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(54) Title: MICROSPORIDIA ISOLATE

(57) Abstract: The present invention is directed to a novel isolate of *Encephalitozoon cuniculi*, methods for the diagnosis and treatment of disease in animals, and methods for assessing the risk of abortion, foetal loss or still birth in animals.

Microsporidia isolate

Field of the Invention

The present invention relates to a novel isolate of microsporidia and extracts thereof. The novel isolate is useful in the development of diagnostic assays for the detection of microsporidial infections in animals. In addition, the present invention relates to a method for assessing the probability or risk of abortion, foetal loss or still births in pregnant animals, such as cows. The present invention also relates to pharmaceutical compositions for the treatment and prevention of microsporidial infections in animals.

Background of the Invention

Microsporidia are single-celled, spore forming, obligate intracellular protozoan parasites.

At least thirteen species of microsporidia that are parasitic in mammals have been described, and they are now well recognised as opportunistic pathogens of immunocompromised mammals. For example, infections with microsporidia have been recognised in increasing numbers of HIV-infected patients.

There are a number of parasites known to cause abortion and foetal loss in cattle, including *Neospora caninum*, *Sarcocystis cruzi*, and *Trichomoniasis foetus*.

Although recent work suggests dogs, rodents, pigs, rabbits and a number of other mammals may be infected with microsporidia, the role these parasites play in foetal loss in livestock such as cattle or horses is controversial and no definitive conclusion that any microsporidia are abortifacients has been reached (Reetz, 1995; Eckert, 1995; Van Rensburg *et al*, 1991; and Halanova *et al*, 1999).

Summary of Invention

The present inventors have isolated a protozoan parasite from an aborted bovine foetus, obtained from a dairy herd with a history of abortion. The parasite was identified as a microsporidian species, and more specifically as *Encephalitozoon cuniculi* strain I. The novel isolate has been called "Kangaloon".

The present inventors have also demonstrated that this isolate is associated with foetal loss in cattle and have developed a novel method for assessing the risk of foetal loss in cattle that is based on the diagnosis of microsporidial infection.

A sample of the Kangaloon isolate was deposited under the provisions of the Budapest Treaty on 21 June 2001 with the Australian Government Analytical Laboratories (AGAL) and accorded AGAL Accession No NM01/22337.

Accordingly, in a first aspect the present invention provides an isolate of *Encephalitozoon cuniculi* having the characteristics of the isolate deposited as AGAL Accession No. NM01/22337.

In a preferred embodiment of the first aspect, the isolate is that deposited as AGAL Accession No NM01/22337.

In a second aspect, the invention provides an antibody raised against an isolate according to the first aspect. Preferably, the antibody is a monoclonal antibody.

In a third aspect, the invention provides a vaccine composition comprising an isolate of the first aspect, wherein the isolate is in the form of killed parasites or live attenuated parasites.

The present invention also provides a vaccine composition comprising an extract of an isolate according to the first aspect. Preferably, the extract is selected from the group consisting a cell lysate, an antigenic polypeptide and a polynucleotide encoding an antigenic polypeptide.

In a fourth aspect, the invention provides an isolated polynucleotide probe that hybridises specifically to the sequence set out in SEQ ID NO:1 or to a sequence complementary to the sequence set out in SEQ ID NO:1.

In a preferred embodiment of the fourth aspect, the probe is at least 16 nucleotides in length.

In a further preferred embodiment, the probe is conjugated to a label.

In a fifth aspect, the invention provides a method of diagnosing a parasitic disease in an animal, the method comprising identifying the presence of the isolate according to the first aspect of the invention in the animal, or in a clinical specimen from the animal.

Preferably the clinical specimen is a biopsy, stool specimen, blood sample or foetal tissue.

In a sixth aspect, the invention provides a method for the treatment or prevention of infection or disease in an animal, the method comprising administering to the animal a vaccine composition according to the third aspect.

In a seventh aspect, the invention provides a method for the treatment or prevention of infection or disease in an animal, the method comprising administering to the animal an antibody according to the second aspect.

In a preferred embodiment of the fifth, sixth or seventh aspects, the parasitic disease is caused by the presence of microsporidia, and more preferably by the presence of *Encephalitozoon cuniculi* in the animal.

In a further preferred embodiment of the fifth, sixth or seventh aspects, the animal is a human or livestock animal. More preferably, the livestock animal is a cow.

In an eighth aspect, the present invention provides a method for the isolation and enrichment of parasite populations, the method comprising:

(a) inoculating a sample containing the parasite in an immunocompromised or immunosuppressed mouse;

(b) allowing the parasite to grow and divide in the mouse; and

(c) obtaining the parasite from the mouse.

In a preferred embodiment of the eighth aspect, the sample is selected from of the group consisting of cattle tissue, cattle foetal tissue, horse tissue and horse foetal tissue.

In a further preferred embodiment of the eighth aspect, the immunocompromised mouse is an interferon-γ receptor knockout (IFN-γRKO) mouse.

In a ninth aspect, the invention provides a method for *in-vitro* cultivation of a biologically pure culture of bovine *Encephalitozoon cuniculi*, the method comprising:

(a) inoculating a sample of the *Encephalitozoon cuniculi* in a culture of vero (monkey kidney) cells;

(b) incubating the inoculated vero cells in the presence of RPMI tissue culture medium supplemented with horse or new born calf serum such that the *Encephalitozoon cuniculi* grows in the cells.

