Nucleic Acids Encoding *Sarcocystis Neurona* Antigen and Uses Thereof

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NUCLEIC ACIDS ENCODING SARCOCYSTIS NEURONA ANTIGEN AND USES THEREOF

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Field of Classification Search .......... 435/320.1; 536/23.7, 24.1, 24.2, 24.32 See application file for complete search history.

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Attorney, Agent, or Firm—King & Schickli, PLLC

ABSTRACT

The present invention provides novel isolated nucleic acids encoding antigenic proteins derived from Sarcocystis neurona, or unique fragments thereof. In particular, the invention provides novel isolated nucleic acids encoding membrane-associated polypeptides SnsAG2, SnsAG3, and SnsAG4. Also provided are purified antigenic polypeptide fragments encoded by the novel nucleic acid sequences set forth herein that encode for SnsAG2, SnsAG3, and SnsAG4. Also provided are isolated nucleic acids capable of selectively hybridizing with the nucleic acid from Sarcocystis neurona. The invention also provides vectors comprising the nucleic acids of the invention encoding an antigenic protein derived from Sarcocystis neurona or a unique fragment thereof and provides the vector in a host capable of expressing the polypeptide encoded by that nucleic acid. Finally, the invention provides purified polyclonal and/or monoclonal antibodies specifically reactive with Sarcocystis neurona and a method of detection of Sarcocystis neurona utilizing the antibodies of the invention.

17 Claims, 8 Drawing Sheets
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**FIG. 2**
FIG. 3
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NUCLEIC ACIDS ENCODING SARCOCYSTIS NEURONA ANTIGEN AND USES THEREOF

The present application is a continuation of U.S. utility patent application Ser. No. 10/569,430, filed on Feb. 19, 2003 now U.S. Pat. No. 7,056,733, which claims the benefit of priority of U.S. provisional patent application No. 60/357,479, filed Feb. 15, 2002, the disclosures of which are incorporated herein in their entirety by reference.

TECHNICAL FIELD

The present invention relates to nucleic acids of Sarcocystis neurona. In particular, the present invention relates to nucleic acids of Sarcocystis neurona and to nucleic acid reagents and antibodies for use in methods of detection and prevention of Sarcocystis neurona infection. More particularly, the present invention relates to novel nucleic acid sequences of Sarcocystis neurona and to utilization thereof including primers, probes, antigen/antibody diagnostic kits, vectors for production of peptides, encoding the novel nucleic acids, and to antigenic proteins and vaccines against Sarcocystis neurona.

BACKGROUND OF THE INVENTION

Sarcocystis neurona is an apicomplexan parasite that is the primary cause of equine protozoal myeloencephalitis (EPM; Dubey et al., 1991), which is a common and debilitating infectious disease that affects the central nervous system of horses. S. neurona is related to the human and animal pathogen Toxoplasma gondii and to the important veterinary pathogen Neospora spp. The geographic range of S. neurona appears to be limited to the western hemisphere, thus EPM primarily affects horses in the Americas.

Definitive antemortem diagnosis of EPM remains exceedingly difficult, for a variety of reasons. Horses afflicted with EPM exhibit signs that are similar to a number of different neurological disorders (MacKay et al., 2000). Furthermore, S. neurona infection does not equate to disease, since only a small proportion of seropositive horses will suffer from EPM (MacKay et al., 2000); as a consequence, the detection of anti-S. neurona antibodies in serum provides little diagnostic information other than indicating previous exposure to the parasite. Analysis of cerebrospinal fluid (CSF) to reveal intrathecal antibody production has improved the predictive value of antibody detection for EPM diagnosis. However, interpretation of CSF antibody presence can be confounded by contamination of the CSF sample with minute amounts of serum antibodies (Miller et al., 1999).

Other contemporary diagnostic assays provide only mediocre predictive value for EPM diagnosis. Western blot analysis (a.k.a., immunoblot) of crude S. neurona lysate remains the principal immunodiagnostic test that is used to detect antibodies in suspect EPM horses (Granstrom et al., 1993). The assay relies on the recognition of several antigens, primarily in the low molecular weight range, by serum/CSF antibodies (Dubey et al., 2001b; Granstrom et al., 1993; MacKay et al., 2000). Unfortunately, Western blot analysis is primarily a research tool that is relatively laborious and somewhat hindered by subjectivity, so any improvements to the immunoblot are of limited value. While the immunoblot has been utilized for a number of years to help diagnose EPM, it is a first-generation test that needs to be replaced with improved assays based on simplified, and thus more reliable, techniques that are more appropriate for diagnostic use.

Nucleic acid amplification assays (polymerase chain reaction; PCR) for S. neurona detection have been developed based on the S. neurona ribosomal RNA genes (Fenger et al., 1994; Marsh et al., 1996). These PCR-based assays detect the presence of S. neurona DNA, and therefore the parasite, in the horse, so they can provide a definitive indication of active infection. However, prior to the present invention, these nucleic acid-based tests have been inherently unreliable. Specifically, parasites may be very few or non-existent in a CSF sample, so there will be few or no available target molecules (i.e., parasite genomic DNA) for PCR amplification. More importantly, the general use of PCR for diagnosis is still suspect. Although measures can be taken to improve the reliability of PCR, the technique continues to be troubled by both false positive and false negative results.

The selection of an antigen for development of a diagnostic test can be somewhat subjective since any particular pathogen is composed of numerous antigenic proteins. Logically, the target molecule in a diagnostic assay must elicit a detectable antibody response in the infected animal. In this regard, surface antigens of the Coccidia, such as the primary surface antigens of Toxoplasma gondii (Handman and Remington, 1980; Sharma et al., 1983) and Neospora caninum (Howe et al., 1998), are exceedingly immunogenic. These surface antigens have been designated SAGs and SAG-related sequences (SRSs). Significantly, the TgSAG1 surface antigen of T. gondii has been shown to protect mice against acute toxoplasmosis (Bulow and Boothroyd, 1991), and the NcSAG1 (p29) major surface antigen of N. caninum has been used to develop an ELISA for detection of Neospora infection in cattle (Howe et al., 2002). Collectively, these previous studies demonstrate that coccidian SAGs are at least candidate proteins for the development of both diagnostic assays and protective vaccines.

Despite the foregoing art, prior to the present invention it had not been shown that the surface antigens of S. neurona (i.e., SnSAG2, SnSAG3, and SnSAG4) are effective target molecules for examining immune responses in infected horses and for developing improved assays for EPM diagnosis. Such molecules would also provide the basis for improved vaccines and diagnostic kits, including antigen and antibody kits, for fast and reliable diagnosis of S. neurona infection.

SUMMARY OF THE INVENTION

The present invention satisfies the aforementioned need in the art by providing a novel isolated nucleic acid encoding an antigenic protein derived from Sarcocystis neurona, or a unique fragment thereof. In one embodiment, the invention provides novel isolated nucleic acids encoding membrane-associated polypeptides SnSAG2, SnSAG3, and SnSAG4.

The present invention also provides purified antigenic polypeptide fragments encoded by the novel nucleic acid sequences set forth herein that encode for Sarcocystis neurona. In one embodiment, the invention provides purified antigenic proteins or purified antigenic polypeptide fragments encoded by the novel nucleic acid sequences set forth herein that encode for SnSAG2, SnSAG3, and SnSAG4. In another embodiment, the present invention provides a purified antigenic polypeptide fragment encoded by the nucleic acid sequences set forth herein or a selective portion thereof, in a pharmaceutically acceptable carrier.

The present invention also provides isolated nucleic acids capable of selectively hybridizing with the nucleic acid from Sarcocystis neurona including, but not limited to, primers and probes for utilization in polymerase chain reaction
(PCR) and other nucleic acid amplification techniques. The isolated nucleic acids of the present invention are capable of hybridizing under conditions of low, moderate, and high stringency with a nucleic acid from Sarcozystis neurona.

Further, the present invention provides vectors comprising the isolated nucleic acids, or degenerate variants thereof, set forth herein encoding Sarcozystis neurona or a unique fragment thereof and provides the vector in a host capable of expressing the polypeptide encoded by that nucleic acid.

Still yet further, the present invention also provides a purified polyclonal and/or a monoclonal antibody specifically reactive with Sarcozystis neurona and a method of detection of Sarcozystis neurona utilizing the antibodies of the present invention.

The above-described embodiments provided by the present invention, provide a method for detecting Sarcozystis neurona in a biological sample, comprising detecting the presence in the sample of an antibody or fragment thereof which specifically binds to a polypeptide comprising an isolated amino acid sequence selected from the group set forth in the Sequence Listing as SEQ ID NO: 24, SEQ ID NO: 26, and SEQ ID NO: 28. In one embodiment of the method, the biological sample is serum. In another embodiment, the present invention provides a method as described for detecting Sarcozystis neurona in cerebrospinal fluid (CSF).

Finally, the present invention provides a kit for detecting Sarcozystis neurona in a biological sample, comprising at least one isolated amino acid sequence selected from the group set forth in the Sequence Listing as SEQ ID NO: 24, SEQ ID NO: 26, and SEQ ID NO: 28, and a reporter molecule for detecting a first antibody or fragment thereof which specifically binds to a polypeptide comprising the at least one isolated amino acid sequence. The reporter molecule may be any suitable detectable second antibody or fragment thereof, which binds to the first antibody or fragment thereof, and which is labeled with a detectable moiety or bound to a substrate.

FIG. 3 shows a Western blot analysis of the SnSAGs in S. neurona merozoites. The SnSAG genes were expressed in E. coli, and monospecific polyclonal antisera were generated against the recombinant proteins. Western blot analysis of reduced antigen revealed that each SnSAG migrated significantly higher than its predicted molecular weight, consistent with what has been observed for the T. gondii SAGs/SRSs, SnSAG1 and SnSAG4 co-migrated and corresponded to the immunodominant band at about 30-32 kDa. SnSAG2 corresponded to an immunodominant band at approximately 18-20 kDa.

FIG. 4 shows the SnSAGs are membrane-associated in Sarcozystis neurona merozoites. Triton X-114 partitioning assays indicated that the SnSAGs are associated with membranes, consistent with their surface localization via glycolipid anchoring. Western blot analysis of the partitioned proteins with the SnSAG-specific polyclonal antisera revealed that all four SnSAGs were separated exclusively into the detergent phase (D). The control protein, SnMIC10, was partitioned into the aqueous phase (A), as expected.

FIG. 5 shows that the four SnSAGs are displayed on the surface of Sarcozystis neurona merozoites. Surface biotinylation of S. neurona merozoites indicated that the four SnSAGs are displayed on the surface of the parasite. Western blot analysis with the SnSAG-specific antisera revealed each of the SnSAGs in the biotinylated protein fraction precipitated with immobilized streptavidin. The SnSAGs were not present in the non-labeled parasites, thus indicating that the streptavidin precipitation was specific for biotin-labeled proteins. The negative control protein (actin) was not detected in the biotin-labeled/streptavidin-precipitated protein fraction.

