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(57) Abstract

The invention relates to isolated polynucleotide molecules encoding *Neospora caninum* antigens, isolated *N. caninum* polypeptides, antibodies to the polypeptides and uses of the polynucleotide molecules and polypeptides to immunise mammals against neosporosis.

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Parasite Antigens

Technical Field

The present invention is directed to parasite antigens, particularly *Neospora* antigens and uses thereof.

Background Art

Neospora caninum is a cyst-forming coccidian parasite which was described during a retrospective study of dogs previously diagnosed with fatal toxoplasmosis in 1988. The genus *Neospora* was established in the family Sarcocystidae of the phylum Apicomplexa because of the close similarity in morphology between *Neospora* and other cyst-forming coccidia such as *Toxoplasma*. The complete life cycle of *Neospora* is not known but congenital transmission has been recorded in dogs, cats, sheep, cattle, goats and horses.

Abortion is a major cause of economic loss to both the dairy and beef industries, estimated at 2-5% annually on most farms in Australia but occasionally reported to be as high as 20-30% per annum. In a review of 500 abortions, suspected neosporosis was found to be the major cause of abortion in cattle, particularly in northern coastal NSW. Protozoal abortion is estimated to cause at least $0.5 million and $1 million in direct losses annually to north eastern dairy farmers and beef producers respectively, with estimated potential losses of $85 million and $25 million to the Australian dairy and beef industries, respectively. In Australia and other countries of the world a diagnosis of *Neospora* abortion in cattle is made when there is evidence of focal or multifocal nonsuppurative inflammation in the brain, heart, liver and occasionally other organs, and less commonly, demonstration of tachyzoites or cysts in tissue sections. Parasites may be found adjacent to necrotic foci, but they are difficult to locate in routine haematoxylin and eosin (H&E) stained sections. Parasites are more readily demonstrated by an immunoperoxidase test performed on formalin-fixed, paraffin-embedded tissue using specific anti-*N. caninum* sera made in rabbits. Using a mixture of these techniques *N. caninum* was demonstrated in the brains of aborted calves from cases collected from NSW and Tasmania, confirming that neosporosis is the main cause of bovine abortion in Australia. However, the immunoperoxidase procedure is not very sensitive and depends on the presence of large numbers of organisms in tissue sections to make a firm diagnosis. There are also cross reaction problems associated with its use.

Consequently, during 1994 and 1995 the present inventors and others developed a prototype diagnostic test based on the polymerase chain reaction (PCR) for the detection of *Neospora* DNA and DNA derived from formalin-fixed paraffin embedded tissue sections that will detect as little as 1 fg of target DNA by a single-tube nested PCR .

Methods for the serological diagnosis of *Neospora* infections in dogs and cattle have also been described. An IFAT test was developed although cross-reactivity with *T. gondii* and *Hammondia hammondi* has been demonstrated in sera from cattle believed to be infected with *Neospora*. This test has subsequently been superseded by an ELISA test based on cell-free lysates of *Neospora*.

Neospora caninum is recognised as a major cause of bovine abortion and morbidity in dogs worldwide. Losses may be extremely significant in areas with large dairy and beef cattle industries such as the USA, Europe and Australia. Commercial vaccines against *N. caninum* are not yet available, although a vaccine based on a crude lysate has been conditionally released in the United States of America by the Bayer Corporation. Several antigens from *N. caninum* have also been described including NcSAG1 (Ncp29/NCp36), NcSRS-2 (Ncp35/NCp43), NCGRA6 (NCDG2), NCGRA7 (NCDG1), NC-p65, and more recently, NCGRA2. These antigens have all been cloned and most appear associated with the parasite cell membrane or localised to dense granules. Homologues to some of these antigens in *Toxoplasma gondii*, (a close relative to *N. caninum*), have been extensively evaluated for diagnosis or for the induction of protective immunity. However, the selection of appropriate candidates for vaccine development requires careful consideration. For example, antigens which induce proliferation of humoral and cellular immune responses in *Neospora*-infected calves may be considered suitable candidates for further study.

The present inventors have isolated and characterised a new gene from *N. caninum* (called *NCP20*), which was isolated by immunoscreening a cDNA library with pooled sera from mice made resistant to a lethal challenge of *N. caninum*. The gene encodes a polypeptide that is antigenic and has been found to be a good candidate for immunising animal against neosporosis.

Disclosure of Invention

In a first aspect, the present invention consists in an isolated polynucleotide molecule encoding a *N. caninum* antigen, the polynucleotide molecule having a sequence as set out in Figure 1 (SEQ ID NO: 1), or polynucleotide molecules which hybridise thereto under stringent conditions.

When used herein, stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0/1% $NaDodSO_4$ at 65^0C; (2) employ during hybridisation a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42^0C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS and 10% dextran sulfate at 42^0C in 0.2 x SSC and 0.1% SDS.

Also provided are vectors including such polynucleotides, host cells transformed with such vectors, and recombinant polypeptides encoded by such polynucleotides. The vectors may be adapted to be used in bacterial or mammalian cells as known to the art. Due to differences in the mechanism of gene expression between prokaryotic and eukaryotic cells, it will be appreciated that different polypeptides may be produced from the same polynucleotide using the two expression systems.

In a second aspect, the present invention provides an isolated *N. caninum* polypeptide encoded by the isolated polynucleotide according to the first aspect of the present invention, or functionally equivalent polypeptides, or immunogenic fragments thereof .

In one preferred embodiment of the second aspect of the present invention, the isolated *N. caninum* polypeptide is produced in a prokaryotic expression system, preferably using *Escherichia coli*, such that the polypeptide has amino acid sequence substantially as shown in Figure 2 (SEQ ID NO: 2), or functionally equivalent amino acid sequences.

In another preferred embodiment of the second aspect of the present invention, the isolated *N. caninum* polypeptide is produced in a eukaryotic expression system from the polynucleotide molecule shown in Figure 1 (SEQ

ID NO: 1). As eukaryotic cells express genes differently from prokaryotes, the polypeptide produced by such a system as shown in Figure 2 (SEQ ID NO: 2) may be post-translationally modified in some way.

The polypeptide produced by the bacterial expression of the cDNA sequences according to the first aspect of the present invention react with antibodies present in mice that have been infected by *N. caninum*. Accordingly, it will be appreciated that the polypeptide will have the same or similar antigenic epitopes as present on the native polypeptide of *Neospora*. Thus, the polypeptide according to the second aspect of the present invention, whether produced by in prokaryotic or eukaryotic expression systems, should be a good candidate for antigens to raise protective antibodies to *N. caninum* in animals.

In a third aspect, the invention provides a composition for use in raising an immune response in animals against neosporosis, the composition comprising a carrier and a polypeptide having an amino acid sequence substantially as the sequence shown in Figure 2 (SEQ ID NO: 2), functionally equivalent polypeptides, or immunogenic fragments thereof.

It will be appreciated by those skilled in the art that a number of modifications may be made to the polypeptides and fragments of the present invention without deleteriously effecting the biological activity (immunogenicity) of the polypeptides or fragments. This may be achieved by various changes, such as sulfation, phosphorylation, nitration and halogenation; or by amino acid insertions, deletions and substitutions, either conservative or non-conservative (eg. D-amino acids, desamino acids) in the peptide sequence where such changes do not substantially alter the overall biological activity of the peptide. Preferred substitutions are those which are conservative, i.e., wherein a residue is replaced by another of the same general type. As is well understood, naturally-occurring amino acids can be subclassified as acidic, basic, neutral and polar, or neutral and nonpolar. Furthermore, three of the encoded amino acids are aromatic. It is generally preferred that encoded peptides differing from the determined polypeptide contain substituted codons for amino acids which are from the same group as that of the amino acid replaced. Thus, in general, the basic amino acids Lys, Arg, and His are interchangeable; the acidic amino acids Asp and Glu are interchangeable; the neutral polar amino acids Ser, Thr, Cys, Gln, and Asn are interchangeable; the nonpolar aliphatic amino acids Gly, Ala, Val, Ile,

and Leu are conservative with respect to each other (but because of size, Gly and Ala are more closely related and Val, Ile and Leu are more closely related), and the aromatic amino acids Phe, Trp and Tyr are interchangeable.