In a tenth aspect, the invention provides a method for assessing the risk of abortion, foetal loss or still birth in a pregnant animal, the method

comprising determining the presence, absence or exposure to microsporidia in the pregnant animal.

In a preferred embodiment of the tenth aspect, the animal is a human or livestock animal, more preferably the livestock animal is a cow.

Preferably, the microporidia is a strain of *Encephalitozoon cuniculi*. More preferably, the strain has the characteristics of the isolate deposited as AGAL Accession No NM01/22337.

Brief Description of the Figures

Figure 1. A histogram demonstrating the frequency distribution of *E. cuniculi* ELISA absorbance values from serum collected from cows in the study herd. The histogram shows that approximately half of the herd had absorbances of 0.45 or less, while another 5% of the herd had relatively high absorbances of 0.9 or greater.

Figure 2. Scatterplot demonstrating the relationship between *E. cuniculi* ELISA absorbances and IFAT results for a selection of herd sera. Both serum samples categorised as positive and negative by IFAT have high and low ELISA absorbance readings. There is no obvious absorbance at which only positive IFAT samples have high readings and only negative IFAT samples have low readings, thus allowing the development of a cut-off for ELISA positives and negatives.

Detailed Description of the Invention

The parasite described herein was initially isolated by infection of a IFN-γRKO mouse with brain homogenate from the aborted foetus. The parasite was successfully grown in tissue culture, following the repeated passage of brain material through naïve IFN-γRKO mice. This method of isolation proved very successful, as the potentially low parasite load in the foetus was increased with each passage through a naïve mouse. Sequence analysis identified the parasite as *E. cuniculi*. The presence of three 5'-GTTT-3' consecutive repeats in the intergenic spacer region between the small subunit and large subunit rDNA, further identified the parasite as a strain I type of *E. cuniculi*. This isolate has been named "Kangaloon", based on the locality of the farm from which the foetus was obtained. This Kangaloon

isolate represents the first isolation of a microsporidian from a bovine foetus and is therefore particularly useful in the development of diagnostic tools and vaccines for cattle.

The Kangaloon isolate of the present invention may be used to develop diagnostic tools or aids for the detection or diagnosis of parasitic disease in animals. Such diagnostic tools or aids include antigenic polypeptides derived from the isolate, antibodies raised against the isolate and molecular probes or primers derived from the genome of the isolate.

Standard protein purification techniques can be used to isolate antigenic polypeptides from the novel Kangaloon strain of the present invention. Such techniques include selective precipitation with such substances as ammonium sulphate, column chromatography, immunopurification methods, and the like. See, for instance, R. Scopes, Protein Purification: Principles and Practice, Springer-Verlag: New York (1982). Proteins and portions thereof isolated from the strain can be sequenced according to standard techniques as described for instance in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Publish., Cold Spring Harbour, NY 2nd Ed (1989).

Antibodies raised against the strain may be polyclonal or monoclonal antibodies.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) may be immunised with an immunogen preferably a purified protein mixed with an adjuvant. Serum from the immunised animal is collected and treated according to known procedures. Further fractionation of the antisera to enrich for antibodies reactive to the isolated proteins of the invention can be done if desired.

Monoclonal antibodies may be obtained by various techniques by one skilled in the art. Briefly, spleen cells in an animal immunised with a desired immunogen are immortalised, commonly by fusion with a myeloma cell (See, Koller & Milstein, Eur. J. Immunol. 6:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalised cells are screened for production of antibodies of the desired specificity and affinity for the desired antigen.

Antibodies, both monoclonal and polyclonal, which are directed against epitopes are particularly useful in diagnosis, and those which are

neutralising are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies. Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the antigen of the agent against which protection is desired.

Techniques for raising anti-idiotype antibodies are known in the art. These anti-idiotype antibodies may also be useful in therapy.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv).

In one embodiment, the antibodies of the present invention bind specifically to the Kangaloon isolate of the present invention.

Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

Molecular probes or primers may be generated by recombinant or synthetic means. In a preferred embodiment of the present invention, the probe or primer is a polynucleotide that hybridises specifically to the sequence set out in SEQ ID NO:1 or to a sequence complementary to the sequence set out in SEQ ID NO:1.

The phrase "hybridises specifically to" refers to a hybridisation between, for example, a probe and a target sequence in which the probe binds substantially only to the target sequence when the target is in a heterogeneous mixture of polynucleotides. Such hybridisation is determinative of the presence of the target sequence. Although the probe may bind other unrelated sequences, at least 90%, preferably 95% or more of the hybridisation complexes formed are with the target sequence.

Preferably, the polynucleotide is at least 16 nucleotides in length, more preferably at least 20, 25, 30 or 40 nucleotides in length.

The polynucleotide may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, or a probe e.g. conjugated by conventional means to a radioactive or non-radioactive label.

Diagnosis may be achieved by detecting the presence of the parasite or by assaying for antibodies to the parasite in the animal. As there is now concern that humans are susceptible to these type of parasite infections, the present invention includes human applications.

