. This technique is more commonly used in larger organisms, such as plants and animals, but its use in bacteria and in eukaryotic cell cultures has been described.

(0036) In plants, another common method of transfection is with the use of the pathogen *Agrobacterium tumefaciens*, a gram-negative bacterium that affects plants. This bacterium has a plasmid that contains genes which are tumorigenic in plants; in order to be able to use it, the plasmid is modified such as to preserve the region responsible for the integration into the genome and to add to it the DNA sequences that one seeks to study and to add to the genome of the affected cells.

(0037) Also noteworthy is a quite relevant technique involving virus-mediated transfection, wherein a retrovirus is modified so as to contain the sequences of interest of the researcher, and is then used to infect the cell line of interest. Due to the nature of this type of virus, it integrates its DNA into the DNA of the host cell.

(0038) The expression vector may comprise a promoter, a termination signal, a selection agent, a reporter gene, a genome integration region, a terminator, and other regions common in the art.

(0039) The process of transformation of the strain can be carried out by techniques common in the art, such as, for example, electroporation.

(0040) In the context of the present invention, the term "pathogenic microorganisms" is defined to mean microorganisms capable of multiplying in the host organism, which may cause infections and other complications.

(0041) In the context of the present invention, the term "disease vector" is defined as a species capable of transmitting an infectious agent to a host, with the species being different from the selected species of the pathogenic microorganism.

(0042) According to present invention, the parasite is selected from a different species from the selected species of the pathogenic microorganism. Examples of parasites are nematodes, cestodes, and arthropods (this is not an exhaustive list).

(0043) In the context of the present invention, the term "signal peptide" is defined as an N-terminal extension of a newly synthesized membrane protein or secretory protein. Such sequences usually have a size of about 16 to 30 amino acids, containing: a hydrophilic region (generally positively charged), a hydrophobic domain in the central region, and a C-terminal region containing a cleavage site for a peptidase. In eukaryotes (as is the case with babesia), signal sequences direct the insertion of proteins into the membrane of the endoplasmic reticulum, wherewith the signal peptide is cleaved, and in most cases, rapidly degraded. According to an embodiment, the addition of a signal peptide of a protein of the protozoon *Babesia bovis* to the coding sequence of GSTH1 allows the chimera to be effectively recognized by the cellular mechanism of Babesia, and then enables its routing to the surface of the cell membrane.

Exemplary embodiments:

(0044) The examples presented herein are for the sole purpose of exemplifying one of numerous techniques for carrying out the invention, and do not limit the scope of the invention.

Example 1:

(0045) One method of transforming *Babesia bovis* is by an electroporation procedure, wherewith it is possible to induce the insertion of exogenous genetic material (derived from a plasmid) into the babesia genome. The details of the technique for construction of the plasmid and the subsequent transfection in order to establish the recombinant babesia line of interest are described below. As termination signals, the termination regions of the RAP and MSA 1 proteins were chosen. Regarding the reporter genes, the open reading frame (ORF) of the green fluorescent protein (GFP) was chosen, which confers a green color on the parasite when it is stimulated with ultraviolet light. In order to allow selection of the transfected agents, the coding sequence of the enzyme blasticidin deaminase was inserted into the plasmid, which gives the transfected parasite the ability to survive in a medium containing the antibiotic blasticidin.

(0046) For the construction of the plasmid to be used for the transfection of *Babesia bovis* and for development of a vaccine, the GFP-BSD plasmid was used as the basis for the construction of the plasmid MSASignal-GSTHI-GFP-BSD, since it already contains in its structure the promoter region, the coding region for green fluorescent reporter (Green-GFP), and blasticidin resistance proteins, as well as the termination and targeting regions for insertion into the protozoan genome. Initially, for the development of the plasmid, the chimeric sequence containing the coding region for MSA-1 and GST HI was constructed. For this purpose, the coding sequence for the signal peptide of the MSA-1 protein containing the restriction sites for the BamHI, NotI, and SacII enzymes was obtained by chemical synthesis and was subsequently amplified with primers based on the 5' and 3' regions of the chimeric sequence. The amplicon obtained was cloned into the cloning vector pCR2.1-TOPO, and this material was used for transformation of *E. coli* Top 10. The plasmids obtained were extracted by the mini-preparation technique, and the nucleic acid sequences were analyzed by sequencing, to ensure the absence of errors in synthesis. The plasmid selected at the end of this process was designated "MSASignal-TOPO".