It should further be noted that if polypeptides are made synthetically, substitutions by amino acids which are not naturally encoded by DNA may also be made. For example, alternative residues include the omega amino acids of the formula $NH_2(CH_2)_nCOOH$ wherein n is 2-6. These are neutral, nonpolar amino acids, as are sarcosine, t-butyl alanine, t-butyl glycine, N-methyl isoleucine, and norleucine. Phenylglycine may substitute for Trp, Tyr or Phe; citrulline and methionine sulfoxide are neutral nonpolar, cysteic acid is acidic, and ornithine is basic. Proline may be substituted with hydroxyproline and retain the conformation conferring properties.

In a further preferred embodiment of the third aspect of the invention, the composition further includes a suitable adjuvant. Preferred adjuvants include Freund's complete adjuvant, Freund's incomplete adjuvant, glucosaminylmuramyl dipeptide, Quil A, DEAE Dextran/mineral oil, Alhydrogel, Auspharm adjuvant and Algammulin.

In a fourth aspect, the present invention provides a method of obtaining a protective effect against neosporosis in mammals, the method comprising inoculating the mammal with a polypeptide according to the second aspect of the present invention.

In a fifth aspect, the present invention relates to use of the polypeptide according to the second aspect of the present invention in methods for detecting antibodies reactive or specific to *N. caninum*.

One particularly suitable use would be in a recombinant ELISA assay where detection of antibodies in a serum or blood sample from an animal that bind to one or more of the polypeptides would be indicative of the exposure to and/or infection of that animal with *N. caninum*. Screening of animal herds for the presence of an immune response to *Neospora* can be carried out using the polypeptide according to the present invention in suitable immunological assays known to the art. Such tests would also be useful to determine whether immunisation with a vaccine according to the third aspect of the present invention of an animal was successful at raising antibodies to the *N. caninum*.

Furthermore, antibodies raised against the polypeptides according to the present invention could be used in assays to identify or diagnose the

presence of *N. caninum*. The antibodies could be raised in animals, for example laboratory animals, and purified for use by standard techniques. Similarly, monoclonal antibodies could also be produced in the usual manner from rodents immunised with a polypeptide so as to produce antibodies specific to *N. caninum*.

In a sixth aspect, the present invention consists in a suitable vector for the replication and/or expression of a polynucleotide molecules according to the first aspect of the present invention.

The vector may be, for example, plasmid, virus or phage vector provided with an origin of replication, and preferably a promoter for the expression of the polynucleotide, and optionally a regulator of the promoter. The vector may contain one or more selectable markers, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian expression vector. The vector may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

In a seventh aspect of the present invention relates to host cells transformed or transfected with the vector according to the sixth aspect of the present invention.

Suitable host cells for cloning or expressing the protein(s) disclosed herein are the prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Escherichia coli*, *Bacilli* such as *B. subtilis* or *B. thuringiensis*, *Pseudornonas* species such as *P. aeruginosa*, *Salmonella typhimurium* or *Serratia marcescens*.

Eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for expressing the protein(s) of the present invention. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as e.g. *K. lactis*; filamentous fungi such as, e.g. *Neurospora*, or *Penicillium*; and *Aspergillus* hosts such as *A. nidulans* and *A. niger*. Other parasites, such as *T. gondii*, are also appropriate hosts for expressing the protein according to the present invention.

Suitable higher eukaryotic host cells can be cultured vertebrate, invertebrate or plant cells. Insect host cells from species such as *Spodoptera frugiperda*, *Aedes aegypti*, *Aedes albopictus*, *Drosophila melanogaster*, and *Bombyx mori* can be used. Plant cell cultures of cotton, corn, potato, soybean, tomato, and tobacco can be utilised as hosts. Typically, plant cells are transfected by incubation with certain strains for the bacterium *Agrobacterium tumefaciens*.

Propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture); baby hamster kidney cells (BHK ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO); mouse sertoli cells, monkey kidney cells (CV1 ATCC CCL 70); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK ATCC CCL 34), and a human hepatoma cell line (Hep G2). Preferred host cells are canine kidney cells and Chinese hamster ovary cells.

Host cells are transfected and preferably transformed with expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells.

In a eighth aspect, the present invention consist in a process for preparing a polypeptide according to the second aspect of the present invention, the process comprising cultivating a host cell transformed or transfected with an (expression) vector of the seventh aspect of the present invention under conditions providing for expression of the polynucleotide encoding the polypeptide, and recovering the expressed polypeptide.

Such cells can be used for the production of commercially useful quantities of the encoded protein.

In a ninth aspect, the present invention provides an oligonucleotide probe or primer, the probe or primer having a nucleotide sequence that

hybridises selectively to a polynucleotide molecule according to the first aspect of the present invention.

In a preferred embodiment of the ninth aspect, the oligonucleotide probe or primer includes at least 15 nucleotides, more preferably at least 18 nucleotides and more preferably at least 25 nucleotides.

In a further preferred embodiment, the oligonucleotide probe or primer is used as a detectable probe where the oligonucleotide is conjugated with a label such as a radioisotope, an enzyme, biotin, a fluorescent molecule or a chemiluminescent molecule.

In a tenth aspect, the present invention provides an isolated antibody, or fragment thereof, reactive to the polypeptide according to the second aspect of the present invention.

The present inventors have obtained a new gene of *N. caninum* coding an antigen recognised by sera from mice that are immune to infection by this parasite. Thus:

a) proteins encoded by this gene have value for diagnosis of neosporosis in animals by virtue of their antigenicity;

b) proteins encoded by this gene are useful for vaccination of animals against neosporosis;

c) DNA encoding these antigenic proteins are useful for vaccination of animals against neosporosis in the form of a DNA vaccine;

d) DNA encoding these proteins are suitable to form the basis of a DNA-based detection system for *N. caninum* by the development of DNA probes, PCR or other applicable technology; and

(e) antibodies to the protein have value in diagnosis of neosporosis by providing a means of directly demonstrating the presence of the parasite in mammalian tissues.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, 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.

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

Brief Description of Drawings

Figure 1 shows the DNA sequence of *N. caninum NCP20* (5'-3') cDNA (SEQ ID NO: 1).

Figure 2 shows the predicted amino acid sequence coded by the *N. caninum* gene for *NCP20* (SEQ ID NO: 2) .

Figure 3 shows the results of a vaccination trial using recombinant NCP20 protein. The graph shows a plot of mean group body weight (MGW) against days post infection (DPI) with *N. caninum*.

Figure 4 shows ELISA optical density (absorbance) reading for three independent test sera of mice for the presence of antibodies to NCP20.

Modes for Carrying Out the Invention

General molecular biology

Unless otherwise indicated, the recombinant DNA techniques utilised in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (Editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present) and are incorporated herein by reference.

Gene/DNA isolation

The DNA encoding a protein may be obtained from any cDNA library prepared from tissue believed to express the gene mRNA and to express it at a detectable level. DNA can also be obtained from a genomic library.

Libraries are screened with probes or analytical tools designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal or polyclonal antibodies that recognise and specifically bind the protein; oligonucleotides of about 20-80 bases in length that encode known or suspected portions of cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments thereof that encode the same or a hybridising gene. Appropriate probes for screening genomic DNA libraries

include, but are not limited to, oligonucleotides; cDNAs or fragments thereof that encode the same or hybridising DNA including expressed sequence tags and the like; and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook *et al.*

An alternative means to isolate a gene encoding is to use polymerase chain reaction (PCR) methodology as described in section 14 of Sambrook *et al.* This method requires the use of oligonucleotide probes that will hybridise to the gene.