The parasite may be detected by culturing the parasite from a clinical specimen and identifying the parasite by microscopy, or may be identified by the use of antibodies to the parasite or by detecting a portion of the genome of the parasite by molecular biological techniques. The clinical specimen may be a biopsy, stool specimen, blood sample, foetal tissue or the like. It will be appreciated that the discovery of the parasite and its association with parasitic disease will allow its detection by any of the known methods of the art. It will also be appreciated that molecular detection methods like polymerase chain reaction (PCR) can also be used to identify the presence of the parasite in a clinical specimen.

Immunological and immunoassay procedures in general, are described in Basic And Clinical Immunology 7th Ed. (D. Stites and A. Terr ed.) 1991. According to the present invention immunoassays may by performed in any of several configurations, which are reviewed extensively in Enzyme Immunoassay, E. T. Maggio, ed., CRC Press, Boca Raton, Fla. (1980); Practice and Theory of Enzyme Immunoassays," P. Tijssen, Laboratory Techniques In Biochemistry And Molecular Biology, Elsevier Science Publishers B. V. Amsterdam (1985). For example, a person skilled in the art would understand that the proteins and antibodies according to the present invention can be used in ELISA, immunoblot analysis and agglutination assays.

In brief, immunoassays to measure antibodies or antigens are either competitive or noncompetitive binding assays. In competitive binding assays, the sample analyte (e.g., anti-*Encephalitozoon cuniculi* antibodies) competes with a labelled analyte (e.g., anti-*Encephalitozoon cuniculi* monoclonal antibody) for specific binding sites on a capture agent (e.g., isolated *Encephalitozoon cuniculi* protein) bound to a solid surface. The concentration of labelled analyte bound to the capture agent is inversely proportional to the amount of free analyte present in the sample.

Noncompetitive assays are typically sandwich assays, in which the sample analyte is bound between two analyte-specific binding reagents. One of the binding agents is used as a capture agent and is bound to a solid surface. The second binding agent is labelled and is used to measure or detect the resultant complex by visual or instrument means.

Alternatively, the immunoassay is carried out in liquid phase and a variety of separation methods are employed to separate the bound labelled

component from the unbound labelled components. These methods are known to those skilled in the art and include, but are not limited to, immunoprecipitation, column chromatography, adsorption, addition of magnetisable particles coated with a binding agent and other similar procedures.

In another alternate procedure, an immunoassay is carried out in liquid phase without a separation procedure. Typically, in these procedures, the assayed protein or other analyte competes with a compound for binding to an antibody attached to a label. The binding of the analyte to an antibody, but not the binding of the compound to the antibody, causes a change in the signal emitted by the label, so that analyte binding is measured without separating the bound from the unbound labelled component.

Western blot (immunoblot) analysis is also used to detect the presence of antibodies to *E. cuniculi* in a biological sample. This technique is a reliable method for confirming the presence of antibodies against a particular protein in the sample.

Pharmaceutical compositions prepared using extracts of the isolate of the present invention may be used for the treatment and/or prevention of parasitic infections, particularly microsporidia infections.

Preferably, the extract is selected from the group consisting of live attenuated parasites, killed and fixed parsites, cell lysates, antigenic polypeptides and polynucleotides encoding antigenic polypeptides.

Vaccines of the invention may comprise a crude extract of the Kangaloon isolate. Killed and/or chemically fixed parasites or cells can also be used. Vaccines may also comprise partially or completely purified polypeptide preparations derived from the Kangaloon isolate. The polypeptide may be an antigen produced by recombinant DNA technology.

In addition, polynucleotide sequences derived from the Kangaloon isolate may be used to transform viruses that transfect host cells in animals. Live attenuated viruses, such as vaccinia or adenovirus, are convenient alternatives to vaccines because they are inexpensive to produce and are easily transported and administered.

The preparation of vaccines which contain an immunogenic polypeptide(s) as active ingredient(s), is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid

prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine.

Further examples of adjuvants and other agents include aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, *Corynebacterium parvum* (*Propionobacterium acnes*), *Bordetella pertussis*, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Michigan).

Typically, adjuvants such as Quil A, Amphigen (oil-in-water), Alhydrogel (aluminum hydroxide), or a mixture of Amphigen and Alhydrogel are used. Other immunostimulatory molecules such as cytokines (for example, γ-interferon) may also be used.

The proportion of immunogen and adjuvant can be varied over a broad range so long as both are present in effective amounts. For example, aluminum hydroxide can be present in an amount of about 0.5% of the vaccine mixture (Al_2O_3 basis). Conveniently, the vaccines are formulated to contain a final concentration of immunogen in the range of from 0.2 to 200 μg/ml, preferably 5 to 50 μg/ml, most preferably 15 μg/ml.

After formulation, the vaccine may be incorporated into a sterile container which is then sealed and stored at a low temperature, for example 4°C, or it may be freeze-dried. Lyophilisation permits long-term storage in a stabilised form.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include

suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer.

Capsules, tablets and pills for oral administration to a patient may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.

Vaccine compositions of the present invention may be administered to animals susceptible to or otherwise at risk of infection to elicit an immune response against the parasite and thus enhance the animals own immune response capabilities. Such an amount is defined to be an "immunogenically effective amount". In this use, the precise amount depends on the judgement of the prescribing veterinarian or doctor and would include consideration of the patient's state of health and weight, the mode of administration, the nature of the formulation, and the like.