(0047) The plasmid MSASignal-TOPO was subjected to hydrolysis with the restriction enzyme BamHI to release the MSASignal insert and to bring about ligation of it to the pBlueScript vector. For this purpose, the MSASignal-TOPO plasmid and the pBlueScript plasmid were hydrolyzed with the BamHI enzyme at 37 °C for 4 hours, after the pBlueScript plasmid was dephosphorylated, and both were subjected to an electrophoresis process in agarose gel and were purified. The MSASignal was cloned into pBlueScript using the enzyme DNA ligase. The resulting plasmid was used for transformation of *E. coli* Top 10. The plasmid obtained was extracted by the mini-preparation technique, and the nucleic acid sequences were analyzed to ensure the absence of mutations. The plasmid obtained at the end of this process was designated "MSASignal-pBlue".

(0048) After obtaining the MSASignal-pBlue plasmid, the GSTHI coding region was amplified using primers specific for the 5' and 3' region of the coding sequence of GSTHI. As the template for the reaction, the plasmid pET43a-GSTHI was used. BamHI, SacII, and PstII enzyme restriction sites were added, via primer. In addition, a 657G> A point mutation was carried out, via primer, where there was no change in the amino acid to be encoded. The amplicon generated with the designated primers was cloned into pCR2.1-TOPO, thus obtaining the plasmid GSTHI-TOPO. This plasmid was hydrolyzed with the restriction enzymes SacII and PstI, to release the GSTHI insert; this process was carried out at 37 °C for 4 hours, after which the product (the insert) was subjected to electrophoresis, and was purified from the agarose gel. This insert was cloned into the MSASignal-pBlue plasmid which had been previously treated with the same restriction enzymes, and was purified. The ligation between the two materials was carried out using the enzyme DNA ligase, and this (the resulting) material was used for transformation of *E. coli* Top 10. The plasmids obtained were extracted by the mini-preparation technique, and the nucleic acid sequences were analyzed in order to guarantee the absence of mutations. The plasmid obtained at the end of this process was designated "MSASignal-GSTHI-pBlue".

(0049) The plasmid MSASignal-GSTHI1-pBlue was subjected to hydrolysis with the restriction enzymes NotI and PstI, to release the insert corresponding to the chimera formed by the coding sequences of the MSA-1 signal peptide and the GSTHI protein. The plasmid GFP-BSD was also

subjected to the same cleavage procedure, and after hydrolysis it was dephosphorylated with the enzyme CIAP for one hour. The chimera was cloned into the GFP-BSD vector using the enzyme DNA ligase. This material was used for transformation of *E. coli* Top 10. The plasmids obtained were extracted by the mini-preparation technique, and the nucleic acid sequences were analyzed to ensure the absence of mutations. The transfection plasmid thus obtained was designated "MSASignal-GSTHI-GFP-BSD".