The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimised. The actual nucleotide sequence(s) is usually based on conserved or highly homologous nucleotide sequences or regions of the gene. The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage in that species is known. The oligonucleotide must be labelled such that it can be detected upon hybridisation to DNA in the library being screened. The preferred method of labelling is to use ^{32}P-labelled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labelling.

Nucleic acid having all the protein coding sequence is obtained by screening selected cDNA or genomic libraries, and if necessary, using conventional primer extension procedures as described in section 7.79 of Sambrook *et al.*, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Another alternative method for obtaining the gene of interest is to chemically synthesise it using one of the methods described in Fingels *et al.* (Agnew Chem. Int. Ed. Engl. *28:* 716-734, 1989). These methods include triester, phosphite, phosphoramidite and H-Phosphonate methods, PCR and other autoprimer methods, and oligonucleotide syntheses on solid supports. These methods may be used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available, or alternatively, if the target amino acid sequence is

known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue.

Mutants, variants and homology - nucleic acids

Mutant polynucleotides will possess one or more mutations which are deletions, insertions, or substitutions of nucleotide residues. Mutants can be either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagensis on the DNA). It is thus apparent that polynucleotides of the invention can be either naturally occurring or recombinant (that is to say prepared using recombinant DNA techniques).

An allelic variant will be a variant that is naturally occurring within an individual organism.

Nucleotide sequences are homologous if they are related by divergence from a common ancestor. Consequently, a species homologue of the polynucleotide will be the equivalent polynucleotide which occurs naturally in another species. Within any one species a homologue may exist as numerous allelic variants, and these will be considered homologues of the polynucleotide. Allelic variants and species homologues can be obtained by following standard techniques known to those skilled in the art. Preferred species homologues include those obtained from representatives of the same Phylum, more preferably the same Class and even more preferably the same Order.

A polynucleotide at least 70% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) Ad. Appl. Math., 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol., 48: 443-453), to the that of the present invention are included in the invention, as are proteins at least 80% or 90% and more preferably at least 95% identical to the polynucleotide of the present invention. This will generally be over a region of at least 60, preferably at least 90, contiguous nucleotide residues.

Mutants, variants and homology - proteins

Mutant polypeptides will possess one or more mutations which are deletions, insertions, or substitutions of amino acid residues. Mutants can be either naturally occurring (that is to say, purified or isolated from a natural source) or synthetic (for example, by performing site-directed mutagensis on the encoding DNA). It is thus apparent that polypeptides of the invention can be either naturally occurring or recombinant (that is to say prepared using recombinant DNA techniques).

An allelic variant will be a variant that is naturally occurring within an individual organism.

Protein sequences are homologous if they are related by divergence from a common ancestor. Consequently, a species homologue of the protein will be the equivalent protein which occurs naturally in another species. Within any one species a homologue may exist as numerous allelic variants, and these will be considered homologues of the protein. Allelic variants and species homologues can be obtained by following standard techniques known to those skilled in the art. Preferred species homologues include those obtained from representatives of the same Phylum, more preferably the same Class and even more preferably the same Order.

A protein at least 50% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) Ad. Appl. Math., 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol., 48: 443-453), to the that of the present invention are included in the invention, as are proteins at least 70% or 80% and more preferably at least 90% identical to the protein of the present invention. This will generally be over a region of at least 20, preferably at least 30, contiguous amino acids.

Protein variants

Amino acid sequence variants can be prepared by introducing appropriate nucleotide changes into DNA, or by *in vitro* synthesis of the desired polypeptide. Such variants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final protein product possesses the desired characteristics. The amino acid changes also may alter post-translational processes such as changing the number or position of

glycosylation sites, altering the membrane anchoring characteristics, altering the intra-cellular location by inserting, deleting or otherwise affecting the transmembrane sequence of the native protein, or modifying its susceptibility to proteolytic cleavage.

In designing amino acid sequence variants, the location of the mutation site and the nature of the mutation will depend on characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of other ligands adjacent to the located site.

A useful method for identification of residues or regions for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (*Science* (1989) **244**: 1081-1085). Here, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimise the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. These may represent naturally occurring alleles or predetermined mutant forms made by mutating the DNA either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon the characteristic to be modified.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues.

Amino acid sequence insertions include amino and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Other insertional variants include the fusion of the N- or C-terminus of the proteins to an immunogenic polypeptide e.g. bacterial polypeptides such as betalactamase or an enzyme encoded by the *E. coli trp* locus, or yeast protein, bovine serum albumin, and chemotactic polypeptides. C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, are included.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the protein molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s). Other sites of interest are those in which particular residues obtained from various species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

Substantial modifications in function or immunological identity are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydro-phobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

(1) hydrophobic: norleucine, met, ala, Val, Leu, Ile;
(2) neutral hydrophilic: Cys, Ser, Thr;
(3) acidic: Asp, Glu;
(4) basic: Asn, Gln, His, Lys, Arg;
(5) residues that influence chain orientation: Gly, Pro; and
(6) aromatic: Trp, Tyr, Phe

TABLE 1 Preferred amino acid substitutions

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Lys; Arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe norleucine	Leu
Leu (L)	norleucine, Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile;	Leu
Phe (F)	Leu; Val; Ile; Ala	Leu
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe Ala; norleucine	Leu

Non-conservative substitutions will entail exchanging a member of one of these classes for another.

Substantially purified

By "substantially purified" the present inventors mean a polypeptide that has been separated from lipids, nucleic acids, other polypeptides, and other contaminating molecules.

Active fragment

By "active fragment" the present inventors mean a fragment of an amino acid sequence shown in Figure 2 (SEQ ID NO: 2) which retains immuno-activity of the native polypeptide.

MATERIALS AND METHODS

Parasite culture

N. caninum isolates NC-Liverpool (Barber *et al.* 1995, *Parasitology* **111**, 563-568) and NC-SweB1 (Stenlund *et al.* 1997, *Parasitology Research* **83**, 214-219) were propagated *in vitro* in Vero host cells according to established procedures (Barber *et al.* 1995).

Immunoblotting

Female Balb/C mice were made resistant to an acute, lethal infection of NC-Liverpool by 2 infections of NC-SweB1 as described (Atkinson *et al.* 1999, *Parasitology* **118**, 363-370). Immunoblotting was used to compare antibody responses of these resistant mice and a separate group of acutely infected, naive mice (Atkinson *et al.* 1999).

Affinity purified antibodies (APAbs) were prepared (Hemphill *et al.* 1997b, *Parasitology* **115**, 371-380) by immunoblotting 100 μg of NC-Liverpool antigen separated by SDS-PAGE onto PDVF. A portion of the PDVF was cut out from either side of the PVDF membrane and a section covering the 15-30 kDa size range were visualised by immunoscreening. The portions were then realigned with the main strip of PVDF and the region spanning antigens 15-30 kDa excised. Polyclonal antibody from the resistant mice (n=5, pooled) was then bound to the excised PVDF for 6 hours at RT at a dilution of 1/10. The polyclonal antibody was removed and the antigen strip was washed thoroughly in Tris-buffered saline/Tween (TBS-Tween) 3 times for 20 mins. Bound antibody was eluted in a low pH buffer (50 mM Tris, 50 mM Glycine; pH 2.8) for 5 mins at RT. After neutralisation with 1/10 volumes of 1 M Tris, the eluate was diluted with TBS and 5% skim milk powder to a final volume of approximately 10 ml for use in immunoscreening a cDNA library.