A variety of vaccination regimes may be effective in immunising cattle and other animals. Preferably, female cattle are vaccinated just prior to or at the time of breeding so as to prevent abortion and reduce the possibility of congenital infections. A second immunisation may be given at other time periods before and during gestation. Calves and adult males may also be vaccinated, if desired. Animals that have previously been exposed to microsporidia or have received colostral antibodies from the mother may require booster injections. The booster injection is preferably timed to coincide with times of maximal challenge and/or risk of abortion.

The present invention also provides methods for the isolation and enrichment of parasite populations. One such method is based on the finding

that parasite populations may be enriched by passaging through an immunocompromised or immunosuppressed mouse. An example of a suitable immunocompromised mouse is an IFN-γRKO mouse. Other knock-out mice, such as IFNγ, SCID and Nude mice may also be used, and all are commercially available. Mice may be immunosuppressed, for example, by the addition of Dexamethasone to drinking water or by the injection of Cortisone Acetate or Prednisolone Acetate.

An alternative method for *in-vitro* cultivation of a biologically pure culture of bovine *Encephalitozoon cuniculi* comprises:

(a) inoculating a sample of the parasite in a culture of vero (monkey kidney) cells;

(b) incubating the inoculated vero cells in the presence of RPMI tissue culture medium supplemented with horse or new born calf serum such that the parasite grows in the cells.

Proliferation of the parasites in tissue culture may occur about 42 days post inoculation of the flask. Virtually all cells in the monolayer become heavily infected and scraping the monolayer liberates individual parasites. The addition of approximately 1/5 of these parasites to a 24 hr Vero cell monolayer may result in proliferation of parasites to a fully infected monolayer of cells within 7 days. *In vitro* growth may be maintained by subsequent passage into fresh Vero cells. The parasite may be frozen in liquid nitrogen for cryopreservation and subsequently re-established in culture following thawing.

In order that the present invention may be more clearly understood preferred forms will be described with reference to the following examples and drawings.

Example 1: Brain Preparation

The brain was removed from the bovine foetus, placed in antibiotic saline (0.9% saline containing 200U/ml Penicillin/200mg/ml Streptomycin) and homogenised. The homogenate was passed through repeatedly decreasing needle sizes from 18 G to 21 G. 0.05% trypsin was added to the homogenate and it was incubated at 37°C for 30 mins. The homogenate was then centrifuged at 1200 g for 10 mins and resuspended in antibiotic saline. The pellet was washed twice in antibiotic saline with centrifugation. Following the final spin, the homogenate was resuspended in antibiotic

saline and 1 ml was injected intraperitoneally into each of 10 IFN-γRKO mice. The mice were monitored for clinical signs of disease. One mouse was observed with a ruffled coat and lethargic appearance 51 days post inoculation with homogenate. It was euthanased with carbon dioxide and the brain removed. The brain was homogenised and processed as above, for passage back into two naïve IFN-γRKO mice and also for infection of tissue culture cells.

Example 2: Tissue Culture

Brain homogenate was resuspended in RPMI medium supplemented with 2% NBS and 1% Penicillin/Streptomycin after the final spin, and overlaid onto a 24 hr Vero cell monolayer. Homogenate was removed after 24 hr and replaced with fresh supplemented medium.

Example 3: SDS-PAGE and Western Blotting

Parasites were recovered from tissue culture and reduced to protein extracts by resuspension in lysis solution (20 mM Tris pH 7.5, 0.15 M NaCl, 1% Triton X-100, 1 mM ethylenediaminetetraacteic acid, 1 mM phenylmethylsulphonyl fluoride, 1 mM benzimidine HCl and 2 mM dithiothreitol) and disruption by sonication at 50W/20kHz for 10 secs. The extract was analysed by SDS-PAGE using 15% Tris-glycine gels under denaturing conditions. After electrophoresis, proteins were transferred to PVDF membrane (Millipore) following standard procedures (see for example Towbin H *et al* (1978)). The membrane was stained with Ponceau S stain following transfer to visualise the proteins and the low molecular weight markers (Biorad). The filter was then cut into 0.5 cm strips and incubated in 0.03% PBS/Tween-20 containing 5% skim milk powder for 30 mins to block the membrane. After washing, membrane strips were incubated with primary antibody (sera from the dam prior to and after abortion, sera from dogs on the farm and sera from mice injected with brain homogenate) at a dilution of 1/100 for 2 hrs. After washing with 0.03% PBS/Tween-20, strips were incubated with anti-bovine AP conjugate (Sigma), anti-dog AP conjugate (Sigma), or anti-mouse AP conjugate (Sigma) at a dilution of 1/1000 for 1 hr. After further washing with 0.03% PBS/Tween-20, bands were visualised by incubation with SigmaFast BCIP/NBT tablets (Sigma) dissolved in water.

Example 4: Electron Microscopy

Parasites grown in tissue culture were placed in three changes of 0.1 M cacodylate buffer and post-fixed in 1% aqueous osmium tetroxide for 2 hrs. After washes in water to remove excess osmium fixative, the samples were dehydrated in ethanol, transferred to anhydrous acetone and infiltrated and embedded in Epon resin. The blocks were cut and ultra thin sections examined with a JEM 1010 transmission electron microscope operating at 80 kV.