(0050) To carry out the transfection procedure, 20 g of the plasmid MSASignal-GSTHI-GFP-BSD was dehydrated and then resuspended in a buffer containing 120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄ (pH 7.6), 25 mM HEPES, 2 mM EGTA, 5 mM MgCl₂, with final pH 7.6. The solution containing the plasmid was added to a solution containing infected erythrocytes, and was then subjected to electroporation using 0.2 cm cuvettes in an electroporator, with the following settings: 1.2 kV, 200 Ω , and fixed capacitance of 25. Immediately after the electroporation the material was incubated in a 24-well culture plate, containing 1 mL of HL-1 medium and 100 μ L of erythrocytes in each well, at 37 °C with 5% CO₂. After 4 hours, the culture medium was changed, and the selection agent, blasticidin, was added to the new medium, at a concentration of 4 μ g /mL, with the plates being continuously kept in an incubator at 37 °C with 5% CO₂. After this, the medium was changed every second day, always in the presence of the selection agent. The parasitemia of the culture was evaluated twice weekly by a blood smear technique using 4 μ L of the cell culture. The slide was stained with "rapid panoptic" stain, and counting was performed under an optical microscope.

(0051) Using the cultures which were positive for babesia infection, tests were performed to verify if they were effectively capable of expressing GST. For this purpose, the insertion of the material from the transfection plasmid into the genome of the protozoon was initially confirmed by PCR using a pair of primers, one of which was capable of annealing to a genomic sequence and the other of which was capable of annealing to the GST sequence from the plasmid. As a template for this reaction, genomic DNA obtained from a cell culture well containing 450 μ L of culture medium and 50 μ L of infected erythrocytes was used. The material was centrifuged at 400 g for 5 minutes, the supernatant was discarded, and the erythrocytes were washed with 500 μ L of PBS. The washing process was repeated one more time and the mass of erythrocytes was

frozen for 16 hours at -20 °C, for lysis. After the lysis, the cells were washed with 500 µL of PBS, and were centrifuged at 2000 g to sediment the merozoites; and the supernatant was discarded. This process was repeated until the reddish pigmentation was completely removed, obtaining merozoites for the DNA extraction process. The merozoites were lysed and added to 25 µL of a 20 mg/mL proteinase K solution. This mixture was incubated for 16 hours at 56 °C, and then cooled on ice to achieve protein precipitation, with 200 µL of protein precipitation solution (provided by Qiagen). The material was centrifuged, and the supernatant was collected and precipitated with isopropanol for 16 hours at -20 °C. The material was then centrifuged at 12,000 g for 10 minutes, the supernatant was discarded, and the pellet was resuspended in 10 µL of hydration solution. The re-suspended material is comprised of DNA, for use in PCR or Southern blot techniques.

(0052) Fig. 1 shows the result of the PCR, demonstrating the amplification with the specific primers, and confirming the insertion of a sequence. To confirm the identity of the insertion of the amplicon obtained, it was cloned into a pCR2.1-TOPO vector and was sequenced.

(0053) The insertion was also verified by the Southern blot technique. For this purpose, 500 ng of genomic DNA was digested with the restriction enzyme BgIII at 37 °C for 4 hours, and was subjected to separation of the fragments by electrophoresis in agarose gel. The DNA was transferred to a membrane, and was analyzed with probes for the insertion site (elongation factor) and for the reporter gene (GFP). Fig. 2 shows that there was a change in the size of the region corresponding to the elongation factor, which indicates the genomic insertion and the presence of the region corresponding to the reporter gene, in the transfected material.

(0054) To verify the expression of GST, samples were resolved with electrophoresis on polyacrylamide gel, followed by Western blotting, and were probed with rabbit serum immunized with GST. The tested material was obtained from a cell culture well containing 450 µL of culture medium and 50 µL of infected erythrocytes. The material was centrifuged at 400 g for 5 minutes, the supernatant was discarded, and the erythrocytes were washed with 500 µL of PBS. The washing process was repeated one more time, and the erythrocyte mass obtained was frozen for 16 hours at -20 °C, for lysis. After the lysis, the cells were washed with 500 µL of

PBS, and were centrifuged at 2000 g to precipitate the merozoites, with the supernatant being discarded. This process was repeated until all reddish pigmentation was completely removed, thereby obtaining a pool of merozoites. The pool of merozoites was mixed into a sample buffer, with reduction, and was subjected to processes of ultrasound treatment and boiling, which prepared it for application to polyacrylamide gel. Fig. 3 shows the expression of the protein of interest in the material tested.