FIG. 6 shows reciprocal antibody titers in serum of EPM-confined horses, determined by an ELISA using the recombinant surface antigens of the present invention.

FIG. 7 shows reciprocal antibody titers in CSF of EPM-confined horses, determined by an ELISA using the recombinant surface antigens of the present invention.

FIG. 8 shows expression of S. neurona surface antigens in COS-1 (green monkey kidney) cells, detected by immunofluorescent labeling with fluorescein isothiocyanate.

DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the examples included therein. As used in the claims, “a” can mean one or more. As can be appreciated by one of skill in the art, methods and materials similar or equivalent to those described herein can be used in the practice of the present invention. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety in order to more fully describe the state of the art to which this invention pertains. It is noted that the abbreviated citations of literature referenced herein are set forth fully in U.S. patent application Ser. No. 10/369,430, the disclosure of which is also incorporated herein in its entirety by reference.

Although the present invention has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims. In the case of a conflict with incorporated references, the present specification, including definitions, will control.
In addition, the particular embodiments discussed below are illustrative only and not intended to be limiting.

The present invention satisfies the long felt need in the art by providing novel isolated nucleic acid sequences which encode antigenic proteins derived from *Sarcocystis neurona*, or which encode unique antigenic protein fragments thereof. As used herein, a “nucleic acid” means a chain of at least two or more nucleotides such as DNA (deoxyribonucleic acid) or RNA (ribonucleic acid). As used herein, a “purified” nucleic acid is one that is substantially separated from other nucleic acid sequences in a cell or organism in which the nucleic acid naturally occurs. Likewise, by “isolated” nucleic acid is meant separated from at least some of other nucleic acids found in the naturally-occurring organism. The nucleic acids of the present invention can include positive and negative strand RNA as well as DNA. The above terms encompass double-stranded DNA, single-stranded DNA, and RNA and are meant to include genomic and subgenomic nucleic acids found in the naturally-occurring *Sarcocystis neurona* organism. The nucleic acids contemplated by the present invention include a nucleic acid having sequences from which a *Sarcocystis neurona* cDNA can be transcribed; or allelic variants and/or homologs of thereof. By “capable of selectively hybridizing” is meant a sequence which does not hybridize with other nucleic acids to prevent an adequate positive hybridization with nucleic acids from *Sarcocystis neurona* and is meant to include stringent hybridization conditions including low, moderate and high stringency conditions. Such stringency conditions are known in the art, e.g., in US Patent Publication No.: 2002/0115828 A1. By “unique fragment” is meant a fragment of the nucleic acids set forth in the Sequence Listing that is less than the full length that can selectively hybridize with a RNA, DNA or cDNA sequence derived from the novel sequences set forth herein or that can selectively hybridize with nucleic acids from *Sarcocystis neurona*. Modifications to the nucleic acids of the invention are also contemplated as long as the essential structure and function of the polypeptide encoded by the nucleic acids is maintained. Likewise, fragments used as primers or probes can have substitutions so long as enough complementary bases exist for selective hybridization (Kunkel et al. Methods Enzymol. 1987: 154-367, 1987). As one of skill in the art can appreciate, there can be naturally occurring allelic variants and non-naturally occurring variants or modifications of the nucleic acids of the invention. For example, homologs or naturally occurring allelic variants of the nucleic acids of the invention having from about 50% and up to about 99% sequence identity are contemplated by the invention. Likewise, it is contemplated that non-naturally occurring variants or modifications of the nucleic acids of the invention can range from about 50% to about 99% sequence identity to native *S. neurona* are contemplated.

In particular, one embodiment of the present invention provides isolated nucleic acids derived from *Sarcocystis neurona* cluster sequences, namely Sn Cluster 144, Sn Cluster 21 and Sn Cluster 4, which comprise the nucleotide sequences set forth in the Sequence Listing as SEQ ID NO: 1, 3, and 29 respectively and the sequences complimentary thereto. Also provided by the invention are the corresponding protein or polypeptide amino acid sequences for these three *Sarcocystis neurona* cluster sequences. The polypeptide sequence comprising Sn Cluster 144 is set forth in the Sequence Listing as SEQ ID NO: 2. The polypeptide sequence comprising Sn Cluster 21 is set forth in the Sequence Listing as SEQ ID NO: 4 and the polypeptide sequence comprising Sn Cluster 4 is set forth in the Sequence Listing as SEQ ID NO: 30. As used herein, the terms “polypeptide” and “protein” are used interchangeably and are meant to include any peptide-linked chain of amino acids, regardless of length or post-translational modification, e.g., glycosylation or phosphorylation. By “purified” polypeptide is meant a polypeptide that has been substantially purified and isolated away from other polypeptides in a cell, organism, or mixture in which the polypeptide occurs.

Similar to other members of the Apicomplexa, *S. neurona* is an obligate intracellular pathogen that utilizes a number of unique structures and molecules (i.e., virulence factors) to support its parasitic lifestyle. Parasite surface molecules are virulence factors that are typically novel and undoubtedly important since they are responsible for the initial interactions with the host cell surface and host immune response. In *Toxoplasma gondii*, for example, an extensive family of 25+ surface antigens has been identified, which are developmentally regulated and exhibit various levels of sequence similarity to either of the major *T. gondii* surface antigens TgSAG1 or TgSAG2. These surface molecules appear to be involved in receptor/ligand interactions with the host cell surface, and there is increasing evidence that some of the *T. gondii* SAGs are involved in modulation of host immune responses.

In one embodiment, the present invention provides identity and characterization of certain of the virulence factors of *S. neurona*. In particular, the present invention provides four isolated nucleic acids of *S. neurona* (genes) that encode parasitic surface antigens. A sequencing project was conducted that generated approximately 8500 expressed sequence tags (ESTs) from this organism. Examination of this sequence database has revealed a family of at least four *S. neurona* surface antigens that are orthologues of the *SAG/SRS* family of surface proteins in *T. gondii*. Each protein is predicted to contain an amino-terminal signal peptide and a carboxyl-terminal glycolipid anchor addition site, indicating surface localization, and Triton X-114 partitioning and surface biotinylation assays confirmed that all four proteins are membrane-associated and displayed on the *S. neurona* merozoite surface (See, FIGS. 4 and 5). Additionally, these novel *S. neurona* proteins possess multiple conserved cysteine residues that have been described previously for *T. gondii* SAGs and which are likely important for the tertiary structure of the proteins (See, FIGS. 1 and 2). Due to their surface localization and relative homology to *T. gondii* surface antigens, these *S. neurona* proteins have been designated SnSAG1, SnSAG2, SnSAG3, and SnSAG4.

Accordingly, one embodiment of the present invention comprises an isolated nucleic acid as set forth in the Sequence Listing as SEQ ID NO: 21. The nucleic acid identified in SEQ ID NO: 21 comprises an 828-nucleotide open reading frame of the SnSAG1 gene of *Sarcocystis neurona* which encodes a 276 amino acid polypeptide set forth in the Sequence Listing as SEQ ID NO: 22. The polypeptide encoded by SEQ ID NO: 22 has a predicted amino-terminal signal peptide (indicating expression via the secretory pathway) and a glycolipid anchor addition site at the carboxy-terminal end (indicating surface localization). Database searches with the predicted protein sequence of SnSAG1 (rSnSAG1) revealed significant similarity (alignment score=80; E value=2x10^-14) to a 31 kDa surface antigen from *Sarcocystis muris*.

A recombinant form of the *Sarcocystis neurona* SnSAG1 (rSnSAG1) has been expressed in *E. coli*. Western blot analysis of rSnSAG1 demonstrated that the recombinant antigen is recognized by antisera from a rabbit that was immunized with *S. neurona* merozites and by antibodies in
cerebrospinal fluid (CSF) from an EPM (Sarcocystis neurona infected) horse (See, e.g., FIG. 3).

Another embodiment of the present invention comprises an isolated nucleic acid as set forth in the Sequence listing as SEQ ID NO: 23. The nucleic acid identified in SEQ ID NO: 23 comprises an 975 nucleotide open reading frame of the SnSAG2 gene of Sarcocystis neurona which encodes a 168 amino acid polypeptide set forth in the Sequence Listing as SEQ ID NO: 24.

The present invention also provides an isolated nucleic acid as set forth in the Sequence listing as SEQ ID NO: 25. The nucleic acid identified in SEQ ID NO: 25 comprises an 1585 nucleotide open reading frame of the SnSAG3 gene of Sarcocystis neurona which encodes a 281 amino acid polypeptide set forth in the Sequence Listing as SEQ ID NO: 26.

Also provided by the present invention is an isolated nucleic acid as set forth in the Sequence listing as SEQ ID NO: 27. The nucleic acid identified in SEQ ID NO: 27 comprises an 1111 nucleotide open reading frame of the SnSAG4 gene of Sarcocystis neurona which encodes a 287 amino acid polypeptide set forth in the Sequence Listing as SEQ ID NO: 28.

As set forth more fully below, these genes have been expressed as recombinant proteins in E. coli. The recombinant SnSAG proteins can be implemented into antibody-capture ELISAs and used to detect the presence of S. neurona antibodies in a sample. Likewise, the recombinant proteins provided by the invention can be used as reagents for use in vaccines against S. neurona.

Another embodiment of the present invention includes the discovery of additional novel expressed sequence tags (ESTs) that encode novel antigenic peptides for utilization in the vaccines and diagnostic kits as disclosed by this invention. In particular, cluster analysis of the Sarcocystis neurona expressed sequence tags (ESTs) generated from the csn1 cDNA library has revealed a gene family that encodes at least eight homologous proteins. Of the approximately 8500 S. neurona ESTs that have been generated thus far, roughly 540 sequences can be placed in this gene family, which has been provisionally designated SnGF1 (S. neurona Gene Family 1). Based on its relative abundance in the collection of S. neurona ESTs, SnGF1 encodes a set of similar proteins (at least eight) that are highly expressed and most likely play significant roles in the biology of S. neurona (i.e., parasite virulence factors). In addition to their biological importance, the abundance of these proteins would suggest that they elicit significant immune responses in infected animals. Collectively, the characteristics of the novel nucleic acids of SnGF1, and the encoded proteins therefrom, make this gene family well suited for the development of improved diagnostics and/or vaccines for EPM as set forth herein.