Example 5: PCR and Sequencing of Parasite DNA

PCR was carried out using genomic DNA extracted from parasites grown in tissue culture. The PCR reaction and cycling conditions were similar to those described in Vossbrinck CR *et al* (1993). Primers used were 530f (5' GTGCCATCCAGCCGCGG) and 580r (5' GTCCGTGTTTCAAGACGG). The PCR products were pooled and run on a 1% agarose gel to confirm amplification and size. A band of approximately 1300 bp was cut from the gel and purified using a Qiaquick gel purification kit (Qiagen). Sequencing was performed by cycle sequencing using an ABI automated sequencer. Two sequences were obtained using the forward primer 530f and two were obtained using the reverse primer 580r. From these a consensus sequence was obtained. A BlastN search was performed using the Genbank database of nucleic acids, to identify any previously reported sequences with homology.

Example 6: Infection of Mice

Six (6) to eight (8) week old BALB/c mice were inoculated subcutaneously with saline, 10^4 or 10^6 parasites. Mice were monitored for evidence of infection (ruffled fur, weight loss) for a period of 68 days. Following euthanasia both serum and brains were collected from mice. Brains were processed using routine histological methods, and following haematoxylin and eosin staining were analysed for pathological evidence of infection. Sera were used for western blot analysis.

Example 7: Herd Serology: *E. cuniculi* prevalence

ELISA . The dairy herd was screened for antibodies to the parasite using an indirect ELISA protocol (see Hollister *et al* 1987). Tissue culture derived parasites were used at a concentration of 1.2×10^6 parasites/well.

Serum samples were diluted 1:100 in blocking buffer and incubated at 4°C overnight. Anti-bovine AP conjugated IgG (Sigma) was used as the secondary antibody, diluted 1:6000 in blocking buffer and was incubated at 4°C overnight. Development was performed by the addition of ρ-nitrophenylphosphate at a concentration of 1mg/ml, and incubation for 30 mins at 37°C. As no known positive and negative samples were available to use as controls, a cut-off absorbance between negative and positive readings was not readily available. In an attempt to overcome this two other methods of serology testing were used to try and identify positive and negative samples. The dairy herd was also screened for antibodies to the cattle abortifacient *N. caninum* using a commercial ELISA kit (IDEXX). The relationship in antibody levels between *N. caninum* and the isolated parasite was analysed using a Spearman correlation.

Western Blotting. Serum samples from cows in the herd were analysed by western blotting using the method described above.

IFAT. Eight (8) well diagnostic slides (Bellco) were coated with parasites freshly isolated from tissue culture. The parasites were resuspended in PBS following centrifugation, to a volume that yielded approximately 40 spores per field on a 100X objective, when 20 µl was dropped onto a slide well. Slides were left to dry, after which they were fixed in acetone for 10 mins. Following fixation, slides were stored at -20°C until use. Slides were rinsed in PBS and then blocked with human hyperimmune serum for 1 hr at room temperature in a moist chamber. Following incubation slides were washed in PBS for 10 mins and diluted herd serum samples were added to the wells. Serum was diluted 1:16 and 1:64 in PBS and slides were incubated for 1 hr at room temperature in a moist chamber. After a 10 min wash in PBS the wells were coated with anti-bovine FITC conjugated IgG (Sigma), diluted 1:160 in PBS. Following incubation for 1 hr at 37°C in a moist dark chamber, Anti-fade (Calbiochem) and coverslips were applied to the slides. Slides were viewed using an Olympus BX51 microscope with U25ND25 fluorescence attachment. Grading of fluorescence was performed similarly to the methods described in Chalupsky *et al* (1978). Sera with a grade of + or ++ at a 1:64 dilution were considered positive. IFAT results were compared to the ELISA absorbance readings using a Spearman correlation.

Herd serology: abortion analysis. As an example, the herd sera may be analysed for a statistical relationship between antibody response, to *N. caninum* or the isolated parasite, and reproductive history. Cattle are preferably categorised as no abortion or abortion (cows that had aborted or those that are repeat breeders). A Student T-test may be performed using the ELISA absorbance values.

Example 8: Pathology of aborted foetus

Autolysis was moderate to advanced in the majority of tissues examined. Pathologic examination showed no evidence of lesions in the brain, however non suppurative epicarditis was observed in the heart. This did not exclude a diagnosis of *N. caninum* infection in the foetus.

Example 9: Growth of parasite *in vitro*

In vitro growth was established using the brains obtained from the two naïve IFN-γRKO mice which were infected with brain homogenate from the original infected mouse (2nd passage). These mice developed clinical signs of infection 12 days post inoculation, they had enlarged intestines and histological examination identified large necrotic lesions in the liver.