(0055) To obtain a homogenous line, the cell culture was cloned, with use of a "FACS Vantage SE" flow cytometer. For this 50 μ L of a growing culture was washed in HL-1 culture medium and was used for the plating of 1 cell per well in a 96-well plate. 4 plates were prepared with 200 μ L of culture medium and 20 μ L of erythrocytes. These plates were incubated in an incubator at 37 °C with 5% CO₂ and 3% O₂ for 21 days, and then were tested for the presence of PCR-infected erythrocytes.

(0056) A clonal line capable of expressing GST was selected and was used to test the ability of the transfected babesia to induce an immune response against the heterologous protein (GST) produced by it. For this, three Holstein calves were inoculated intravenously with 5×10^6 parasites. The inoculums were prepared with the indicated amount of infected erythrocytes, diluted in 5 mL of HL-1 culture medium.

(0057) The animals were monitored to verify if the transfected parasites remained capable of leading to the development of the clinical picture characteristic of babesiosis. For this, the temperature and hematocrit were measured daily for the first 15 days after the infection, and after re-establishment of the disease, these parameters were measured 2 times per week. Fig. 4 presents data from the infected animals, showing that the animals were infected, but that they recovered from the disease in its acute phase.

(0058) The development of the humoral response was analyzed by the Western blot technique, and anti-GST antibodies were detected on the 12th day following the immunization. Fig. 5 shows the Western blot result, with the recognition of the GSTHI protein by antibodies present in

the serum of animals immunized with the babesia clonal line expressing GSTHI, and non-recognition by the control animals immunized with babesia without expressed GST.

(0059) The recombinant strain comprising the expression vector contains: a coding region for glutathione S-transferase of *H. longicornis* (GSTHI), fused to the coding sequence of the signal peptide of the surface antigen protein of merozoite 1 (MSA-1); a coding region of the green fluorescent protein; and a coding region of the blasticidin deaminase, the expression of which is controlled via the bidirectional promoter of the elongation factor 1-alpha. This construction allows the expression of the *H. longicornis* GST fused to the signal peptide or MSA-1, as well as the green fluorescent proteins (GFP) and blasticidin deaminase, the latter two being, respectively, the reporter protein and the enzyme responsible for resistance to the selection agent. GSTHI is a protein with no known biological functions, which was originally isolated from tissues of the tick *H. longicornis*. When inoculated into animals, it was able to induce a protective immune response against the bovine tick *Rhipicephalus microplus*. After the transfection process, the parasite maintains its capacity for infection and reproduction in the animals that are exposed to the hemoparasite. In addition, when using this protozoon as a live vaccine vector, it was possible to induce the generation of antibodies against the protein GST-HI.

(0060) Persons skilled in the art will appreciate the teaching presented herein, and will be able to reproduce the invention in the embodiments presented, as well as in other variant embodiments, within the scope of the attached claims.

CLAIMS:

1. A recombinant strain of a pathogenic microorganism; characterized in that it is comprised of an expression vector of an antigen derived from a parasite or a vector of diseases.
2. The recombinant strain of a pathogenic microorganism according to claim 1; characterized in that it is comprised of an expression vector of an antigen derived from a parasite or a vector of diseases.
3. The recombinant strain of a pathogenic microorganism according to claim 1 or 2; characterized in that the said antigen is fused to a signal peptide.
4. The recombinant strain of a pathogenic microorganism according to claim 3; characterized in that the said antigen is glutathione S-transferase, and the said signal peptide is MSA-1.
5. The recombinant strain of a pathogenic microorganism according to one of claims 1 to 4; characterized in that said pathogenic microorganism is *Babesia bovis*.
6. The recombinant strain of a pathogenic microorganism according to one of claims 1 to 5; characterized in that the aforesaid parasite or disease vector is a tick.
7. A double vaccine; characterized in that it is comprised of the recombinant strain as defined in one of claims 1 to 6; wherein the said recombinant strain is attenuated.
8. The double vaccine according to claim 7; characterized in that it is comprised of adjuvants, preservatives, antibiotics, stabilizers, or a combination of these.
9. The double vaccine according to claim 7; characterized in that it is a booster vaccine.
10. The double vaccine according to claim 7; characterized in that it is a live vaccine.