The eight SnGF1 isoforms identified thus far have been designated SnGF1a-h. These genes are predicted to encode proteins of, e.g., 109 amino acids, 106 amino acids, and 107 amino acids in length, and the proteins share approximately 70% to 80% sequence identity. These proteins have a predicted N-terminal signal peptide and a predicted transmembrane domain near the C-terminus. The SnGF1 members show no similarity to sequences in the current public gene databases, suggesting that SnGF1 is relatively unique to S. neurona.

Accordingly, one embodiment of the present invention provides an isolated nucleic acid designated SnGF1a which comprises the nucleic acid set forth in SEQ ID NO: 5 and sequences complimentary thereto. Another embodiment of the invention comprises the polypeptide sequence encoded by SnGF1a set forth in the Sequence Listing as SEQ ID NO: 6.

Another embodiment of the present invention provides an isolated nucleic acid designated SnGF1b which comprises the nucleic acid set forth in SEQ ID NO: 7 and sequences complimentary thereto. Another embodiment of the invention comprises the polypeptide sequence encoded by SnGF1b set forth in the Sequence Listing as SEQ ID NO: 8.

Yet another embodiment of the present invention provides an isolated nucleic acid designated SnGF1c which comprises the nucleic acid set forth in SEQ ID NO: 9 and sequences complimentary thereto. Another embodiment of the invention comprises the polypeptide sequence encoded by SnGF1c set forth in the Sequence Listing as SEQ ID NO: 10.

Still another embodiment of the present invention provides an isolated nucleic acid designated SnGF1d which comprises the nucleic acid set forth in SEQ ID NO: 11 and sequences complimentary thereto. Another embodiment of the invention comprises the polypeptide sequence encoded by SnGF1d set forth in the Sequence Listing as SEQ ID NO: 12.

The present invention also provides an isolated nucleic acid designated SnGF1e which comprises the nucleic acid set forth in SEQ ID NO: 13 and sequences complimentary thereto. Another embodiment of the invention comprises the polypeptide sequence encoded by SnGF1e set forth in the Sequence Listing as SEQ ID NO: 14.

Another embodiment of the present invention provides an isolated nucleic acid designated SnGF1f which comprises the nucleic acid set forth in SEQ ID NO: 15 and sequences complimentary thereto. Another embodiment of the invention comprises the polypeptide sequence encoded by SnGF1f set forth in the Sequence Listing as SEQ ID NO: 16.

Yet another embodiment of the present invention provides an isolated nucleic acid designated SnGF1 g which comprises the nucleic acid set forth in SEQ ID NO: 17 and sequences complimentary thereto. Another embodiment of the invention comprises the polypeptide sequence encoded by SnGF1 g set forth in the Sequence Listing as SEQ ID NO: 18.

Still another embodiment of the present invention provides an isolated nucleic acid designated SnGF1 h which comprises the nucleic acid set forth in SEQ ID NO: 19 and sequences complimentary thereto. Another embodiment of the invention comprises the polypeptide sequence encoded by SnGF1 h set forth in the Sequence Listing as SEQ ID NO: 20.

The present invention provides isolated nucleic acids as set forth in the Sequence Listing and nucleic acid reagents derived therefrom which can be utilized to diagnose and prevent infection of S. neurona. Purified polypeptides encoded by the nucleic acids are also provided. These polypeptides can be utilized in methods of diagnosis or as vaccine components for prevention of infection. Vectors are also provided which comprise the nucleic acids of the present invention. The vectors can be utilized in host expression systems to produce antigenic peptide reagents for diagnostic and prophylactic applications. The present invention also provides purified antibodies selectively reactive with S. neurona. These antibodies can be used in various diagnostic methods or as a therapeutic.

In one embodiment, the invention provides purified antigenic polypeptides encoded by the nucleic acids set forth in the Sequence Listing. The invention also provides these antigenic polypeptides in a pharmaceutically acceptable
carrier. The amino acid sequence of these polypeptides can be deduced from the nucleotide sequences set forth in the Sequence Listing.

Purified antigenic polypeptide fragments encoded by the nucleic acids of the present invention are also contemplated. As used herein, "purified" means the antigen is at least sufficiently free of contaminants or cell components with which the antigen normally occurs to distinguish the antigen from the contaminants or components. Purified antigenic polypeptides of S. neurona and antigenic fragments thereof of the present invention are also referred to herein as "the antigen" or "the S. neurona antigen." It is contemplated that the antigenic fragments can be encoded from any portion of the nucleic acid encoding S. neurona as set forth in the Sequence Listing, but especially from fragments encoded by the open reading frames set forth in SEQ ID NO: 24, 26 and 28 as described herein. Specifically, one example provides an approximately 12 kDa antigenic polypeptide encoded by an open reading frame of SEQ ID NO: 24 consisting essentially of the amino acids encoded by the nucleotide as sequence set forth in the Sequence Listing as SEQ ID NO: 23.

An antigenic fragment of the antigen can be isolated from the whole antigen by chemical or mechanical disruption. The purified fragments thus obtained can be tested to determine their antigenicity and specificity by the methods taught herein. Antigenic fragments of the antigen can also be synthesized directly. An immunoreactive fragment is generally an amino acid sequence of at least about five consecutive amino acids derived from the antigen amino acid sequence.

The polypeptide fragments of the present invention can also be recombinant proteins obtained by cloning nucleic acids encoding the polypeptide in an expression system capable of producing the antigenic polypeptide or fragments thereof.

Once the amino acid sequence of the antigen is provided, it is also possible to synthesize, using standard peptide synthesis techniques, peptide fragments chosen to be homologous to immunoreactive regions of the antigen and to modify these fragments by inclusion, deletion or modification of particular amino acids residues in the derived sequences. Thus, synthesis or purification of an extremely large number of peptides derived from the antigen is possible.

The amino acid sequences of the present polypeptides can contain an immunoreactive portion of the S. neurona antigen attached to sequences designed to provide for some additional property, such as solubility. The amino acid sequence of an S. neurona antigen can include sequences in which one or more amino acids have been substituted with another amino acid to provide for some additional property, such as to remove/add amino acids capable of disulfide bonding, to increase its bio-longevity, alter enzymatic activity, or alter interactions, e.g., with gastric acidity. In any case, the peptide should possess a bioactive property, such as immunoreactivity, immunogenicity, etc.

The purified polypeptide fragments thus obtained can be tested to determine their immunogenicity and specificity. Briefly, various concentrations of a putative immunogenically specific fragment are prepared and administered to an animal and the immunological response (e.g., the production of antibodies or cell mediated immunity) of an animal to each concentration is determined. The amounts of antigen administered depend on the subject, e.g. a horse or a guinea pig, the condition of the subject, the size of the subject, etc. Thereafter an animal so inoculated with the antigen can be exposed to the parasite to test the potential vaccine effect of the specific immunogenic fragment. The specificity of a putative immunogenic fragment can be ascertained by testing sera, other fluids or lymphocytes from the inoculated animal for cross reactivity with other closely related Sarcozystis spp.

A vector comprising the nucleic acids of the present invention is also provided. The vectors of the invention can be in a host capable of expressing the antigenic polypeptide fragments contemplated by the present invention. There are numerous E. coli expression vectors known to one of ordinary skill in the art useful for the expression of the antigen. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilis, and other enterobacteriaaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters can be present, such as the lac promoter system, a tryptophan (Trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences for example, for initiating and completing transcription and translation. If necessary, for example, an amino terminal methionine can be provided by insertion of a Met codon 5' and in-frame with the antigen. Also, the carboxyterminal extension of the antigenic fragments can be removed using standard oligonucleotide mutagenesis procedures.

Additionally, yeast expression can be used. There are several advantages to yeast expression systems. First, evidence exists that proteins produced in a yeast secretion systems exhibit correct disulfide pairing. Second, post-translational glycosylation is efficiently carried out by yeast secretory systems. In one example, the Saccharomyces cerevisiae pre-pro-alpha-factor leader region (encoded by the MF.alpha.-1 gene) is routinely used to direct protein secretion from yeast (Brake et al., 1984). The leader region of pre-pro-alpha-factor contains a signal peptide and a pre-segment which includes a recognition sequence for a yeast protease encoded by the KEX2 gene: this enzyme cleaves the precursor protein on the carboxyl side of a Lys-Arg dipetide cleavage-signal sequence. The antigen coding sequence can be fused in-frame to the pre-pro-alpha-factor leader region. This construct is then put under the control of a strong transcription promoter, such as the alcohol dehydrogenase I promoter or a glycolytic promoter. The antigen coding sequence is followed by a translation termination codon which is followed by transcription termination signals. Alternatively, the antigen coding sequences can be fused to a second protein coding sequence, such as SJ26 or .beta.-galactosidase, used to facilitate purification of the fusion protein by affinity chromatography. The insertion of protease cleavage sites to separate the components of the fusion protein is applicable to constructs used for expression in yeast.

Mammalian cells permit the expression of proteins in an environment that favors important post-translational modifications such as folding and cysteine pairing, addition of complex carbohydrate structures, and secretion of active protein. Vectors useful for the expression of antigen in mammalian cells are characterized by insertion of the antigen coding sequence between a strong viral promoter and a polyadenylation signal. The vectors can contain genes conferring either gentamicin or methotrexate resistance for use
as selectable markers. The antigen and immunoreactive fragment coding sequence can be introduced into a Chinese hamster ovary cell line using a methotrexate resistance-encoding vector. Presence of the vector DNA in transformed cells can be confirmed by Southern analysis and production of a cDNA or opposite strand RNA corresponding to the antigen coding sequence can be confirmed by northern analysis. A number of other suitable host cell lines capable of secreting intact proteins have been developed in the art, and include the CHO cell lines, HeLa cells, myeloma cell lines, Jurkat cells, etc. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma Virus, etc. The vectors containing the nucleic acid segments of interest can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts.

Alternative vectors for the expression of antigen in mammalian cells can be employed, similar to those developed for the expression of human gammainterferon, tissue plasminogen activator, clotting Factor VIII, hepatitis B virus surface antigen, protease, Nexin, and eosinophil major basic protein. Further, the vector can include CMV promoter sequences and a polyadenylation signal available for expression of inserted nucleic acid in mammalian cells (such as COS7). The nucleic acid sequences can be expressed in hosts after the sequences have been operably linked to, i.e., positioned to ensure the functioning of an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors can contain selection markers, e.g., tetracycline resistance or hygromycin resistance, to permit detection and/or selection of those cells transformed with the desired nucleic acid sequences (see, e.g., U.S. Pat. No. 4,704,362).

Polynucleotides encoding a variant polypeptide may include sequences that facilitate transcription (expression sequences) and translation of the coding sequences such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art. For example, such polynucleotides can include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a ribosome binding site, and, optionally, an enhancer for use in eukaryotic expression hosts, and, optionally, sequences necessary for replication of a vector.