cDNA library construction

Total cellular RNA was isolated from tachyzoites of *N. caninum* (NC-Liverpool) which were grown in Vero cells *in vitro*. Essentially, tachyzoites were lysed in lysis buffer containing 5.7 M guanidinium thiocyanate, 100 mM sodium acetate pH 5.2, 10 mM EDTA containing 100 mM 2-mercaptoethanol. Insoluble debris was removed by centrifugation at 10,000g for 10 min at room temperature. The supernatant was collected and 0.1 volumes of 3 M sodium acetate pH 5.2 was added to it along with 0.75 volumes of ethanol. After precipitation overnight, RNA was pelleted at 12,000g for 15 min at 4°C. The pellet was dissolved in lysis buffer containing 4.5 M guanidinium and the RNA solution was extracted with an equal volume of phenol/chloroform. After centrifugation to break the phases, the aqueous phase was mixed with 2 volumes of ethanol and RNA precipitated overnight. The RNA was collected by centrifugation, washed in 70% ethanol and stored at -70°C until required. RNA pellets from storage were centrifuged (20 minutes, 10000g, 4°C), pooled and resuspended in 5 ml of TS buffer (10 mM Tris, 0.1% SDS) for 15 minutes at 65°C. The solution was then cooled rapidly on ice and sodium chloride added to a final concentration of 400 mM. Purification of mRNA followed an established procedure. Briefly, the solution was passed through a sterile syringe containing oligo-dT cellulose (Clontech), eluate was collected in baked cuvettes and the A_{260} of consecutive fractions was read on a spectrophotometer. After the major peak of poly-A- (rRNA) was eluted off, the bound poly-A^+ (mRNA) was collected by flushing the column with TS buffer. Fractions containing the poly-A^+ A_{260} peaks were then precipitated and stored at -70°C.

Reagents and equipment for cDNA library construction were supplied by Stratagene. Approximately 4 µg of mRNA was centrifuged and resuspended in DEPC-treated water for 15 minutes at 65°C. The solution was cooled rapidly on ice and single stranded cDNA was synthesised in a buffer containing a poly-dT primer with an internal *Xho*1 restriction site. Second strand synthesis, blunt-ending, addition of *EcoR*1 adaptors and *Xho*1 digestion were performed following instructions provided by the manufacturer. Double stranded cDNA was then size fractionated on a Sephacryl S-500 column (Clontech) to remove molecules less than approximately 400 bp in size. cDNA was ligated into *EcoR*1/*Xho*1 digested

arms of the UNI-ZAP XR bacteriophage vector and packaged into viable phage using Gigapack Gold III packagene extracts. The titre of the cDNA library was determined by plating serially diluted aliquots onto *Escherichia coli*. The primary cDNA library contained 1.1 x 10^6 recombinant clones.

Immunoscreening cDNA library

The APAb was used to screen 10,000 clones of the cDNA library following standard immunoscreening procedures (Sambrook, Fritz & Maniatis, 1989, Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York). The clones were adsorbed to IPTG impregnated PVDF membranes (2 h, RT). Membranes were then probed with the APAb (45 min, RT). After washing (3 x10 min) in TBS-Tween the membranes were placed in anti-mouse IgG (Sigma) diluted in 1/1000 in TBS and 5% skim milk powder (45 min, RT). Washing was repeated and development took place in alkaline phosphatase buffer containing nitroblue tetrazoleum and 5-bromo-4-chloro-3-indolyl-phosphate (Sambrook, Fritz & Maniatis, 1989). Positive clones were rescreened until plaque pure.

Characterisation of cDNA clones

Plaque pure positive clones were picked, placed in 100 µl sterile water, boiled and subject to PCR and sequencing (Ellis *et al.* 2000, *Parasitology* **120**, 383-390). Sequences were blasted (BlastN and TblastX) using the Australian Genome Information Service (ANGIS) against the GenBank or NR Nucleic Acid database. This latter database is compiled by ANGIS and contains non-redundant data from GenBank, EMBL and PDB. Matches were considered significant if scores were returned with a probability greater than 10^6. DNA sequences were also blasted against the *Toxoplasma* Database of Clustered ESTS (ToxoDB; http://www.cibil.upenn.edu/agi-bin/ParaDBs/Toxoplasma/index.html).

Protein structure predictions

The protein sequence of NCP20 was submitted to the PSA server (http://bmerc-www.bu.edu/psa/) and a secondary structure prediction made using a Type-1 analysis and the DSM model of Stultz *et al.* (1993, *Protein Science* 2, 305-314) which presumes the protein is a monomeric, single-domain, globular, water-soluble protein.

Expression of NCP20 in *E. coli*

Since there was three potential start codons in the mRNA encoding NCP20, the open reading frame (ORF) of *NCP20* was PCR amplified from EST clone P06 with either

P20-ATG1F (5'ACGTATGGATCCGTTTTGTCAGGTGTTCTTG3') (SEQ ID NO: 3) ;

P20-ATG2F (5'ACGTATGGATCCGGCTTTGTCTACGATGAAC3') (SEQ ID NO: 4);

P20-ATG3F (5'ACGTATGGATCCGAACAAGCCCGGGCCGTTT3') (SEQ ID NO: 5); or

P20-pTrcR (5'ACGCATGAATTCTGTTTCTGAGTTCCCGCT3') (SEQ ID NO: 6).

These primers place unique BamH1 and EcoR1 restriction sites on the five and three prime sides of the *NCP20* ORF, respectively. The PCR products obtained were checked on a 1% agarose gel for size and purified using a Qiaquick PCR purification kit. DNA from the purified PCR product and pTrcHisB vector (Invitrogen) were then digested with both BamHI and EcoR1 restriction enzymes for three hours at 37^{O}C. The digested DNA were purified using a Qiaquick column and checked on a 1% agarose gel. The three PCR products of *NCP20* were then ligated separately into the pTrcHisB vector and transformed into *E. coli* DH5α. Individual recombinants were screened for inserts by PCR using primers pTrcHisFwd (5'GAGGTATATATTAATGTATCG3') (SEQ ID NO: 7) and P20-pTrcR. The sequence of the constructs made were confirmed by cycle sequencing. This strategy ensured the initiation codon of *NCP20* was cloned in-frame into the pTrcHisB vector, which following transcription and translation should produce a polypeptide. Subsequently, *E. coli* containing recombinant DNA were grown in LB medium containing ampicillin and at mid-log phase were induced with 1 mM IPTG. After several hours, the bacteria were collected by centrifugation and solubilised in guanidinium lysis buffer. His-tagged protein was purified using Ni-NTA (Qiagen) resin following the manufacturer's instructions for preparation of denatured *E. coli* cell lysate. Proteins were analysed on 14% SDS-PAGE gels by staining with Coomassie blue.

Identification of native NCP20 antigen

One microgram of recombinant NCP20 purified from *E. coli*, was injected subcutaneously into five, 9 week-old QS mice with Freund's complete or Freund's incomplete adjuvant 4 weeks apart. Mice were bled from the tail vein 3 weeks after the boost. Sera were pooled and used in immunoblotting against reduced tachyzoite antigen.

Vaccination trial

Ten groups of 9 mice were injected twice, subcutaneously in the scruff of the neck, 4 weeks apart, with one of the following treatments (ANZCCART 1998, ISBN 0 646 24923 1):

Group 1: 0.1 ml Freund's complete adjuvant (FCA) followed by a boost with 0.1 ml Freund's incomplete adjuvant (FIA) only;
Group 2: 0.1 ml FCA plus 1 μg NCP20;
Group 3: 0.1 ml FIA only;
Group 4: 0.1 ml FIA plus 1 μg NCP20;
Group 5: 0.1 ml FIA plus 25 μg glucosaminylmuramyl dipeptide (GMDP);
Group 6: 0.1 ml FIA plus 25 μg GMDP plus 1 μg NCP20;
Group 7: 0.1 ml 0.9% NaCl containing 10 μg Quil A only;
Group 8: 0.1 ml 0.9% NaCl containing 10 μg Quil A plus 1 μg NCP20;
Group 9: saline (+ve control)
Group 10: no treatment (-ve control).