Example 10: Antibody response to infection

Western Blotting using serum recovered from the dam of the aborted foetus showed she had an antibody response to parasite antigens with molecular weights of 23, 29, 31 and 45 kDa. The 31 and 45 kDa antigens are probably polar tubule proteins (Keohane *et al*, 1994; Delbac *et al*, 2001). This occurred in sera taken from her prior to and after abortion. Sera from a dog from the farm was positive for the parasite with an antigen profile that was virtually identical to that of the dam. The same antigens that are recognised by the dam are also recognised by serum from IFN-γRKO mice injected with brain homogenate from previously infected IFN-γRKO. The mouse sera recognise a number of other antigens in addition to those recognised by the dam including 3 low molecular weight bands. These are not recognised by sera from the dam, are very faint in the first passage of brain homogenate through IFN-γRKO mice but are quite prominent after further passage through IFN-γRKO mice.

Example 11: Electron Microscopy

Electron microscopy confirmed that the parasite was a microsporidian species, of the genus *Encephalitozoon*. The spore size was 1-1.5 μM and a 3-5 coiled, single tiered polar filament was visible. Spore development was occurring within a parasitophorous vacuole.

Example 12: Parasite Identification using PCR and DNA sequencing

A BlastN search of the Genbank nucleic acid database revealed 98% homology across 503 bp, between the PCR product and the rDNA of *Encephalitozoon cuniculi* (accession number AJ005581). This homologous sequence incorporated a region of the small subunit rDNA, the intergenic spacer, and a region of the large subunit rDNA. The parasite is therefore probably a strain of *E. cuniculi*. DNA sequencing of the ribosomal DNA has previously identified the species to possess 3 strain types (I, II and III). The presence of three 5'-GTTT-3' consecutive repeats in the intergenic spacer region identifies the parasite as being a type I. The nucleotide sequence data is set out in SEQ ID NO 1.

Example 13: Dose response in a murine infection

Mice infected with Kangaloon displayed no signs of infection, and a weight increase was observed over the period of the experiment (data not shown). Examination of brains from infected mice revealed very mild non-suppurative encephalitis, necrotic lesions and gliosis. Lesion severity was similar between mice receiving 10^4 or 10^6 parasites. Infected mice produced an anti-Kangaloon IgG response during the experiment, not observed in saline injected mice. No difference in response was observed between sera from mice infected with 10^4 or 10^6 parasites.

Example 14: Herd Serology: *E. cuniculi* prevalence

ELISA – Approximately half of the herd had absorbances of 0.45 or less. Another 5% of the herd had relatively high absorbances of 0.9 or greater (Figure 1). When a selection of both high and low absorbance serum samples were re-run on an ELISA, using doubling dilutions from 1:50 to 1:1600, high absorbance samples titrated out whereas very low absorbance samples did not vary in absorbances between dilutions, indicating a negative result in this test (data not shown). This indicated that values obtained at a single dilution

of 1:100 were a true indication of the serum samples responsiveness to the parasite. Comparison of the ELISA absorbance readings for *E. cuniculi* and *N. caninum* revealed a positive correlation. This correlation did not occur due to cross reactivity of epitopes in the ELISA assays, as some cows with high readings to *E. cuniculi* had low readings to *N. caninum* and vice versa.

Example 15: Western Blotting

A Western Blot was performed on samples with a range of absorbances from 0.2355-1.8505. Nine (9) of the ten (10) serum samples tested produced antigen profiles. The sample that did not produce a profile had a high ELISA absorbance reading of 0.8955. Although the strength of the response of the sera to the antigen on the blot did vary, they did not appear to correlate with the ELISA absorbance readings.

Example 16: IFAT

Samples of herd sera tested using immunofluorescence showed varying grades of fluorescence. Using the grading system at a 1:64 dilution, 11 of the 26 serum samples were considered positive. A Spearman correlation indicated that there was no statistical relationship between the ELISA absorbances and the IFAT results (Figure 2).

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or any other country before the priority date of each claim of this application.

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

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the

invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

References:

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Halanova M, Letkova V, Macak V, Stefkovic M, Halan M. The first finding of antibodies to *Encephalitozoon cuniculi* in cows in Slovakia. *Veterinary Parasitology* 1999; 82: 167-171.

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Keohane EM, Orr GA, Takvorian PM et al. Polar tubule proteins of microsporidia of the family Encephalitozoonidae. *Journal of Eukaryotic Microbiology* 1999; 46: 1-5.

Reetz J. Microsporidia as a cause of abortions in cattle. *Tierarztliche Umschau* 1995; 50: 550-554.

Towbin H, Staehlin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proceedings of the National Academy of Sciences USA* 1978; 76: 4350-4354.

Van Rensburg IBJ, Volkmann DH, Soley JT, Stewart CG. *Encephalitozoon* infection in a stillborn foal. *Journal of the South African Veterinary Association* 1991; 62: 130-132.

Vossbrinck CR, Baker MD, Didier ES, Debrunner-Vossbrinck BA, Shadduck JA. Ribosomal DNA sequences of *Encephalitozoon hellem* and *Encephalitozoon cuniculi* - species identification and phylogenetic construction. *Journal of Eukaryotic Microbiology* 1993; 40: 354-362.

CLAIMS:

1. An isolate of *Encephalitozoon cuniculi* having the characteristics of the isolate deposited as AGAL Accession No. NM01/22337.