One presently preferred vector system for expression of the peptides of the invention comprises the use of Alphaviruses vector constructs, for example, as set forth in U.S. Pat. Nos. 5,643,576; 5,843,723; 6,156,558; and 6,242,259, the teachings of which are hereby incorporated herein by reference.

A purified monoclonal antibody specifically reactive with S. neurona is also provided. The antibodies can be specifically reactive with a unique epitope of the antigen or they can also react with epitopes of other organisms. The term “reactive” means capable of binding or otherwise associating non randomly with an antigen. “Specifically reactive” as used herein refers to an antibody or other ligand that does not cross react substantially with any antigen other than the one specified, in this case, S. neurona. Antibodies can be made as described in the Examples (see also, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988). Briefly, purified antigen can be injected into an animal in an amount and in intervals sufficient to elicit an immune response. Antibodies can either be purified directly, or spleen cells can be obtained from the animal. The cells are then fused with an immortal cell line and screened for antibody secretion. The antibodies can be used to screen clone libraries for cells secreting the antigen. Those positive clones can then be sequenced (see, for example, Kelly et al., BioTechnology, 10: 163-167, (1992) and Bebington et al., BioTechnology, 10: 169-175, (1992)).

The antibody can be bound to a substrate or labeled with a detectable moiety or both bound and labeled. The detectable moieties contemplated by the present invention include, but are not limited to fluorescent, enzymatic and radioactive markers.

A purified S. neurona antigen bound to a substrate and a ligand specifically reactive with the antigen are also contemplated. Such a purified ligand specifically reactive with the antigen can be an antibody. The antibody can be a monoclonal antibody obtained by standard methods and as described herein. The monoclonal antibody can be secreted by a hybridoma cell line specifically produced for that purpose (Harlow and Lane, 1988). Likewise, nonhuman polyclonal antibodies specifically reactive with the antigen are within the scope of the present invention. The polyclonal antibody can also be obtained by the standard immunization and purification protocols (Harlow and Lane, 1988).

The present invention provides a method of detecting the presence of S. neurona in a subject, comprising the steps of contacting an antibody-containing sample from the subject with a detectable amount of the antigenic polypeptide fragment of the present invention and detecting the reaction of the fragment and the antibody, the reaction indicating the presence of the S. neurona or a previous infection with S. neurona.

One example of the method of detecting S. neurona is performed by contacting a fluid or tissue sample from the subject with an amount of a purified antibody specifically reactive with the antigen as defined herein, and detecting the reaction of the ligand with the antigen. It is contemplated that the antigen will be on intact cells containing the antigen, or will be fragments of the antigen. As contemplated herein, the antibody includes any ligand which binds the antigen, for example, an intact antibody, a fragment of an antibody or another reagent that has reactivity with the antigen. The fluid sample of this method can comprise any body fluid which would contain the antigen or a cell containing the antigen, such as blood, plasma, serum, cerebrospinal fluid, saliva, feces and urine. Other possible examples of body fluids include sputum, mucus, gastric juice and the like.

Enzyme immunosassays such as immunofluorescence assays (IFA), enzyme linked immunosorbent assays (ELISA) and immunoblotting can be readily adapted to accomplish the detection of the antigen. An ELISA method effective for the detection of the antigen can, for example, be as follows: (1) bind the antibody to a substrate; (2) contact the bound antibody with a fluid or tissue sample containing the antigen; (3) contact the above with a secondary antibody bound to a detectable moiety (e.g., horseradish peroxidase enzyme or alkaline phosphatase enzyme); (4) contact the above with the substrate for the enzyme; (5) contact the
above with a color reagent; (6) observe color change. The above method can be readily modified to detect antibody as well as antigen.

Another immunologic technique that can be useful in the detection of S. neurona or previous S. neurona infection utilizes monoclonal antibodies (MAbs) for detection of antibodies specifically reactive with S. neurona antigen. Briefly, sera or other body fluids from the subject is reacted with the antigen bound to a substrate (e.g. an ELISA 96-well plate). Excess sera is thoroughly washed away. A labeled (enzyme-linked, fluorescent, radioactive, etc.) monoclonal antibody is then reacted with the previously reacted antigen serum antibody complex. The amount of inhibition of monoclonal antibody binding is measured relative to a control (no patient serum antibody). The degree of monoclonal antibody inhibition is a very specific test for a particular variety or strain since it is based on monoclonal antibody binding specificity. MAbs can also be used for detection directly in cells by IFA.

A micro-agglutination test can also be used to detect the presence of S. neurona in a subject. Briefly, latex beads (or red blood cells) are coated with the antigen and mixed with a sample from the subject, such that antibodies in the tissue or body fluids that are specifically reactive with the antigen crosslink with the antigen, causing agglutination. The agglutinated antigen-antibody complexes form a precipitate, visible with the naked eye or capable of being detected by a spectrophotometer. In a modification of the above test, antibodies specifically reactive with the antigen can be bound to the beads and antigen in the tissue or body fluid thereby detected.

In addition, as in a typical sandwich assay, the antibody can be bound to a substrate and reacted with the antigen. Thereafter, a secondary labeled antibody is bound to epitopes not recognized by the first antibody and the secondary antibody is detected. Since the present invention provides S. neurona antigen for the detection of infectious S. neurona or previous S. neurona infection other serological methods such as flow cytometry and immunoprecipitation can also be used as detection methods.

In the diagnostic methods taught herein, the antigen can be bound to a substrate and contacted by a fluid sample such as serum, cerebrospinal fluid, urine, saliva, feces or gastric juice. This sample can be taken directly from the patient or in a partially purified form. In this manner, antibodies specific for the antigen (the primary antibody) will specifically react with the bound antigen. Thereafter, a secondary antibody bound to, or labeled with, a detectable moiety can be added to enhance the detection of the primary antibody. Generally, the secondary antibody or other ligand which is reactive, either specifically with a different epitope of the antigen or nonspecifically with the ligand or reacted antibody, will be selected for its ability to react with multiple sites on the primary antibody. Thus, for example, several molecules of the secondary antibody can react with each primary antibody, making the primary antibody more detectable.

The detectable moiety will allow visual detection of a precipitate or a color change, visual detection by microscopy, or automated detection by spectrometry, radiometric measurement or the like. Examples of detectable moieties include fluorescein and rhodamine (for fluorescence microscopy), horseradish peroxidase (for either light or electron microscopy and biochemical detection), biotin-streptavidin (for light or electron microscopy) and alkaline phosphatase (for biochemical detection by color change). The detection methods and moieties used can be selected, for example, from the list above or other suitable examples by the standard criteria applied to such selections (Harlow and Lane, 1988).

The antigen, e.g., a purified antigenic polypeptide fragment encoded by the Sequence Listing of this invention can be used in the construction of a vaccine comprising an immunogenic mount of the antigen and a pharmaceutically acceptable carrier. The vaccine can be the entire antigen, the antigen on an intact S. neurona organism, E. coli or other strain, or an epitope specific to the antigen. The vaccine can also be potentially cross-reactive with antibodies to other antigens. The vaccine can then be used in a method of preventing EPM or other complications of S. neurona infection.

Immunogenic amounts of the antigen can be determined using standard procedures. Briefly, various concentrations of a putative specific immunoreactive epitope are prepared, administered to an animal and the immunological response (e.g., the production of antibodies) of an animal to each concentration is determined.


An adjuvant can also be a part of the carrier of the vaccine, in which case it can be selected by standard criteria based on the antigen used, the mode of administration and the subject (Arnon, R. (Ed.), 1987). Methods of administration can be by oral or sublingual means, or by injection, depending on the particular vaccine used and the subject to whom it is administered.

It can be appreciated from the above that the vaccine can be used as a prophylactic or a therapeutic modality. Thus, the invention provides methods of preventing or treating S. neurona infection and the associated diseases by administering the vaccine to a subject.

Nucleic acid vaccines against S. neurona are also contemplated by the invention. The antigenic agent for use in the vaccines of the invention can be any nucleic acid, e.g., as set forth in the Sequence Listing, that can stimulate an immune response against, e.g., SnSAG2, SnSAG3 or SnSAG4 when administered to a subject. Suitable nucleic acids include those that encode the native proteins of S. neurona, e.g., SnSAG2, SnSAG3 or SnSAG4 protein or a variant or antigenic peptide fragment thereof, such as, e.g., the nucleic acid set forth in the Sequence listing as SEQ ID NO:23, SEQ ID NO:25 or SEQ ID NO:27. The nucleic acid used as a vaccine can be e.g., a naked DNA, or the nucleic acid can be incorporated in an expression vector as set forth herein, e.g., in an Alpha virus vector (see, e.g., Rosenberg, S. A., Immunity 10:281, 1999).

The presence of S. neurona can also be determined by detecting the presence of a nucleic acid specific for S. neurona or the antigens of S. neurona encoded by the nucleic acids set forth herein. The present invention provides a method of detecting the presence of S. neurona in a subject, comprising detecting the presence of the nucleic acid encoding an S. neurona antigen. As set forth more fully in the examples below, the specificity of these sequences for S. neurona can be determined by conducting a computerized comparison with known sequences, catalogued in GenBank, a computerized database, using the computer programs Word Search or FASTA of the Genetics Computer Group (Madison, Wis.), which search the catalogued nucleotide sequences for similarities to the nucleic acid in question.

The nucleic acid specific for S. neurona antigen can be detected utilizing a nucleic acid amplification technique, such as polymerase chain reaction or ligase chain reaction.
Alternatively, the nucleic acid is detected utilizing direct hybridization or by utilizing a restriction fragment length polymorphism. For example, the present invention provides a method of detecting the presence of *S. neurona* comprising ascertaining the presence of a nucleotide sequence associated with a restriction endonuclease cleavage site. In addition, PCR primers which hybridize only with nucleic acids specific for *S. neurona* can be utilized. The presence of amplification indicates the presence of *S. neurona* sequence.

In another embodiment a restriction fragment of a nucleic acid sample can be sequenced directly using, techniques known in the art and described herein and compared to the known unique sequence to detect *S. neurona*. In a further embodiment, the present invention provides a method of detecting the presence of *S. neurona* by selective amplification by the methods described herein. In yet another embodiment *S. neurona* can be detected by directly hybridizing the unique sequence with a *S. neurona* selective nucleic acid probe. Furthermore, the nucleotide sequence could be amplified prior to hybridization by the methods described above.

Alternative probing techniques, such as ligase chain reaction (LCR), involve the use of mismatch probes, i.e., probes which are fully complementary with the target except at the point of the mutation. The target sequence is then allowed to hybridize both with oligonucleotides which are fully complementary and have oligonucleotides containing a mismatch, under conditions which will distinguish between the two. By manipulating the reaction conditions, it is possible to obtain hybridization only where there is full complementarity. If a mismatch is present there is significantly reduced hybridization.