Mice (all groups except group 10) were then challenged three weeks after the second injection with 7.5×10^5 culture-derived tachyzoites of NC-Liverpool subcutaneously. Changes in mean group body weight (MGW) between 14 - 27 days post infection (DPI) with *N. caninum* were determined and analysed by a one-factor-repeated measures analysis of variance, with treatment as the factor and time as the repeated measure. All the sampling times were included in the analysis. Two mice (one in group 1 and one in group 6) were removed from the analysis because their changes in body weight were anomalous. The details of the differences among treatments were assessed using a posteriori Tukey HSD multiple comparison test.

Enzyme-linked immunosorbent assay using NCP20

Histidine-tagged, recombinant NCP20 (purified on Ni-NTA resin as described previously) was coated onto 96-well microtitre plates at 1 µg/well diluted in ELISA buffer 1 (70 mM $NaHCO_3$, 29 mM Na_2CO_3, 3.1 mM NaN_3, pH 9.6). Following overnight incubation at 4°C, the plates were washed 3 times in wash buffer (PBS, 0.03% Tween 20, pH 7.2). Serum samples were diluted 1:25 using ELISA buffer 2 (0.5 g bovine haemoglobin, 0.3% Tween 20, 3.1 mM NaN_3, pH 7.2 in PBS), and 100 µl of each sample was added in duplicate. The plates were incubated overnight at 4°C and then washed as before. One hundred µl of biotinylated antibody to mouse IgG (The Binding Site, UK) was added to each well at a dilution of 1:6000 in ELISA buffer 2. Following a 1 hour incubation at 37°C and washing, each well was coated with 100 µl of ExtrAvidin alkaline phosphatase (Sigma, USA) at a dilution of 1:5000 in ELISA buffer 2. After incubation for 1 hour at 37°C the plates were again washed and 100 µl of Alkaline Phosphatase Substrate 104 (Sigma, USA) was added at a concentration of 1 mg/ml in ELISA buffer 3 (58 mM $NaHCO_3$, 42 mM Na_2CO_3, 2 mM $MgCl_2.6H_20$, pH 9.8). The plates were incubated at 37°C for 30 min, allowing sufficient colour development. The absorbance reading of each well at 405 nm was determined using an electronic plate reader (Biorad).

RESULTS

Identification of antigens for APAb preparation

Immunoblotting of sera from resistant (vaccinated) and acutely infected naive Balb/C mice to antigen from NC-Liverpool and NC-SweB1 were studied. A group of antigens were identified by blotting using sera from resistant mice in the range 15-30 kDa which were absent or significantly less immunogenic in tracks probed with sera from acutely infected naive mice. An APAb was therefore prepared from antigens over this specific size range. The specificity of the APAb to *N. caninum* antigen was investigated by immunoblotting. Immunoreactive bands of approx. 17-18 kDa were faintly detected under non-reducing and reducing conditions respectively.

Gene isolation and characterisation

Four positive clones were isolated from the cDNA library by immunoscreening with the APAb. PCR products were obtained from the cDNA clones with sizes of approximately 1200, 1000, 900 and 800 bp. Sequence from the 1200 bp clone was homologous to *NCGRA7*/NCDG1 (Lally

et al. 1996, *Clinical and Diagnostic Laboratory Immunology* **3**, 275-279; Lally *et al.* 1997, *Molecular and Biochemical Parasitology* **87**, 239-243) and not studied further. Sequence from the 1000 bp clone predicted limited protein sequence homology of the gene product with the Gra1 protein of *T. gondii* (Cesbron-Delauw *et al.* 1989, *Proceedings of the National Academy of Sciences of the United States of America* **86**, 7537-7541) which was detected by a TblastX search of the GenBank database. Hence this gene was called NC*GRA*1 (GenBank accession number AF199030) and is described elsewhere.

The remaining two cDNA sequences isolated from the cDNA library were homologous to each other. Eight additional cDNA sequences (ESTs) homologous to these cDNAs, were also identified amongst a small EST database maintained at UTS and a consensus DNA sequence was derived from all of them extending the data to approximately 1088 bp (Figure 1). The final cDNA consensus was 280 bases longer at the 5' end than the original two cDNAs isolated and possessed three potential initiation codons at positions 1, 160 and 175.

NCP20/*NCP20* clustered in the ToxoDB with Ctoxqual_1252, a cluster containing 22 ESTs of *T. gondii*. Of potential significance is that the two most similar *T. gondii* sequences (with the highest probability of a match) were ESTs derived from a bradyzoite cDNA (TgEST zz70do9.r1 and TgESTzz46do3.r1). Thus it was concluded that a *T. gondii* homologue of *NCP20* exists.

Identification of native tachyzoite antigen encoded by NCP20

cDNA sequence analysis predicted the presence of three potential start codons in *NCP20*. Consequently, primers were designed to PCR amplify the ORF from each of these potential start codons and the products were cloned individually, in-frame into pTRcHis. *E. coli* containing PCR product derived from primers P20-ATG3F and P20-pTrcR, encoding the shortest ORF (from 170 through to 480), expressed a protein with a mobility of approximately 30 kDa which could be purified from bacterial extracts by chromatography. No recombinant protein was obtained from *E. coli* containing the other two constructs. Injection of purified protein into QS mice generated IgG antibodies which detected, by immunoblotting, a 20 kDa tachyzoite antigen in NC-SweB1. No antigen was detected by this pooled sera in extracts of NC-Liverpool.

It is believed that NCP20 is encoded by the ORF spanning positions 160 to 480. Translation of this ORF in the DNA sequence of *NCP20* gives a protein of approximately 11.8 kDa (with an isoelectric point of 11.04) containing 107 amino acids (Figure 2) which is predicted to be rich in alanine, leucine and arginine. A prediction of the protein structure indicated NCP20 probably contains several helical regions separated by turns.

Vaccination experiment

The statistical analysis was a one-factor analysis of variance, with the various treatments as the factor. The data analysed were the change in mouse weight through time, calculated using a separate linear regression for each mouse (Figure 3). Only mice with data for all of the days were used in the analysis. The significant factor ($P<0.001$) indicated that the mice change weight differently among some of the groups. A multiple comparison test showed that groups 1 (FCA) and 2 (FCA+NCP20) were not different from 10 (the negative control) in that they maintained their body weight close to their starting weight. Mice in all the other groups behaved like those in group 9 (the positive control) in that they lost weight over the course of the experiment. Thus it was concluded that injection of mice with either FCA with or without recombinant NCP20 provided complete protection against weight loss that normally occurs during an acute infection of *N. caninum*. All mice infected with *N. caninum* showed clinical signs associated with the infection, which were a ruffled coat from day 14 post infection.

Enzyme-linked immunosorbent assay using NCP20

Figure 4 shows the results obtained by ELISA using recombinant NCP20 and three test sera from mice. The negative control serum is derived from a pregnant QS mouse injected with saline at day 8 of gestation whereas the other 2 sera (labeled positive sera) are from pregnant QS mice injected subcutaneously at day 8 of gestation with 1 million tachyzoites of NC-Liverpool. All mice were bled from the heart on day 21 of gestation and sera prepared by standard procedures. The antibody response to NCP20 in the pregnant mice receiving NC-Liverpool, as determined from the ELISA OD readings, was significantly higher than that found in the pregnant mouse receiving saline, thereby indicating the ELISA detected IgG antibodies in those mice infected with *N. caninum*.

DISCUSSION

The isolation and characterisation of a new gene from *N. caninum*, called *NCP20* is reported. The gene was isolated by modifying a strategic immunoscreening technique. The immunoscreening strategy used was devised in order to avoid isolating antigen sequences that are cross-reactive to the dense granule antigen NCDG1 of *N. caninum* which is highly immunogenic. Consequently, antigens blotted onto PVDF membranes that were smaller than 30 kDa were used to select antibody from pooled sera of mice made resistant to a lethal challenge of NC-Liverpool by previous infection with NC-SweB1. The selected antibody was then used to immunoscreen the cDNA expression library.