2. An isolate as claimed in claim 1 which is deposited as AGAL Accession No NM01/22337.

3. An antibody raised against an isolate as claimed in claim 1 or claim 2.

4. An antibody as claimed in claim 3 wherein the antibody is a monoclonal antibody.

5. A vaccine composition comprising an isolate as claimed in claim 1, wherein the isolate is in the form of killed parasites or live attenuated parasites.

6. A vaccine composition comprising an extract of an isolate as claimed in claim 1 or claim 2.

7. A vaccine composition as claimed in claim 6 wherein the extract is selected from the group consisting of cell lysates, antigenic polypeptides and polynucleotides encoding antigenic polypeptides.

8. An isolated polynucleotide probe that hybridises specifically to the sequence set out in SEQ ID NO:1 or to a sequence complementary to the sequence set out in SEQ ID NO:1.

9. An isolated polynucleotide probe as claimed in claim 8 wherein the probe is at least 16 nucleotides in length.

10. An isolated polynucleotide probe as claimed in claim 8 or claim 9 wherein the probe is conjugated to a label.

11. A method of diagnosing a parasitic infection or disease in an animal, the method comprising detecting the presence of an isolate according to claim 1 or claim 2 in the animal, or in a clinical specimen from the animal.

12. A method as claimed in claim 11 wherein the clinical specimen is selected from the group consisting of a biopsy, a stool specimen, a blood sample and foetal tissue.

13. A method for the treatment or prevention of infection or disease in an animal, the method comprising administering to the animal a vaccine composition as claimed in any one of claims 5 to 7.

14. A method for the treatment or prevention of infection or disease in an animal, the method comprising administering to the animal an antibody as claimed in claim 3 or claim 4.

15. A method as claimed in any one of claims 11 to 14 wherein the infection or disease is caused by the presence of microsporidia in the animal.

16. A method as claimed in any one of claims 11 to 15, wherein the animal is a human or livestock animal.

17. A method as claimed in claim 16, wherein the livestock animal is a cow.

18. A method for the enrichment of parasite populations, the method comprising:

(a) inoculating a sample containing the parasite in an immunocompromised or immunosuppressed mouse;

(b) allowing the parasite to grow and divide in the mouse; and

(c) obtaining the parasite from the mouse.

19. A method as claimed in claim 18 wherein the immunocompromised mouse is an interferon-γ receptor knockout (IFN-γRKO) mouse.

20. A method as claimed in claim 18 or claim 19 wherein the parasite is a microsporidian species.

21. A method as claimed in any one of claims 18 to 20, wherein the sample is selected from the group consisting of cattle tissue, cattle foetal tissue, horse tissue and horse foetal tissue.

22. A method for *in vitro* cultivation of a biologically pure culture of bovine *Encephalitozoon cuniculi*, the method comprising:

(a) inoculating a sample of the *Encephalitozoon cuniculi* in a culture of vero (monkey kidney) cells;

(b) incubating the inoculated vero cells in the presence of RPMI tissue culture medium supplemented with new born calf or horse serum such that the *Encephalitozoon cuniculi* grows in the cells.

23. A method for assessing the risk of abortion, foetal loss or still birth in a pregnant animal, the method comprising determining the presence, absence or exposure to microsporidia in the pregnant animal.

24. A method as claimed in claim 23, wherein the animal is a human or livestock animal.

25. A method as claimed in claim 24, wherein the livestock animal is a cow.

26. A method as claimed in any one of claims 23 to 25 wherein the microsporidia is *Encephalitozoon cuniculi*.

27. A method as claimed in claim 26 wherein the *Encephalitozoon cuniculi* has the characteristics of the isolate deposited as AGAL Accession No NM01/22337.

28. A method as claimed in claim 27 wherein the *Encephalitozoon cuniculi* is the isolate deposited as AGAL Accession No NM01/22337.