The polymerase chain reaction (PCR) and reverse transcriptase PCR are techniques that amplify specific nucleic acid sequences with remarkable efficiency. Repeated cycles of denaturation, primer annealing and extension carried out with polymerase, e.g., a heat stable enzyme Taq polymerase, leads to exponential increases in the concentration of desired nucleic acid sequences. Given a knowledge of the nucleotide sequence of *S. neurona* as set forth herein, synthetic oligonucleotides can be prepared which are complementary to sequences which flank the nucleic acid of interest. Each oligonucleotide is complementary to one of the two strands. The nucleic acid can be denatured at high temperatures (e.g., 95 degree C.) and then reannealed in the presence of a large molar excess of oligonucleotides. The oligonucleotides, oriented with their 3' ends pointing towards each other, hybridize to opposite strands of the target sequence and prime enzymatic extension along the nucleic acid template. The end product is then denatured again for another cycle. After this three-step cycle has been repeated several times, amplification of a nucleic acid segment by more than one million-fold can be achieved. The resulting nucleic acid may then be directly sequenced.

In yet another method, PCR may be followed by restriction endonuclease digestion with subsequent analysis of the resultant products. Nucleotide substitutions can result in the gain or loss of specific restriction endonuclease sites. The gain or loss of a restriction endonuclease recognition site facilitates the detection of the organism using restriction fragment length polymorphism (RFLP) analysis or by detection of the presence or absence of a polymorphic restriction endonuclease site in a PCR product that spans the sequence of interest.

For RFLP analysis, nucleic acid is obtained, for example from the blood, cerebrospinal fluid, gastric specimen, saliva, dental plaque, other bodily fluids of the subject suspected of containing *S. neurona*, is digested with a restriction endonuclease, and subsequently separated on the basis of size by agarose gel electrophoresis. The Southern blot technique can then be used to detect, by hybridization with labeled probes, the products of endonuclease digestion. The patterns obtained from the Southern blot can then be compared. Using such an approach, *S. neurona* nucleic acid is detected and their mobility on the gel by determining the number of bands detected and comparing this pattern to the nucleic acid from *S. neurona*.

Similar creation of additional restriction sites by nucleotide substitutions in the disclosed mutation sites can be readily calculated by reference to the genetic code and a list of nucleotide sequences recognized by restriction endonucleases. Single strand conformational analysis (SSCA) offers a relatively quick method of detecting sequence changes which may be appropriate in at least some instances.

In general, primers for PCR and LCR are usually about 20 bp in length and the preferable range is from 15-25 bp. Better amplification is obtained when both primers are the same length and with roughly the same nucleotide composition. Denaturation of strands usually takes place at about 94 degree C. and extension from the primers is usually at about 72 degree C. The annealing temperature varies according to the sequence under investigation. Examples of reaction times are: 20 mins denaturing; 35 cycles of 2 min, 1 min, 1 min for annealing, extension and denaturation; and finally a 5 min extension step.

PCR amplification of specific alleles (PASA) is a rapid method of detecting single-base mutations or polymorphisms. PASA (also known as allele specific amplification) involves amplification with two oligonucleotide primers such that one is allele-specific. The desired allele is efficiently amplified, while the other allele(s) is poorly amplified because it mismatches with a base at or near the 3' end of the allele-specific primer. Thus, PASA or the related method of PAMSA may be used to specifically amplify the mutation sequences of the invention. Where such amplification is done on *S. neurona* isolates or samples obtained from an individual, it can serve as a method of detecting the presence of *S. neurona*. As mentioned above, a method known as ligase chain reaction (LCR) can be used to successfully detect a single-base substitution. LCR probes may be combined or multiplexed for simultaneously screening for multiple different mutations. Thus, LCR can be particularly useful where, as here, multiple mutations are predictive of the same disease.

The present invention is more particularly described in the following examples which are intended as illustrative only, since numerous modifications and variations therein will be apparent to those skilled in the art.

**EXAMPLES**

Identification and Characterization of SnSAG1

Surface biotinylation of extracellular merozoites revealed only two dominant labeled molecules that migrate at about 30 kDa and 16 kDa in SDS-PAGE. Analysis of a *S. neurona* EST database (currently 1800+ sequences) identified an orthologue of the 31-kDa surface antigen from *Sarcocystis muris*. The sequence of the *S. neurona* surface antigen gene, designated SnSAG1, is predicted to encode a 276-residue protein with an amino-terminal signal peptide and a carboxy-terminal GPI anchor addition. Antiserum raised against recombinant SnSAG1 recognized a 25-kDa antigen...
in western blots of non-reduced S. neurona lysates, consistent with the molecular weight predicted for the mature SnSAG1. Under reducing conditions, SnSAG1 migrated abnormally at about 30 kDa, similar to what has been observed in western blot analyses of reduced T. gondii surface antigens. Immunofluorescence labeling of SnSAG1 during intracellular growth of S. neurona indicated that the protein is expressed throughout schizontogenesis. Interestingly, a filamentous staining pattern was observed in intermediate schizonts that likely reflects localization of the surface antigen to previously-described invaginations of the schizont surface membrane.

Parasite Culture

S. neurona strain SN3 [Granstrom, 1992 #1600] merozoites were propagated by serial passage in bovine turbinate (BT) cells and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM sodium pyruvate, Pen/Strep Fungizone (BioWhittaker, Inc.). Extracellular merozoites were harvested and purified from disrupted host cell monolayers by filtration through 3.0 μm membranes, as described previously for Neospora caninum [Howe, 1997 #1372].

Immunoscreen of S. neurona cDNA Library

Construction and analyses of the cSN1 S. neurona merozoite cDNA library has been described previously [Howe, 2001 #1787]. The library was plated for 3 hrs at 42°C on XLI-Blue MRF+ E. coli host cells (Stratagene) grown on 150 mm NY agar plates. When plaques became visible, plates were overlayed with nitrocellulose filters previously soaked in 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for an additional 3 hr incubation at 37°C. Filters were lifted from the plates, washed with TNT buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), and blocked in phosphate buffered saline (PBS), 5% dry milk, 0.5% normal goat serum, 0.05% Tween 20. Antigenic cDNA clones were identified by screening with cerebrospinal fluid (CSF) from a horse that had been naturally infected with S. neurona and exhibited a high titer of intrathecal antibodies against S. neurona in western blot analysis. Prior to screening the S. neurona cDNA library, the CSF was diluted 1:20 in PBS, 0.1% dry milk, 0.1% normal goat serum, 0.05% Tween 20 and incubated for 30 min with filters carrying plaque lifts of a previously-described N. caninum cDNA library [Howe, 1999 #1759] to remove antibodies that were reactive with E. coli and phage proteins. After adsorption of potential cross-reactive antibodies, the diluted CSF solution was incubated for 1 hr with the cSN1 filters. After washing, filters were incubated for 1 hr with goat anti-encephalitogenic IgG conjugated to horseradish peroxidase (HRP) (Jackson Immunoresearch Labs, Inc.) diluted to 1:10,000. Immuno-reactive plaque plaques were picked with sterile pipet tips and suspended in 40 μl of SM buffer (50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 8 mM MgSO₄, 0.01% gelatin). The cDNA inserts were PCR amplified using the T3 and T7 oligonucleotide primers, and the resulting products were analyzed by agarose gel electrophoresis. Sequencing reactions using T3 primer were conducted on the amplified cDNAs to provide a preliminary identification of the immunoreactive clones. Phagemid excision was performed on selected cDNA clones, and plasmids were rescued in SOLR cells according to the manufacturer’s protocol (Stratagene).

S. neurona EST Database Searches and Sequence Analyses

S. neurona homologues to previously-characterized coccidian surface antigens were identified in the S. neurona clustered EST database (See, e.g., paradb.cis.upenn.edu/sarco/index.html) using the BLAST (basic local alignment search tool) set of programs [Altschul, 1990 #616]. At the time the database was searched, it contained 686 consensus sequences that had been generated from 1883 S. neurona ESTs. Selected cDNAs were obtained from the archived collection of EST clones and sequenced using ABI Prism BigDye Terminator Cycle Sequencing reaction mix (Perkin Elmer Applied Biosystems). The reactions were purified using Centri-Sep spin columns (Princeton Separations), and the eluted extension products were resolved and analyzed on an ABI 310 Genetic Analyzer. Sequence analyses were conducted with Genetics Computer Group (GGG) software [Devereux, 1984 #1176] and programs available on the National Center for Biotechnology Information (NCBI) web site (See, e.g., www.ncbi.nlm.nih.gov/) and the Expert Protein Analysis System (ExPASy) server of the Swiss Institute of Bioinformatics (See, e.g., www.expasy.ch/). Multiple sequence alignments were performed using Multalin software [Corpet, 1988 #2046]. The sequence reported herein has been deposited into GenBank under accession number AY053264.

Recombinant SnSAG1 Expression and Generation of Polyclonal Antiserum

The SnSAG1 open reading frame without the predicted amino-terminal signal peptide and the carboxyl-terminal hydrophobic tail was amplified by PCR from the cSN1 cDNA using primers that introduce a Ndel restriction site prior to base 45 (numbered from the initiation codon) and an XhoI site after base 743. The amplification product was digested with Ndel and Xhol, ligated into Ndel/XhoI-digested pET12b expression vector (Novagen), and transformed into IMvcd2 E. coli. The resulting expression plasmid, designated pSnSAG1, was transferred into BL21-CodonPlus E. coli (Stratagene), and a clone that expressed high levels of recombinant SnSAG1 (rSnSAG1) was selected for use. The histidine-tagged rSnSAG1 was purified by nickel-column chromatography according to the manufacturer’s protocol (Novagen), and monospecific polyclonal antisera were produced against the purified protein by immunization of a rabbit and rat (Cocalico Biologicals, Inc.).

Western Blot Analysis

Parasites were lysed in sodium dodecyl sulfate (SDS) sample buffer supplemented with protease inhibitor cocktail (Sigma) and 2% mercaptoethanol, and the lysates were separated in 10% or 12% polyacrylamide gels [Laemmli, 1970 #393]. Proteins were transferred to nitrocellulose membranes by semi-dry electrophoretic transfer in Tris-glycine buffer (pH 8.3). Membranes were blocked with PBS containing 5% nonfat dry milk, 5% goat serum, and 0.05% Tween 20, and then incubated for 1 hr with primary antibody. After washing, membranes were incubated with HRP-conjugated immunoglobulin G secondary antibody (Jackson Immunoresearch Labs, Inc.). Blots were washed, processed for chemiluminescence using Supersignal substrate (Pierce Chemical Company), and exposed to film.