Two genes, previously undescribed from *N. caninum* were isolated by immunoscreening, one of which was homologous to the *GRA*1 gene of *T. gondii* which is described elsewhere. *GRA*1 was the first dense granule antigen of *T. gondii* cloned because it formed a major reactive component of the excretory/secretory fraction from *T. gondii* (Darcy *et al.* 1988, *Parasite Immunology* **10**, 553-567; Cesbron-Delauw *et al.* 1989, *Proceedings of the National Academy of Sciences of the United States of America* **86**, 7537-7541). This fraction contains antigens which induce a protective antibody response and thus prompted the isolation and characterisation of many dense granule antigens from *T. gondii* to date. Homologues of four of the genes coding for these antigens have now been described for *N. caninum*: NC*GRA*1, NC*GRA*2, NC*GRA*6 and NC*GRA*7.

*GRA*1 is released from bradyzoites of *T. gondii* and so is thought to be a marker of chronic infection in toxoplasmosis. In *N. caninum*, a similar function may also apply to NcGra1 and NCP20 since the sera used for the isolation of these clones was derived from mice made resistant to an acute infection of NC-Liverpool by prior infection with NC-SweB1.

The gene encoding NCP20 has not been previously described in any organism. The NCP20 cDNA contained three potential initiation codons, all in the same reading frame. Expression in *E. coli* demonstrated that the third AUG gave rise to a gene product which induced an antibody that recognised the native protein, thereby confirming the reading frame. However, the context of the second AUG codon in the *NCP20* mRNA at position 160 (5'TGCAAAATGG3') is very similar to the consensus sequence derived for the *T. gondii* translation initiation site (5'GNCAAAATGG3': Seeber 1997,

Parasitology Research **83**, 309-311). This leads to the belief that in *N. caninum*, position 160 is the real start of translation initiation for NCP20. This conclusion is also supported by the observation that the ATG codon beginning at position 160 is also conserved in many of the *T. gondii* ESTs analysed, whereas the codon beginning at 175 is not.

NCP20 is a relatively small polypeptide (approximately 12 kDa) which was highly expressed in *E. coli*. Western blot analysis using *N. caninum* extracts demonstrated the size of the native tachyzoite antigen to be approximately 20 kDa. The discrepancy between the predicted and actual sizes of the native NCP20 is presumed to result from some kind of post-translational modification which occurs in the parasite. A homologue of *NCP20* was detected in *T. gondii* by searching the GenBank database with the *NCP20* sequence. Other parasite taxa, such as *Neospora hughesi, Hammondia heydorni* and *Hammondia hammondi* are therefore also expected to contain homologues of *NCP20*, because these taxa are very closely related to *N. caninum*. As the present inventors have shown that the NCP20 protein is suitable to raise high titres of protective antibodies in mice, it will be expected that the protein will act similarly in other animals upon immunisation.

The high prevalence of *N. caninum* in cattle along with its high rate of congenital transmission suggests the development of vaccines are warranted. Prior evidence suggests a role for low molecular weight antigens during the induction of cellular immunity in neosporosis. Antigens in the 15-30 kDa range induce proliferation of $CD4^+$ cells thereby stimulating increased production of gamma interferon which in turn suppresses the growth of *N. caninum* tachyzoites and encourages resistance against *N. caninum*. These low molecular weight antigens also appear more immunogenic in immune mice and outbred (QS strain) mice resistant to clinical disease. Thus low molecular weight antigens such as NCP20 may well have a role in inducing cell mediated immunity against *N. caninum*.

Recombinant NCP20, purified from *E. coli*, was evaluated in a variety of vaccine formulations for its ability to protect susceptible mice against an acute infection of *N. caninum*. Freund's complete adjuvant, with or without recombinant NCP20, was able to provide complete protection against weight loss associated with the acute infection of *N. caninum*.

In summary, a new gene of *N. caninum* have been isolated and characterised, called *NCP20*. Its isolation is significant because this is the first report of using sera, from animals shown to be resistant to an acute infection with *N. caninum*, for immunoscreening of cDNA expression libraries.

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.

CLAIMS:

1. An isolated polynucleotide molecule encoding a *Neospora caninum* antigen, the polynucleotide molecule comprising a DNA sequence as set out in Figure 1 (SEQ ID NO: 1), or a polynucleotide molecule which hybridises under stringent conditions to the DNA sequence as set out in Figure 1 (SEQ ID NO: 1).

2. The isolated polynucleotide molecule according to claim 1 comprising the DNA sequence as set out in Figure 1 (SEQ ID NO: 1).

3. An isolated *N. caninum* polypeptide encoded by the isolated polynucleotide molecule according to claim 1 or 2.

4. The isolated *N. caninum* polypeptide according to claim 3 comprising the amino acid sequence as shown in Figure 2 (SEQ ID NO: 2), functionally equivalent polypeptides, or immunogenic fragments thereof.

5. The isolated *N. caninum* polypeptide according to claim 4 comprising the amino acid sequence as shown in Figure 2 (SEQ ID NO: 2).

6. A composition for use in raising an immune response in a mammal against neosporosis, the composition comprising a carrier and a polypeptide having an amino acid sequence as shown in Figure 2 (SEQ ID NO: 2), functionally equivalent polypeptides, or immunogenic fragments thereof.

7. The composition according to claim 6 wherein the polypeptide comprises the amino acid sequence as shown in Figure 2 (SEQ ID NO: 2).

8. The composition according to claim 6 or 7 further comprising an adjuvant.

9. The composition according to claim 8 wherein the adjuvant is selected form the group consisting of Freund's complete adjuvant, Freund's incomplete adjuvant, glucosaminylmuramyl dipeptide, Quil A, DEAE Dextran/mineral oil, Alhydrogel, Auspharm adjuvant, and Algammulin.

10. A method of obtaining a protective effect against neosporosis in a mammal, the method comprising inoculating the mammal with a polypeptide according to any one of claims 3 to 5.

11. A method of obtaining a protective effect against neosporosis in a mammal, the method comprising inoculating the mammal with a composition according to any one of claims 6 to 9.

12. Use of the polypeptide according to any one of claims 3 to 5 in methods for detecting antibodies reactive or specific to *N. caninum*.

13. The use according to claim 12 comprising assaying for *N. caninum* antibodies in a serum or blood sample from a mammal that bind to the polypeptide and detection of antibodies being indicative of exposure to and/or infection of that animal with *N. caninum*.

14. An isolated antibody, or fragment thereof, reactive to the polypeptide according to any one of claims 3 to 5.

15. A vector comprising the polynucleotide molecule according to claim 1 or 2 for replication and/or expression of the polynucleotide molecule in a host prokaryotic or eukaryotic cell.

16. The vector according to claim 15 selected from the group consisting of plasmid, virus, and phage.

17. The vector according to claim 16 further comprising an origin of replication, a promoter for the expression of the polynucleotide and a regulator of the promoter.

18. A host cell transformed or transfected with the vector according to any one of claims 15 to 17.

19. An oligonucleotide probe or primer comprising a nucleotide sequence that hybridises selectively to the polynucleotide molecule according to claim 1 or 2.