11. The double vaccine according to claim 7; characterized in that it additionally is comprised of other antigens and/or pathogenic microorganisms.

FIGURES

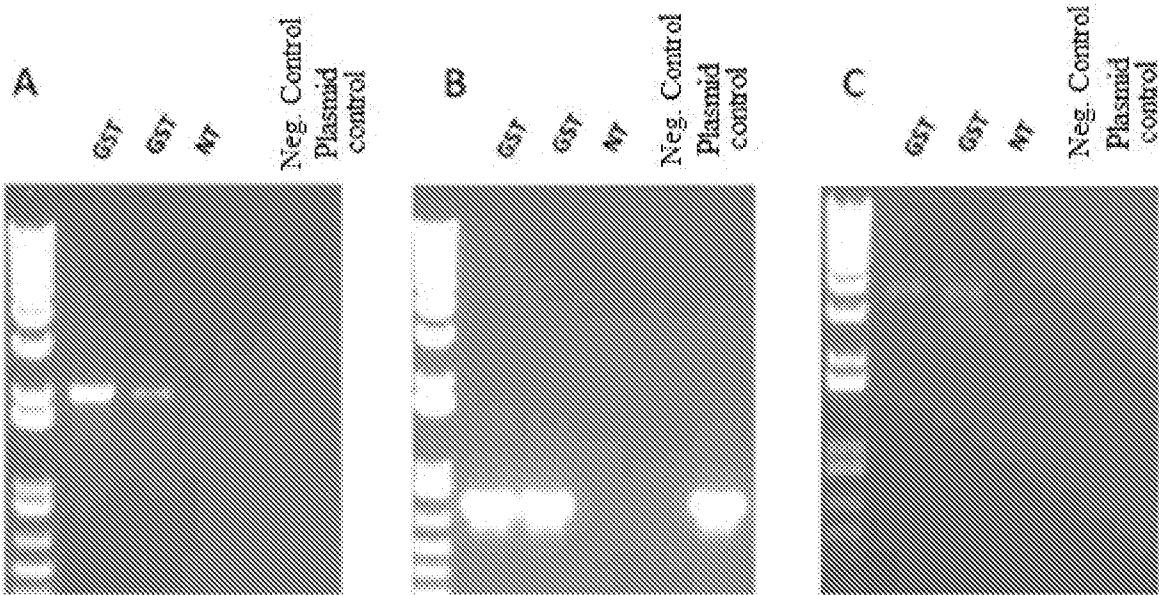


Fig. 1

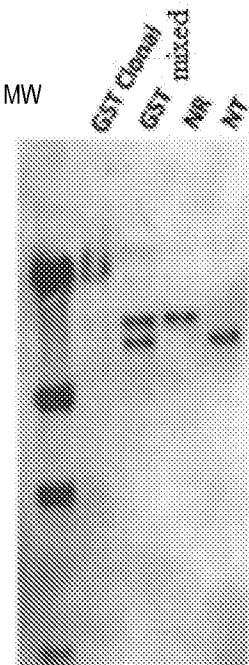


Fig. 2

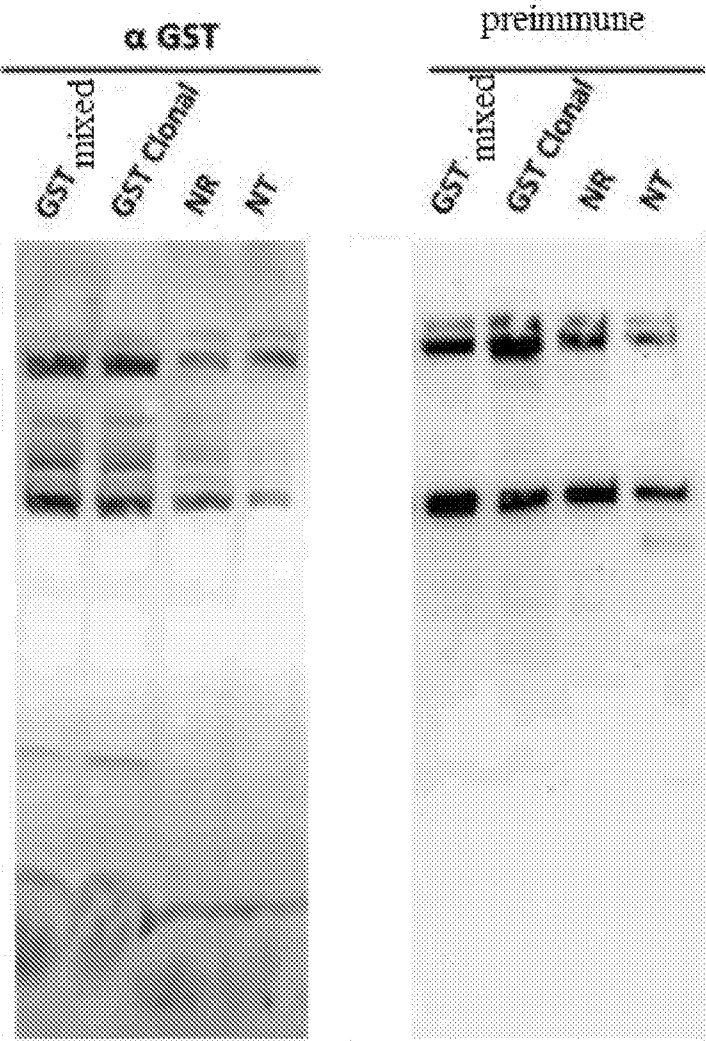
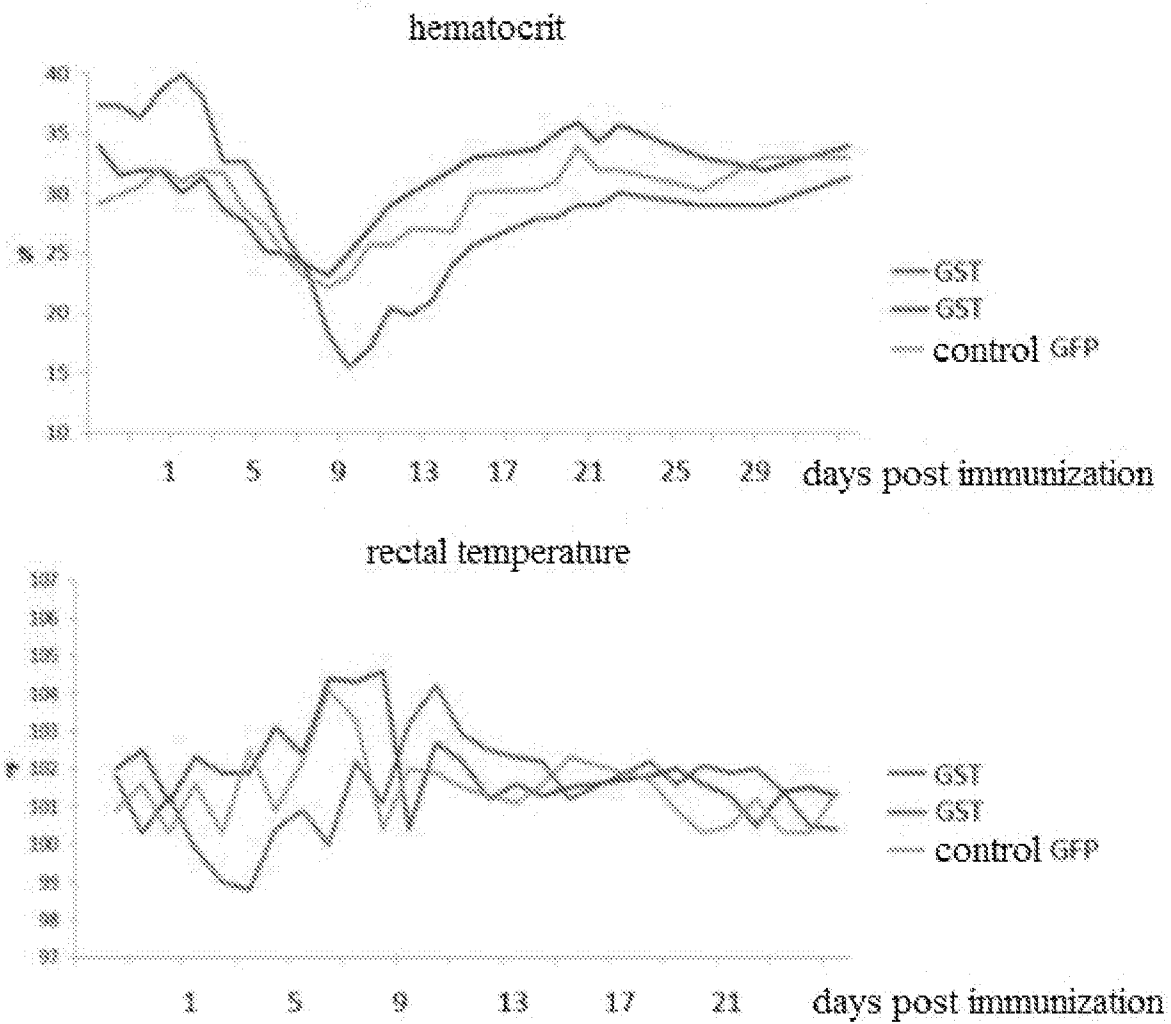