Biotinylation of Surface Proteins and Precipitation with Immobilized Streptavidin

Approximately 3x10⁷ freshly harvested merozoites were resuspended in 1 ml cold PBS (pH 7.8). Sulfo-N-hydroxysuccinimide-biotin (Pierce) was added to a concentration of 0.5 mg/ml and incubated at room temperature for 30 min. The labeled parasites were washed twice with 5 ml of PBS and stored at −20°C.

The labeled parasite pellet was lysed with 1 ml radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 8.0]...
7.5%, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 100 mM NaCl, 5 mM EDTA) supplemented with RNase, DNase, protease inhibitor cocktail, and the sample was centrifuged at 16,000 g to remove the insoluble fraction. The soluble proteins were incubated with UltraLink immobilized streptavidin (Pierce), and the precipitated biotin-labeled protein fraction was analyzed by western blotting, as described above.

Immunofluorescent Labeling of Extracellular and Intracellular Parasites

For detection of SnSAG1 on extracellular parasites and in trails deposited by gliding parasites, freshly lysed merozoites were suspended in fresh RPMI 1640 and incubated on poly-L-lysine-coated slides for approximately 30 min. Slides were washed with PBS, and the parasites were fixed in 2.5% formalin-PBS containing 0.01% glutaraldehyde. For detection of SnSAG1 on intracellular parasites, merozoites were inoculated onto Ht cells grown on Lab-Tek chamber slides (Nunc). At 24 hr, 48 hr, or 72 hr post-inoculation, the cells were fixed in 2.5% formalin-PBS/0.01% glutaraldehyde and permeabilized with 0.2% TritonX-100. After incubation with primary antibody, the slides were rinsed, then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Labs, Inc.). The slides were mounted in Vectashield with DAPI (Vector Laboratories, Inc.) and examined with a Zeiss axio-scope equipped for epifluorescence microscopy.

Results

Isolation and Analysis of Immunoreactive cDNA Clones

A primary screen of the cSn1 cDNA library identified multiple immunoreactive plaques, and a total of 25 plaques were isolated and resuspended in SM buffer. Amplification of the cDNA inserts with T3 and T7 oligonucleotides revealed that 22 of the plaque clones had similar lengths of approximately 1500 base pairs (bp), and sequence analysis using T3 primer indicated that these 22 cDNAs represent the same gene. A secondary screen was performed on five of the selected cDNAs, and two highly reactive plaque clones, designated SnAgL8 and SnAgL19, were chosen for further analyses.

To obtain a preliminary identification of the parasite protein encoded by the selected cDNAs, the SnAgL9 clone was used to affinity purify antibodies that bind the antigen expressed by this clone, and the eluted antibodies were used to probe a western blot of S. neurona merozoite lysate. As shown in Fig. 1, the purified antibodies reacted with an approximately 31-kDa antigen in reduced S. neurona lysate. Furthermore, the antigen revealed by the phage-purified antibodies conformed with a protein that is recognized by equine or rabbit antiserum against S. neurona as the major immunodominant antigen of this parasite (Figs. 1, lanes 2 and 3). This result implies that the 22 matching cDNA clones isolated during the library screen and represented by SnAgL8 and SnAgL9 encode the immunodominant antigen of S. neurona.

Full-length sequence analysis of SnAgL8 revealed a cDNA insert of 1493 nucleotides, with an open reading frame (ORF) that encodes a 276 amino acid protein. Sequence analysis of SnAgL9 indicated that this clone was virtually identical to SnAgL8, although its 3′ untranslated region (UTR) was approximately 160 nucleotides longer due to an alternative polyadenylation site. A hydrophobicity plot of the encoded protein showed hydrophobic domains at both termini, which correspond to a predicted signal peptide at the amino terminus and a GPI anchor addition sequence at the carboxyl terminus (data not shown). The signal peptide cleavage is predicted to occur at Ala5-Arg16 (SignalP; [Nielsen, 1997 #2047], and the most likely GPI transamidase cleavage site is predicted to be at Ala25-Asn28 (DGPI; Swiss Institute of Bioinformatics). A single N-glycosylation site was predicted at residues 140-143. Removal of the N-terminal and C-terminal signal sequences results in a mature protein of 242 amino acids that has a predicted molecular weight of 24.2 kDa before any potential post-translational modifications (e.g., glycolipid anchor addition, glycosylation).

To identify homology to previously characterized sequences, BLAST searches [Altschul, 1990 #616] of the non-redundant GenBank databases were conducted with the SnAgL8 coding sequence as the query. These searches revealed a statistically significant similarity to the 31 kDa major surface antigen of Sarcocystis muris [Eschenbacher, 1992 #1767] and a less significant but recognizable similarity to several SAG2-related surface antigens from T. gondii [Lekakis, 2000 #2049]. In conjunction with the western blot analysis and the predictions of a signal peptide and a GPI-anchor addition, these results suggested that the gene represented by the SnAgL8 and SnAgL9 cDNAs encodes an immunodominant surface antigen of S. neurona; consequently, we tentatively designated this protein SnSGA1, following the genetic nomenclature that is utilized for the related apicomplexan parasites T. gondii and N. caninum [Sibley, 1991 #13; Howe, 1999 #1759].

The sequence analysis for SnSAG2, SnSAG3, and SnSAG4 as well as for the SnGF Cluster sequences provided by the invention and set forth herein have been derived in a fashion similar to that set forth above for SnSAG1. These novel nucleotide sequences and protein sequences of Sar-cocystis neurona can be utilized in the production of vaccines and/or antigen/antibody kits for prevention and diagnosis of Sarcocystis neurona infection. One preferred embodiment of the invention is a vaccine comprised of an alpha virus expression vector and nucleic acid selected from the nucleic acid sequences disclosed herein.

Identification of S. neurona Surface Antigens and Expression as Recombinant Proteins

Analysis of the S. neurona EST database revealed four paralogous proteins that are homologous to the SAG and SRS surface antigens of Toxoplasma gondii. Each S. neurona gene was predicted to encode a protein that possessed an amino-terminal signal peptide and a carboxyl-terminal glycolipid anchor site, consistent with the proteins being surface antigens. Because of their similarity to Toxoplasma SAGs and their probable surface display on merozoites, the four S. neurona proteins were designated SnSAG1, SnSAG2, SnSAG3, and SnSAG4. The four putative surface antigens were each expressed as a recombinant protein in E. coli, and these were used to immunize rabbits and rats for nonspecific polyclonal antiserum production. The resulting polyclonal antisera were used in western blot analysis of reduced (with 2-mercaptoethanol) S. neurona lysate to reveal each of the SnSAGs (See, Fig. 3). The mature forms of native SnSAG1 and SnSAG4 are predicted to be approximately 24 kDa, but these antigens co-migrated at approximately 30-32 kDa and correspond to the immunodominant antigen Sn30 that has been described previously (See, Fig. 3) (Granstrom et al., 1993; Liang et al., 1998). SnSAG1 has also been identified by others as a major surface antigen matching the immunodominant Sn30 band (Ellison et al., 2002), but it is apparent that SnSAG4 likely contributes to the antibody reactivity at this molecular weight. The mature
form of SnSAG2 is predicted to be about 12 kDa, but this antigen migrated at about 18-19 kDa and corresponds to the previously described immunodominant Sn16 antigen (See, FIG. 3) (Granstrom et al., 1993; Liang et al., 1998). Mature SnSAG3 is predicted to be 23 kDa, but migrated at about 28 kDa (See, FIG. 3). The aberrant migration of the SnSAGs under reducing conditions is a characteristic that has been observed previously for the surface antigens of both T. gondii (Burg et al., 1988; Cesbron-Delauw et al., 1994) and N. caninum (Howe et al., 1998). Importantly, the western blot experiments demonstrated that the recombinant forms of the SnSAGs are recognized by antibodies from S. neurona-infected horses. There is strong concordance between antibody recognition of recombinant SnSAG1 (rSnSAG1) and standard western blot analysis of complete parasite antigen (i.e., S. neurona merozoite lysate). Similar results were obtained with rSnSAG2, rSnSAG3, and rSnSAG4. These data demonstrate the utility of using the rSnSAGs in ELISA formats to monitor antibody responses in S. neurona-infected horses.

Enzyme-linked Immunosorbent Assays (ELISAs) Based on Recombinant S. neurona Surface Antigens (rSnSAGs)

The rSnSAGs expressed in E. coli have been shown in western blots to be recognized by equine antibodies; consequently, these recombinant antigens can be utilized as the key reagents for developing ELISAs based on single S. neurona antigens. An ELISA test was developed for each of the four rSnSAGs that have been identified by the invention.

Expression and Purification of Recombinant SnSAGs.

To produce highly purified recombinant forms of the SnSAGs, the genes for each antigen were cloned into the pET22b expression plasmid from Novagen (Madison, Wis.). This plasmid vector provides a carboxyl-terminal fusion to a 6-residue oligohistidine domain (His-Tag), which binds to metal ion affinity columns and allows for the efficient one-step purification of the expressed recombinant protein. Plasmid constructs were transformed into BL21 (DE3) host cells (CodonPlus, Stratagene, Inc.), and expression of recombinant protein was induced by addition of IPTG. Bacterial clones that reliably expressed the recombinant SnSAGs were selected and cryopreserved for future study. The recombinant S. neurona surface antigens have been designated rSnSAG1, rSnSAG2, rSnSAG3, and rSnSAG4.

To obtain recombinant protein, the appropriate bacterial clone was grown to logarithmic phase in LB medium, and protein expression was induction by addition of IPTG to the culture. The recombinant protein was extracted from inclusion bodies with 6 M urea and purified from the host cell lysate by Ni²⁺-column chromatography according to the manufacturer’s protocol (His-Bind resin and buffers, Novagen). Urea was removed by dialysis. If necessary, recombinant proteins was concentrated by centrifugal ultrafiltration in Centricon-10 columns (Amicon).

ELISA Assay

The SN3 strain of S. neurona and the Oregon strain of Neospora hughesi were maintained by serial passage in bovine turbinate cell monolayers. Upon lysis of the host cell monolayer, zoites were dispersed and filtered (0.3 μm Nucleopore membrane filter, Whatman) to remove debris. Harvested parasites were counted, washed, and stored at -20°C.

Concentration of purified recombinant proteins prepared as described above was determined by a calorimetric assay (Coomassie Plus Protein Assay Reagent, Pierce). Purified rSnSAG1, rSnSAG2, rSnSAG3, and rSnSAG4 were diluted in buffer (0.5 M NaCl and 20 mM Tris-Cl) without urea to final protein concentrations of 8.15 μg/ml, 23.0 μg/ml, 14.56 μg/ml, and 10.3 μg/ml, respectively.