20. The oligonucleotide probe or primer according to claim 19 comprising at least 15 nucleotides, preferably at least 18 nucleotides, and more preferably at least 25 nucleotides.

```
1     atgttttgtc aggtgttctt gtgccgaaaa cgacgcccct ccgcgtaccc gttttcgcta
61    gtctctattc cagtaggccg ttctgaatcc gcatcgccgg ataacgtatc tcgacgtttg
121   gcggggcgga cgtctcggct tttgtatttt gtgtgcaaaa tggctttgtc tacgatgaac
181   aagcccgggc cgtttagacg gttgttgggt tatggtctgc tgcttggcgc cgttgtgctc
241   gaagcggcat ttgacctcag cgctcctgcg gaggctgtgg cgctccgaag actagaccaa
301   aaggaaactg tccaggcttt agtggaacag cacaggtttt ctaacgatta cgatcaggag
361   gcccgagtac agaaggcgcc gccaggaact gggaagtcag actccagaag agatcgagga
421   agcaaaacgc aagtacccgc aagcaggtgc ttaaggaaca acaagaagat gaggaattga
481   aaaaaaagac agatgcggtc attgaagagc tgaaaaagac agcagaagag agaggacttc
541   gtcggtacgc cgagcgtgat gaagatcgca ctgacgacca gcagatggat tttgagacac
601   ggcagcggga actcagaaac atggattcag caacaaaagc gcagcttttg aagcagagac
661   agaaagaaaa tgaagagagg aaccgcgtga agcgaaacag cgatgacgtc atggcggagc
721   tcaagcagaa actcgcggcc cgcaagaagg caatgtagaa gacggcaatt cctgctggga
781   aaatgaagtg ttagaggagt gcgttgtcgc cgtctatggc ggaaggttgt tacccagtga
841   gaagtgtctt ttccatttag caggaatgtg gctttatgtg tatataatgt agatactcga
901   gagagggtta gtgccggaaa ccggatgggg tatagaagtt gcagtgcacc actggcttcg
961   acgtaagtgc ctccgccgaa tacgttcttc tgggcgagtc gtctctagtt ggcgttacgt
1021  aratgcgaaa agaacgttaa cctcgacctc gaaagcatcc tcgagggaag ttcaatgact
1081  tcggtctc
```

Figure 1

MALSTMNKPGPFRRLLGYGLLLGAVVLEAAFDLSAPAEAVALRRLDQKETVQALVQHRF

SNDYDQEARVQKAPPGTGKSDSRRDRGSKTQVPASRCLRNNKKMRN

Figure 2

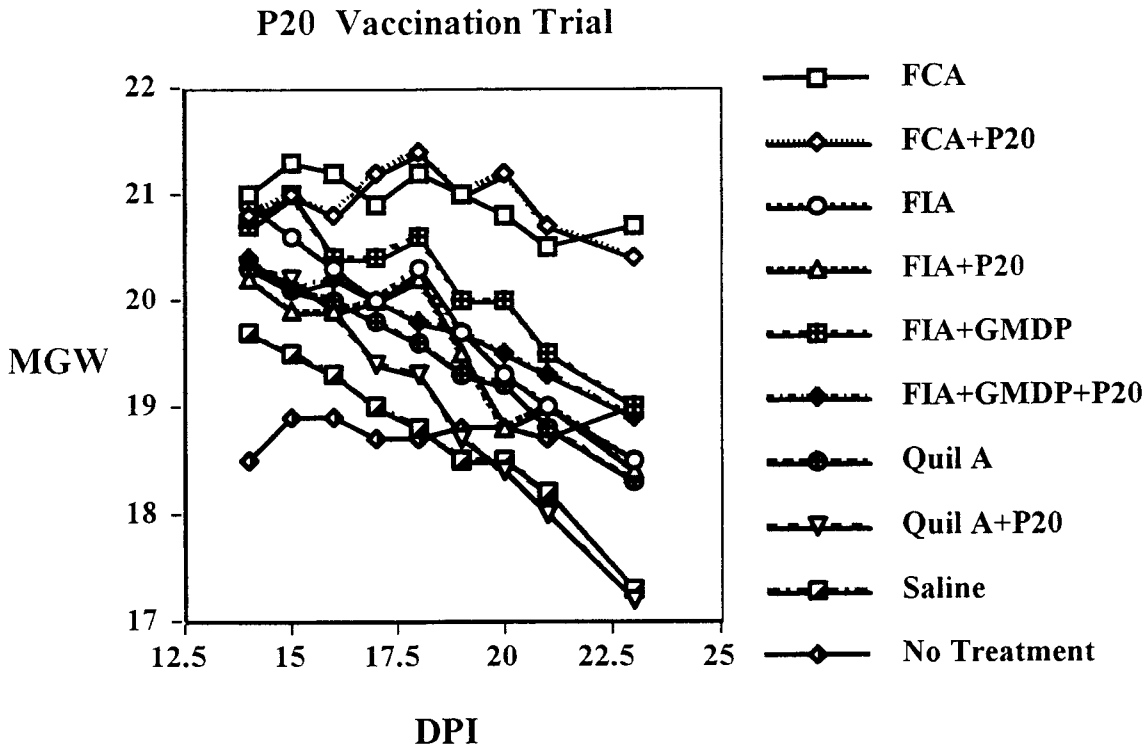
P20 Vaccination Trial
MGW
22
21
20
19
18
17
12.5
15
17.5
20
22.5
25
DPI
FCA
FCA+P20
FIA
FIA+P20
FIA+GMDP
FIA+GMDP+P20
Quil A
Quil A+P20
Saline
No Treatment

Figure 3

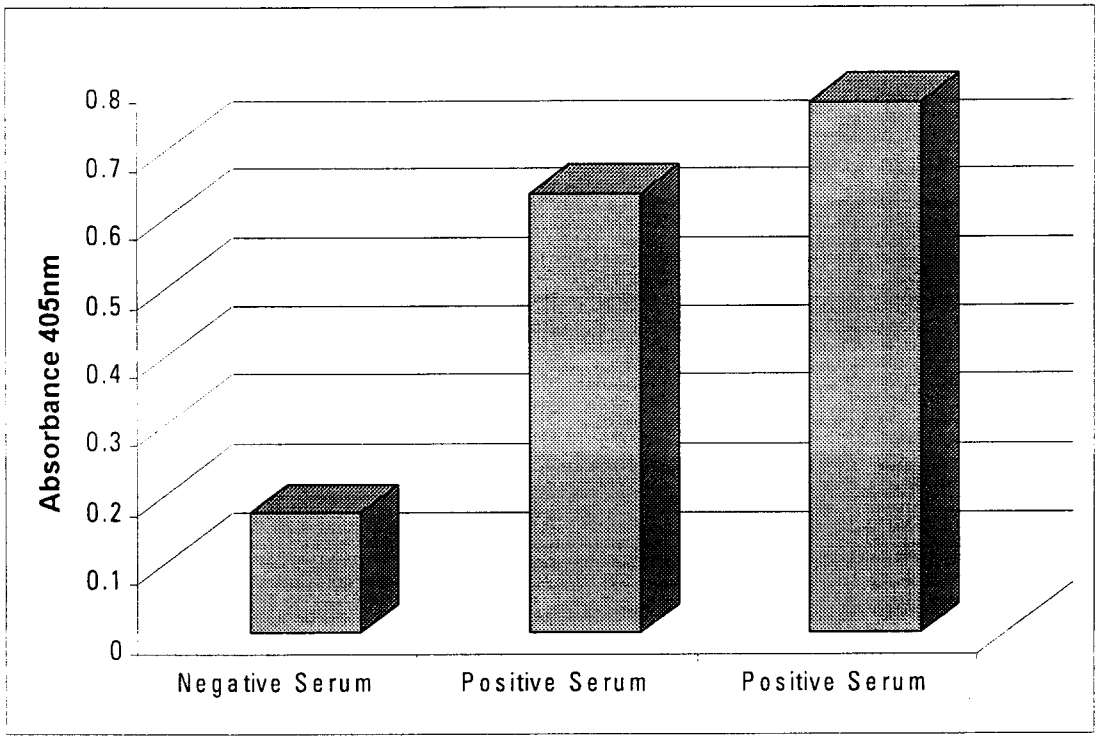
Absorbance 405nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Negative Serum
Positive Serum
Positive Serum