Fig. 3

**Fig. 4**

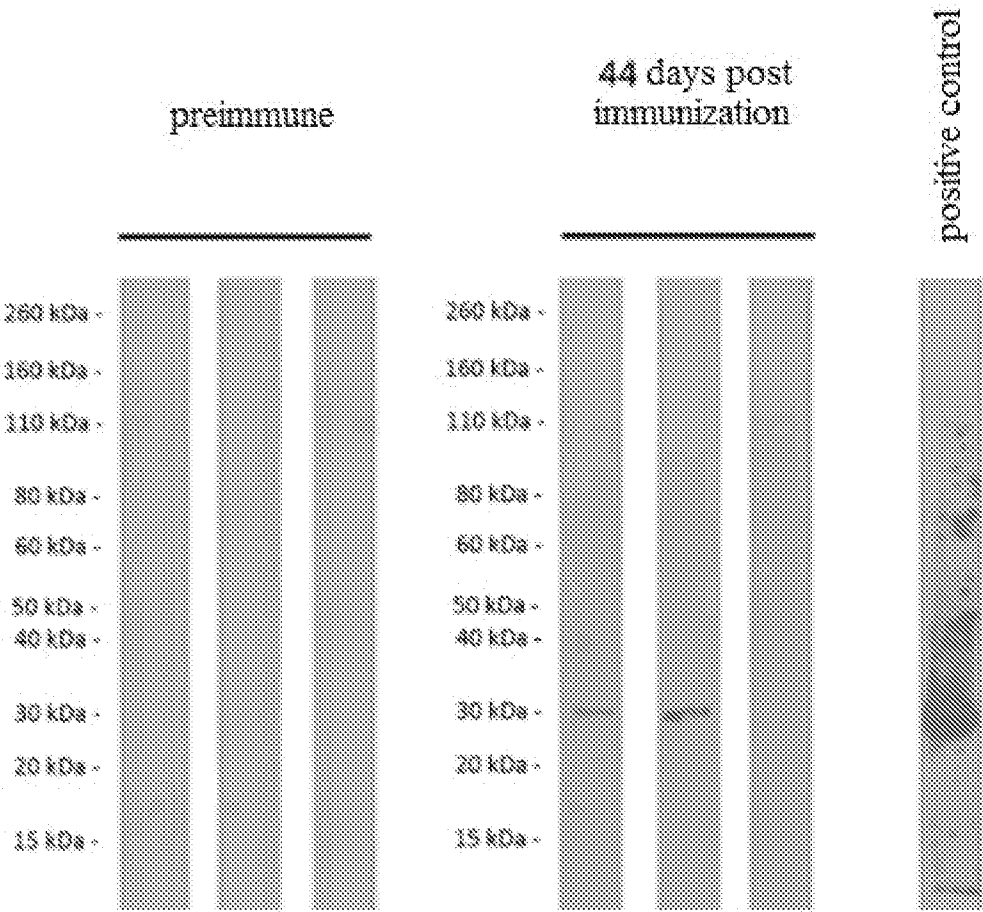


Fig. 5

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/63(2006.01)i, C12N 1/36(2006.01)i, C12N 9/10(2006.01)i, C07K 14/435(2006.01)i, A61K 39/00(2006.01)i, A61K 39/018(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/63; C12N 7/00; A61K 48/00; A61K 39/12; C12N 1/36; C12N 9/10; C07K 14/435; A61K 39/00; A61K 39/018

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & keywords: recombinant strain, expression vector, antigen, parasite, diseases, glutathione S-transferase, MSA-1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAUGHERY et al., `Targeted surface expression of an exogenous antigen in stably transfected Babesia bovis`, PLOS ONE, Vol.9, Issue 5, Article No. e97890, pp.1-10 (2014.05) See p.2, right column; p.4, right column-p.5, left column; figure 3.	1-4
X	WO 2010-005474 A1 (EMERGENT PRODUCT DEVELOPMENT GAITHERSBURG INC.) 14 January 2010 See paragraphs [0116]-[0118]; claims 1-20; figures 1 and 10-13.	1-4
X	US 2007-0160627 A1 (STAIB et al.) 12 July 2007 See claims 1-9.	1-4
A	KIM et al., `Development of a rapid immunochromatographic test for simultaneous serodiagnosis of bovine babesioses caused by Babesia bovis and Babesia bigemina`, The American Journal of Tropical Medicine and Hygiene, Vol.78, No.1, pp.117-121 (2008) See pp.117-118, MATERIALS AND METHODS.	1-4