Positive control serum samples were obtained from two horses with histologically confirmed EPM. The negative control sample for all assays was a preinfection serum sample from a weanling used in an experimental infection trial. Thirty six equine serum samples submitted for S. neurona serology testing were used for standardization of the rSnSAG ELISAs. The samples had previously been classified as positive or negative by Western blot. Twenty-seven samples from horses of confirmed EPM status were obtained from a collection of the University of Kentucky Gluck Equine Research Center. All cases were confirmed by histological examination of central nervous system tissues for the presence of lesions consistent with EPM, as well as Western blot analysis of CSF fluids. Three equine serum samples from an S. fayeri challenge trial were used to examine assay cross-reactivity. An N. hughesi positive control serum sample was also evaluated.

Native and recombinant proteins were suspended in SDS-PAGE buffer supplemented with protease inhibitor cocktail (Sigma) and separated on 12% polyacrylamide gels. For Western blot, proteins were transferred to nitrocellulose membranes by semi-dry electrophoresis. Membranes were blocked with PBS containing nonfat dry milk, 0.1% Tween 20, and 5% normal goat serum, and incubated for 1 hour in primary antibody solution. The membranes were washed, followed by incubation for 45 min. with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Membranes were then exposed to radiochronogenic detection using Chemiluminescent detection using Chemiluminescent detection using SuperSignal Substrate (Pierce) and exposed to radiographic film or documented with a FluorChem 8900 imaging system (Alpha Innotech Corp.).

For rSnSAS ELISAs, high-binding 96-well plates (Corning) were incubated overnight at 4°C with 100 μl purified rSnSAG1, rSnSAG2, rSnSAG3, or rSnSAG4 diluted to 0.20 μg/ml, 1.00 μg/ml, 0.09 μg/ml, and 0.21 μg/ml, respectively. The plates were rinsed with PBS/0.05% Tween 20 and blocked for 1.5 h at room temperature with PBS/1% Tween 20/0.5% normal goat serum/0.001 g/ml nonfat dry milk. Primary sera or CSF was diluted with PBS/1% Tween 20/0.5% normal goat serum/0.001 g/ml nonfat dry milk. One hundred μl aliquots of the sera or CSF containing mixed antibody populations were added to duplicate wells and incubated for 2 h at room temperature. The wells were rinsed, and then incubated for 2 h at room temperature with 150 μl of horseradish peroxidase-conjugated goat anti-horse immunoglobulin G (lgG) secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted to 1:10,000 in PBS/1% Tween 20/0.5% normal goat serum/0.001 g/ml nonfat dry milk. The wells were then again rinsed. The chromogenic substrate o-phenylenediamine dihydrochloride (Sigma) at 0.4 mg/ml (200 μl) was added. After 10 min incubation, the reaction was stopped with 50 μl of 3 M H₂SO₄, and OD₅₇₀ was measured in an Emax microplate reader (Molecular Devices). To account for interplate variation, the OD of each sample was expressed as a percentage of the high positive standard on the plate.

Serum antibody titers against rSnSAG4 were detected in 25 of 26 (96.2%) EPM-confirmed horses (see FIG. 6). The rSnSAG2 and rSnSAG3 ELISAs yielded seropositive results in 24 of 26 (92.3%) EPM-confirmed horses. Only 18 of the 26 (69.2%) horses had detectable serum antibody titers against rSnSAG1. In total, 18 (81.8%), 18 (81.8%), 20 (90.0%), and 21 (95.5%) of the 22 CSF samples had detectable antibody titers against rSnSAG1, rSnSAG2, rSnSAG3, and rSnSAG4, respectively.
SAG3, and rSnSAG4, respectively (see FIG. 7). No significant cross-reactivity of the ELISAs was found when tested against samples containing antibodies to two related pathogens, _S. fayeri_ and _S. hughesi_ (data not shown).

Expression of Recombinant _S. neurona_ Surface Antigens (rSnSAGs) in Mammalian Cells

The open reading frame of each SnSAG as previously described was directionally cloned into the KpnI and XbaI restriction sites of the pVAX1 DNA vaccine plasmid vector (Invitrogen), and the fidelity of the pVAX:SnSAG plasmid constructs was confirmed by expression in COS-1 (green monkey kidney) cells with (+GPI) and without (-GPI) the GPI anchor. Cells were grown on coverslips in 24-well plates. The pVAX:SnSAG plasmids were transfected into the COS-1 cells using the cationic lipid reagent LipoFectamine 2000 (Invitrogen). At 48 hr post-transfection, the coverslips were removed and the cells were fixed with formalin. The cells were then labeled with an anti-rabbit SnSAG serum as appropriate, followed by goat anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC). Cell nuclei were labeled with DAPI for contrast. As shown in FIG. 8, mammalian cells clearly expressed each transfected surface antigen (SnSAG1, SnSAG2, SnSAG3, and SnSAG4).

Accordingly, a simple, reliable assay is provided for detection of _S. neurona_ infection. Importantly, the assays did not cross-react with antisera against related pathogens. The assay described herein provides numerous advantages over current serologic assays, including ease of use, high sample throughput, and more objective interpretation of results. Further, the use of recombinant _S. neurona_ surface antigens obviates the need to propagate parasites in tissue culture. Relative to propagation of the parasite in tissue culture, production of the recombinant proteins described herein is inexpensive and simple.

An important tool is therefore provided for detection of _S. neurona_ infection, as well as for in-depth examination of the equine humoral response to such infection. For example, combining rSnSAGs in a single ELISA, along with investigation using larger sample sets with more negative controls, may prove useful as serodiagnostic tests due to the high sensitivity and specificity exhibited.

The foregoing descriptions have been presented for purposes of illustration and description. The descriptions are not intended to be exhaustive or to limit the invention to the precise form disclosed. Obvious modifications or variations are possible in light of the above testing. The embodiment was chosen and described to provide the best illustration of the principles of the invention and its practical application to thereby enable one of ordinary skill in the art to utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. All such modifications and variations are within the scope of the invention as determined by the claims made in this application when interpreted in accordance with the breadth to which they are fairly, legally and equitably entitled.

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23

Expression of Recombinant _S. neurona_ Surface Antigens (rSnSAGs) in Mammalian Cells

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**SEQ ID NO: 11**
**LENGTH: 994**
**TYPE: DNA**
**ORIGIN: Sarcocystis neurona**

**SEQUENCE: 11**

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<210> SEQ ID NO: 13
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<222> LOCATION: (746)..(746)
<223> OTHER INFORMATION: n is a, c, g, or t

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<212> TYPE: PRT
<213> ORGANISM: Sarcoctisia neura

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35 40 45
Gln Glu Ser Ala Val Glu Asp Gly Thr Glu Ala Asp Ser Asp Leu
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Arg Phe Glu Arg Leu Ala Leu His Ile Val Ser Ala Val Ala Ser
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| caacccctga aacccctctat cacaacagc gcggactcct gttctcttcac ccagacttcg | 180 |
| tctggttgac gcggcaggtgc ctctctcttg tctggttcac gtcggaggaag | 240 |
| atgtacagcg aagttccact acagcgaata ccggcggaa ccagcgggga cccttgaga | 300 |
| atcgccagtc gcggccactg gcggcaggtgc gttgatcgac tcacagga | 360 |
| gacactgtaa gcaggttcgg ctctctctcg gcagctttat cttcttgcc gcgagtcttg | 420 |
| tgcaccttttg gcgcgctgcgc aagcgccgca tcgcagctcg gcagccttcg ccattcttc | 480 |
| tggcggttgc aacggcagca gcggggcag aaggtcttc gagctcgacgc ccacaccaga | 540 |
| gcgcagctgc ccggcagctcg gcggcgcgtt gcgggacgc tcggacgcaa gcgttacctg | 600 |
| cgacacgcag gcgcagccag gcggccctag gttcttccttg tctggttttc ccaggttc | 660 |
| gttggttgtc gcggttcggct aagctcaagt ctggtgacgc gcggccctagct gagctcagc | 720 |
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<210> SEQ ID NO 18
<211> LENGTH: 68
<212> TYPE: PRT
<213> ORGANISM: Sarcocystis neurona

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<210> SEQ ID NO 19
<211> LENGTH: 811
<212> TYPE: DNA
<213> ORGANISM: Sarcocystis neurona

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<211> LENGTH: 127
<212> TYPE: PRT
<213> ORGANISM: Sarcocystis neurona

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Ala Ser Asn Leu Arg Gly Gly Ser Val Tyr Thr Pro Glu Ala Pro
35     40     45
Gln Glu Ser Ala Val Val Glu Ala Gly Thr Glu Glu Ser Gly Val
50     55     60
Ala Thr Leu Glu Leu Arg Asp Ala Leu Ser Glu Val Gly Gln Gly Met
65     70     75     80
Arg Met Ala Leu His Gly Ile Ser Thr Val Val Ser Val Leu Aep Gly
85     90     95
Val Leu Gly Aep Met Phe Pro Ala Thr Ala Glu Gin Arg Gin Pro Ile
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Gln Phe Pro His Leu Gin Arg Leu Leu Arg Arg Leu Ala Met Aep
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<211> LENGTH: 1493
<212> TYPE: DNA
<213> ORGANISM: Sarcocystis neurona

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<211> LENGTH: 276
<212> TYPE: PRF
<213> ORGANISM: Sarcocystis neurona

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Gly Gin Met Gin Val Tyr Ala Thr Ala Val Ala Glu Asn Pro Val Asn
65  70  75  80
Ile Arg Asp Val Leu Pro Gly Ala Ser Tyr Leu Ser Val Gin Asn Val
85  90  95
Pro Thr Leu Thr Val Pro Gin Leu Pro Ala Lys Ala Thr Ser Val Phe
100 105 110
Phe His Cys Gin Gin Gin Pro Asp Asn Gin Cys Phe Ile Gin Val Glu
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Val Ala Pro Ala Pro Arg Leu Gly Pro Asn Thr Cys Ala Ala Leu Gin
130 135 140
Ser Thr Ile Ala Phe Glu Val Gin Gin Ala Asn Glu Thr Ala Val Phe
145 150 155 160
Ser Cys Gly Glu Gly Leu Ala Val Phe Pro Gin Gly Ser Lys Ala Leu
165 170 175
Asp Glu Ala Cys Ser Lys Glu Gin Ala Leu Pro Ser Gly Ala Ala Leu
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Ala Pro Lys Asp Gly Leu His Leu Gly Phe Pro Gin Leu Pro Gin
195 200 205
Gln Ala Met Lys Ile Cys Tyr Ile Cys Thr Asn Gly Val Gin Ala
210 215 220
Glu Ala Ala Gin Arg Cys Glu Val Arg Ile Ser Val Ala Ala Asn Pro
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<211> LENGTH: 168
<212> TYPE: PRT
<213> ORGANISM: Sarcocystis neurona