Figure 4

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Insearch Limited
(B) STREET: Level 2, 187 Thomas Street
(C) CITY: Haymarket
(D) STATE: New South Wales
(E) COUNTRY: Australia
(F) POSTAL CODE (ZIP): 2000

(ii) TITLE OF INVENTION: Parasite Antigens

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1087 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGTTTTGTC AGGTGTTCTT GTGCCGAAAA CGACGCCCCT CCGCGTACCC GTTTTCGCTA 60

GTCTCTATTC CAGTAGGCCG TTCTGAATCC GCATCGCCGG ATAACGTATC TCGACGTTTG 120

GCGGGGCGGA CGTCTCGGCT TTTGTATTTT GTGTGCAAAA TGGCTTTGTC TACGATGAAC 180

AAGCCCGGGC CGTTTAGACG GTTGTTGGGT TATGGTCTGC TGCTTGGCGC CGTTGTGCTC 240

GAAGCGGCAT TTGACCTCAG CGCTCCTGCG GAGGCTGTGG CGCTCCGAAG ACTAGACCAA 300

AAGGAAACTG TCCAGGCTTT AGTGGAACAG CACAGGTTTT CTAACGATTA CGATCAGGAG 360

GCCCGAGTAC AGAAGGCGCC GCCAGGAACT GGGAAGTCAG ACTCCAGAAG AGATCGAGGA 420

AGCAAAACGC AAGTACCCGC AAGCAGGTGC TTAAGGAACA ACAAGAAGAT GAGGAATTGA 480

AAAAAAAGAC AGATGCGGTC ATTGAAGAGC TGAAAAAGAC AGCAGAAGAG AGAGGACTTC 540

GTCGGTACGC CGAGCGTGAT GAAGATCGCA CTGACGACCA GCAGATGGAT TTTGAGACAC 600

GGCAGCGGGA ACTCAGAAAC ATGGATTCAG CAACAAAAGC GCAGCTTTTG AAGCAGAGAC 660

AGAAAGAAAA TGAAGAGAGG AACCGCGTGA AGCGAAACAG CGATGACGTC ATGGCGGAGC 720

TCAAGCAGAA ACTCGCGGCC CGCAAGAAGG CAATGTAGAA GACGGCAATT CCTGCTGGGA 780

AAATGAAGTG TTAGAGGAGT GCGTTGTCGC CGTCTATGGC GGAAGGTTGT TACCCAGTGA 840

GAAGTGTCTT TTCCATTTAG CAGGAATGTG GCTTTATGTG TATATAATGT AGATACTCGA 900

GAGAGGGTTA GTGCCGGAAA CCGGATGGGG TATAGAAGTT GCAGTGCACC ACTGGCTTCG 960

ACGTAAGTGC CTCCGCCGAA TACGTTCTTC TGGGCGAGTC GTCTCTAGTT GGCGTTACGT
1020

AATGCGAAAA GAACGTTAAC CTCGACCTCG AAAGCATCCT CGAGGGAAGT TCAATGACTT
1080

CGGTCTC
1087

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 225 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ala Thr Gly Thr Thr Thr Thr Gly Thr Cys Ala Gly Gly Thr Gly Thr
1 5 10 15

Thr Cys Thr Thr Gly Thr Gly Cys Cys Gly Ala Ala Ala Ala Cys Gly
20 25 30

Ala Cys Gly Cys Cys Cys Cys Thr Cys Cys Gly Cys Gly Thr Ala Cys
35 40 45

Cys Cys Gly Thr Thr Thr Thr Cys Gly Cys Thr Ala Gly Thr Cys Thr
50 55 60

Cys Thr Ala Thr Thr Cys Cys Ala Gly Thr Ala Gly Gly Cys Cys Gly
65 70 75 80

Thr Thr Cys Thr Gly Ala Ala Thr Cys Cys Gly Cys Ala Thr Cys Gly
85 90 95

Cys Cys Gly Gly Ala Thr Ala Ala Cys Gly Thr Ala Thr Cys Thr Cys
100 105 110

Gly Ala Cys Gly Thr Thr Thr Gly Met Ala Leu Ser Thr Met Asn Lys
115 120 125

Pro Gly Pro Phe Arg Arg Leu Leu Gly Tyr Gly Leu Leu Leu Gly Ala
130 135 140

Val Val Leu Glu Ala Ala Phe Asp Leu Ser Ala Pro Ala Glu Ala Val
145 150 155 160

Ala Leu Arg Arg Leu Asp Gln Lys Glu Thr Val Gln Ala Leu Val Gln
165 170 175

His Arg Phe Ser Asn Asp Tyr Asp Gln Glu Ala Arg Val Gln Lys Ala
180 185 190

Pro Pro Gly Thr Gly Lys Ser Asp Ser Arg Arg Asp Arg Gly Ser Lys
195 200 205

Thr Gln Val Pro Ala Ser Arg Cys Leu Arg Asn Asn Lys Lys Met Arg
210 215 220

Asn
225

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACGTATGGAT CCGTTTTGTC AGGTGTTCTT G 31

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ACGTATGGAT CCGGCTTTGT CTACGATGAA C 31

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ACGTATGGAT CCGAACAAGC CCGGGCCGTT T 31

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ACGCATGAAT TCTGTTTCTG AGTTCCCGCT 30

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAGGTATATA TTAATGTATC G 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 00/00354

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[7]: C07K 14/44, 16/20; C07H 21/04; C12N 1/21, 15/63; G01N 33/536; A61K 38/17, 39/012; A61P 33/00, 33/10.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
ANGIS-BLASTP, FASTA (Seq. Id. No. 2 and Figure 2)
STN Subsequence search (Figure 2)
STN (CA, Medline, Biosis, WPIDS) - Keywords: neospor?, caninum, antigen?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, A	WO 99/61046 (Insearch Limited) 2 December 1999.	1-20
P, A	International Journal for Parasitology, Volume 29, 1999, (A. Hemphill et al), "The Antigenic Composition of *Neospora caninum*", pages 1175-1188.	1-20
A	Infection and Immunity, Volume 66, 1998, (D. K. Howe et al.), "The p29 and p35 Immunodominant Antigens of *Neospora caninum* Tachyzoites are Homologous to the Family of Surface Antigens of Toxoplasma Gondii", pages 5322-5328.	1-20

☐ 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

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"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
11 May 2000

Date of mailing of the international search report
24 MAY 2000

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU 00/00354

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

Patent Document Cited in Search Report		Patent Family Member	
WO	99/61046	AU	41229/99

END OF ANNEX

专利名称(译)	寄生虫抗原		
公开(公告)号	EP1179016A4	公开(公告)日	2003-05-28
申请号	EP2000918591	申请日	2000-04-20
[标]申请(专利权)人(译)	悉尼理工大学		
申请(专利权)人(译)	技术，悉尼大学		
当前申请(专利权)人(译)	技术，悉尼大学		
[标]发明人	ELLIS JOHN TIMOTHY ATKINSON ROBERT RYCE CHERYL LOUISE		
发明人	ELLIS, JOHN, TIMOTHY ATKINSON, ROBERT RYCE, CHERYL, LOUISE		
IPC分类号	A61K39/00 A61P33/00 A61P33/10 C07K14/44 C07K16/20 C12N1/21 A61K38/17 A61K39/012 C07H21/04 C12N15/63 G01N33/536		
CPC分类号	C07K16/20 A61K39/00 C07K14/44		
优先权	1999PP9928 1999-04-21 AU		
其他公开文献	EP1179016A1		
外部链接	Espacenet		

摘要(译)

本发明涉及编码新孢子虫抗原的分离的多核苷酸分子，分离的犬新孢子虫多肽，多肽的抗体以及多核苷酸分子和多肽用于免疫哺乳动物抗新孢子虫病的用途。