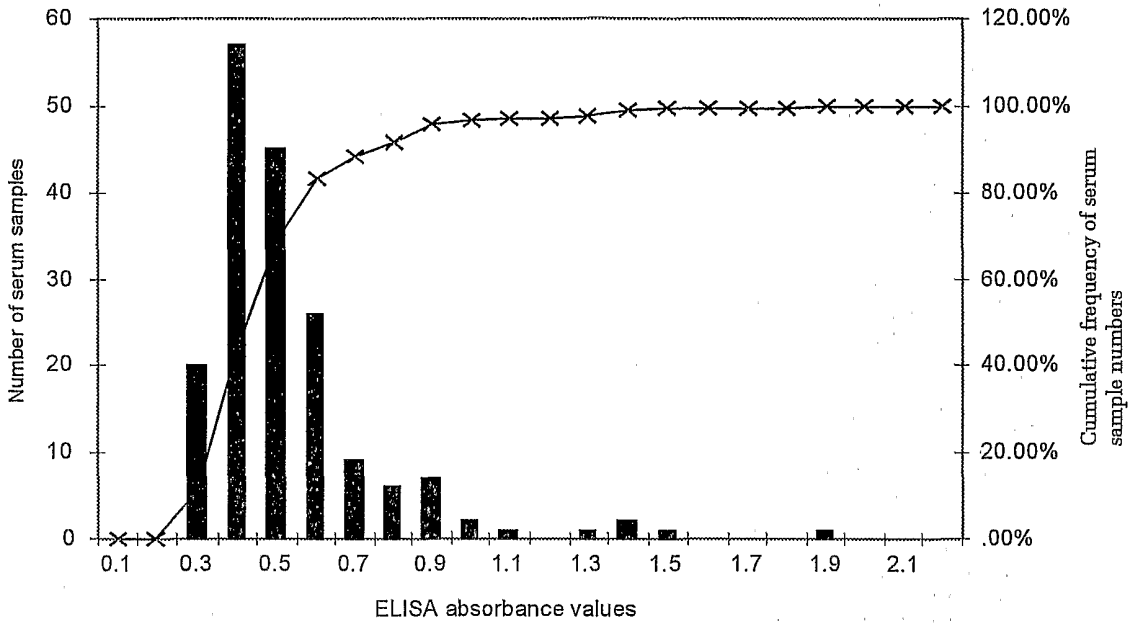

Figure 1

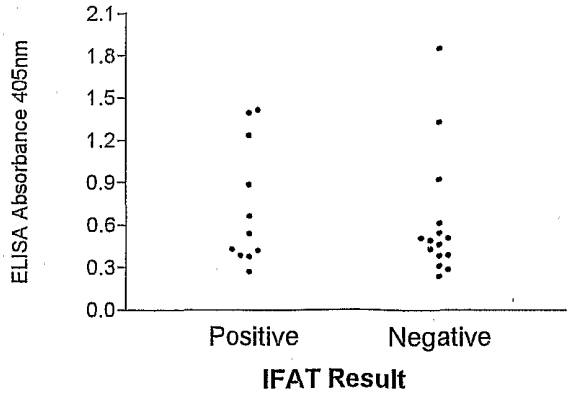

Figure 2

SEQUENCE LISTING

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gccacaaaca caacccgcca aacgcagtta ccacacaggc tcatgccatt tcactcgccg 180
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aagtgcgtca tcttagatag cga 503

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00819

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 1/10, A61K 35/68, A61K 39/002, (C12Q 1/06, C12R 1:90)

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)

WORLD PATENT INDEX (WP) and CHEMICAL ABSTRACTS (CA), KEYWORDS (KW): see electronic database below

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

MEDLINE (ML) and DERWENT BIOTECHNOLOGY ABSTRACTS (DB), KW: see electronic database below

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

ML, CA, WP, DB: KW: Encephalitozoon cuniculi.
GENPEPT, SWISS-PROT, PIR, EMBL, GENBANK - SEQ ID NO: 1.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL database Accession Number L13332 Encephalitozoon cuniculi small ribosomal RNA, 3'end; non-transcribed spacer; large ribosomal RNA, 5'end. 12 May 1993. See whole document, nucleotides 106-399 are 97% identical to nucleotides 503-210 of SEQ ID NO:1	8-10
X	EMBL database Accession Number AJ005581 Encephalitozoon cuniculi complete rDNA unit (16S, 5.8S, 23S, ITS1 and IGS) 17 August 1998. See whole document, nucleotides 2935-3437 are 99% identical to nucleotides 503-1 of SEQ ID NO:1	8-10

[X] 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	Date of mailing of the international search report
21 August 2001	22 August 2001
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**J.H. CHAN** Telephone No : (02) 6283 2340

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00819

Box I 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 : **18 -21**

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:

The scope of these claims is broad and generally not clear. Therefore no meaning full search of these claim could be carried out. However the search with respect to the Encephalitozoon cuniculi parasite of the invention did identify some relevant documents.

3. ☐ Claims Nos :

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

Box II 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 fee, this Authority did not invite payment of any additional fee.
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.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT	International application No. PCT/AU01/00819

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL database Accession Number L29560 Encephalitozoon cuniculi small subunit ribosomal RNA gene, 3' end; 5.8S ribosomal RNA gene, complete sequence; large subunit ribosomal RNA gene, 5'end. 28 April 1995. See whole document, nucleotides 135-299 are 98% identical to nucleotides 378-214 of SEQ ID NO:1	8-10
X	Vet. Parasitol. (1999) Vol 82: 167-171. Halánová, M. *et al.* "The first finding of antibodies to *Encephalitozoon cuniculi* in cows in Slovakia" See page 168 last paragraph.	22
X	J. Clin. Microbiol. (1997) Vol 35 (3): 724-729. Enriquez, FJ. *Et al.* "Simple diagnosis of *Encephalitozoon* sp. Microsporidial infections by using a panspecific antiexospore monoclonal antibody". See page 724 col 2 last paragraph	22
X	J. South African Vet. Assoc. (1991) Vol 62 (3): 130-132. Van Rensburg, IBJ. *et al.* "*Encephalitozoon* infection in a still-born foal". See whole document, particularly page 132 last paragraph.	23-26
X	J. Immunol. (1999) Vol 162: 6086-6091. Khan, IA. *Et al.* "CD8+ CTLs are essential for protective immunity against *Encephalitozoon cuniculi* infection" See whole document, particularly page 6091 last paragraph.	18-21
X	Folia Parasitol. (Praha) (1993) Vol 40 (4): 287-291. Hermanek, J. *et al.* "Prophylactic and therapeutic immune reconstitution of SCID mice infected with Encephalitozoon cuniculi" See whole document, particularly page 288.	18-21