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

13 April 2017 (13.04.2017)

Date of mailing of the international search report

13 April 2017 (13.04.2017)

Name and mailing address of the ISA/KR

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/068897

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	OLDIGES et al., `Transfected Babesia bovis expressing a tick GST as a live vector vaccine`, PLOS Neglected Tropical Diseases, Vol.10, No.12, Article No. e0005152, pp.1-26 (2 December 2016) See the whole document.	1-4

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2016/068897**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 8-11
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 8-11 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).
3. ☒ Claims Nos.: 5-7
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2016/068897

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010-005474 A1	14/01/2010	EP 2162460 A2 EP 2326344 A1 US 2010-0310593 A1 US 2011-0268760 A1 US 8703153 B2 WO 2008-156729 A2 WO 2008-156729 A3	17/03/2010 01/06/2011 09/12/2010 03/11/2011 22/04/2014 24/12/2008 26/02/2009
US 2007-0160627 A1	12/07/2007	AU 2004-276486 A1 AU 2004-276486 B2 BR PI0414874 A CN 1842602 A CN 1842602 B EP 1518932 A1 EP 1668142 A1 US 7767209 B2 WO 2005-030971 A1	07/04/2005 21/01/2010 12/12/2006 04/10/2006 29/06/2011 30/03/2005 14/06/2006 03/08/2010 07/04/2005