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Gln Ser Phe Val Leu Aas Cys Gln Ala Pro Phe Thr Ile Ala Thr Pro 50 55 60
Ala Aas Phe His Thr His Ala Cys Ala Gly Thr Gly Ala Aas Cys Gln 65 70 75 80
Aas Pro Glu Thr Tyr Ala Lys Leu Phe Pro Lys Ala Ser Aas His Val 85 90 95
Trp Val Ser Pro Ala Asp Ser Thr Ser Ala Thr His Thr Trp Thr Ala 100 105 110
Pro Ala Ala Aas Gln Leu Ser Gly Lys Thr Val Phe Ser Val Gly Cys 115 120 125
Thr Ser Thr Gly Asp Pro Ala Gly Ile Cys Ala Val Aas Val Thr Val 130 135 140
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LENGTH: 1585
TYPE: DNA
ORGANISM: Sarcocystis neurona

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LOCATION: (1557)...(1558)
OTHER INFORMATION: n is a, c, g, or t

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<210> SEQ ID NO 27
<211> TYPE: DNA
<213> ORGANISM: Saccharomyces cerevisiae
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (267)...(267)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 27

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cgacactgtg tgtatctgct gttatataac ttgctgttcg ttatacaat gctgtagcacc 180
ggaaccgga gcggcttaca tctgccttcg ggcagaccaac agogyttaacc gatctgtgaa 240
cattggtgg cctctaatt gatgacacaa acggttcacac tttgtaacag gttctatgcg 300
cccgagggcc gcggcgaggg gcgcaggggc gcggatgtgt tttttctctca gatcaagqaga 360
acccagacgg agaggttttcga gaaagtcgggg tgccttgggtc tatacctgca tggagccaaa 420
ataatcagoc caacgttctca aagtaagggc agcgtgctct gggccctcaag cgacatatgt 480
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<210> SEQ ID NO 28
<211> LENGTH: 67
<212> TYPE: PRT
<213> ORGANISM: Sarcocystis neurona
<220> FEATURE:
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<222> LOCATION: (59) . (59)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 28

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Gln Ala Thr Cys Val Leu Gly Gln Ala Thr Ala Val Thr Glu Leu Val
35    40    45
Thr Phe Gly Gly Leu Asn Ile Val Cys Xaa Asn Gly Ser Thr Leu Gln
50    55    60
Gln Val Pro Ala Ala Pro Gly Ala Ala Asp Gly Ala Gln Gly Ala Gly
65    70    75    80
Tyr Val Phe Ser Ser Asp Glu Gln Arg Gln Gly Val Val Leu Glu
85    90    95
Gln Val Val Pro Gly Ala Ile Phe Ala Val Gly Gln Asn Asn Gln Pro
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Asn Val Leu Asn Val Ala Gln Leu Pro Ser Ala Pro Gln Ser Ile Tyr
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Phe Leu Cys Arg Pro Gln Glu Asn Glu Gln Thr Cys Phe Ile Arg
130   135   140
Val Asn Ile Pro Ala Ser Pro Pro Leu Gly Pro Asn Ala Cys Val Val
145   150   155   160
His Asn Thr Glu Val Gln Phe Lys Ala Gly Ser Ser Asn Ala Thr Val
165   170   175
Gln Phe Ser Cys Gly Asn Ala Ala Leu Gln Pro Gln Gln Ala Thr
180   185   190
Lys Ile Phe Asp Gln Thr Cys Gln Glu Leu Gly Leu Asp Thr Val
195   200   205
Thr Pro Gly Ala Thr Cys Gln Arg Pro Ala Ala Gly Gly Met Val Thr
205   210   215   220
Val Thr Phe Pro Arg Leu Pro Pro Glu Asn Arg Lys Leu Cys Phe Val
225   230   235   240
Cys Thr Arg Gly Gln Glu Asn Cys Lys Val Ile Ile Asp Val Ala Ala
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Ala Leu Gly Ile Val Val Ala Ala Gly Leu Val Gly Val Phe
275   280   285
<400> SEQUENCE: 29

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Gln Glu Tyr Leu Asp Lys Lys Gly Arg Ser Arg Leu Pro Gln Gly Phe
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Ser Asn Lys Ala Val Gln Thr Ala Ser His Val Gly Val Leu Val Leu
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Thr Cys Val Ala Leu Pro Leu Val Leu Leu Met His
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<210> SEQ ID NO 31
<211> LENGTH: 240
<212> TYPE: PRT
<213> ORGANISM: Sarcocystis neurona

<400> SEQUENCE: 31

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Ile His Cys Pro Asn Asn Phe Arg Leu Ala Pro Arg Ala Gly Asn Asp
   35 40 45
Ala Gly Gln Met Gln Val Tyr Ala Thr Ala Val Ala Glu Asn Pro Val
   50 55 60
Asn Ile Arg Asp Val Leu Pro Gly Ala Ser Tyr Leu Ser Val Gln Asn
   65 70 75 80
Val Pro Thr Leu Thr Val Pro Gln Leu Pro Ala Lys Ala Thr Ser Val
   85 90 95
Phe Phe His Cys Gln Gln Gln Pro Asp Asn Gln Cys Phe Ile Gln Val
  100 105 110
Glu Val Ala Pro Ala Pro Arg Leu Gly Pro Asn Thr Cys Ala Ala Leu
  115 120 125
Gln Ser Thr Ile Ala Phe Glu Val Gln Gln Gln Asn Glu Thr Ala Val
  130 135 140
Phe Ser Cys Gly Glu Gly Leu Ala Val Phe Pro Gln Gly Ser Lys Ala
  145 150 155 160
Leu Asp Glu Ala Cys Ser Lys Glu Gln Ala Leu Pro Ser Gly Ala Ala
  165 170 175
Leu Ala Pro Lys Asp Gly Gly Leu His Leu Gly Phe Pro Gln Leu Pro
  180 185 190
Gln Gln Ala Met Lys Ile Cys Tyr Ile Cys Thr Asn Gly Gly Val Gln
  195 200 205
Ala Glu Ala Ala Arg Cys Glu Val Arg Ile Ser Val Ala Ala Asn
  210 215 220
Pro Asp Gly Ser Val Pro Gly Ala Asn Gly Ala Ala Ser Leu Gly Ala
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<210> SEQ ID NO 32
<211> LENGTH: 232
<212> TYPE: PRT
<213> ORGANISM: Sarcocystis neurona

<400> SEQUENCE: 32

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<210> SEQ ID NO 33
<211> LENGTH: 226
<212> TYPE: PRT
<213> ORGANISM: Sarcocystis neurona

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Cys Pro Gly Glu Thr Thr Leu Ala Asn Arg Gin Gin Glu Gin Ala Asp 35 40 45
Asn Gly Pro Thr Ala Glu Val Tyr Ser Glu Ala Asp Ala Gly Lys Asn 50 55 60
Val Ala Leu Asn Thr Leu Val Gly Gin Thr Tyr Val Arg Ala Asp 65 70 75 80
Ala Asn Asp Ala Asn Leu Thr Val Ser Gin Leu Pro Thr Lys Ala Val Thr 85 90 95
Val Leu Phe Leu Cys Asn Arg Gin Pro Gly Pro Gly Gin Val Gly Cys Trp 100 105 110
Thr Ala Val Glu Val Ala Ala Gln Pro Pro Leu Gly Pro Gin Ala Cys 115 120 125
Thr Val Gly Gin Ser Gin Val Thr Leu Thr Val Thr Ala Asn Ala Thr 130 135 140
Thr Ala Gin Phe Ala Cys Ala Ala Thr Lys Asn Val Phe Pro Glu Gly 145 150 155 160
Thr Asn Val Tyr Asn Ser Asp Cys Lys Thr Glu Thr Pro Leu Ser Thr 165 170 175
-continued

Ala Leu Pro Gly Ala Thr Leu Thr Arg Gly Asn Leu Ala Leu Lys
 180 190
Ile Pro Thr Leu Pro Ser Ala Ala Lys Asn Leu Cys Phe Val Cys Ala
 195 205
Thr Asn Val Gly Asp Glu Ala Asn Gln Lys Cys Ser Val Lys Ile Asn
 210 220
Val Ser
225

<210> SEQ ID NO 34
<211> LENGTH: 231
<212> TYPE: PRT
<213> ORGANISM: Sarcocystis muris

<400> SEQUENCE: 34

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Pro Ser Ser Thr Thr Phe Leu Pro Thr Tyr Gly Asp Ala Gly Thr
 10 11 12 13
Gln Thr Val Tyr Leu Thr Gln Asp Gly Ser Ser Thr Glu Lys Leu Gln
 14 15 16 17
Thr Ala Leu Pro Gly Ala Thr Ala Lys Gln Glu Asp Ser Gln Thr Asn
 18 19 20 21
Glu Met Thr Leu Thr Phe Pro Gln Leu Pro Asp Thr Ser Gln Thr Val
 22 23 24 25 26
Tyr Phe His Cys Leu Gly Thr Glu Asn Ile Ala Gly Gln Gly Ser Arg
 27 28 29 30 31
Lys Glu Val Cys Gly Phe Ala Val Thr Leu Thr Ala Pro Pro Pro Gln
 32 33 34 35 36
Gly Pro Gln Ala Cys Val Val Pro Gly Thr Ile Arg Leu Gly Ile
 37 38 39 40 41
Ala Asn Glu Gly Asp Thr Arg Phe Thr Cys Gly Gly Asp Leu Lys
 42 43 44 45 46
Leu Ser Pro Thr Ala Ala Asp Lys Val Phe Lys Glu Asp Cys Ser Thr
 47 48 49 50 51
Glu Glu Ser Leu Lys Asp Leu Lys Arg Ser Glu Asp Lys Asn Ser Tyr
 52 53 54 55 56
Phe Val Leu Thr Ala Thr Lys Thr Pro Ser Lys Thr His Cys Tyr
 57 58 59 60 61
Leu Cys Glu Pro Asp Pro Thr Lys Gly His Asn Asp Lys Asn Cys
 62 63 64 65 66
Ala Val Leu Ile Ala Val Gly
225 226 227 228 229

<210> SEQ ID NO 35
<211> LENGTH: 267
<212> TYPE: PRT
<213> ORGANISM: Toxoplasma gondii

<400> SEQUENCE: 35

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Thr Val Lys Phe Lys Cys Gly Ala Ser Leu Pro Thr Leu Tyr Pro Ala
Thr Val Ser Glu Gly Val Glu Tyr Ser Phe Thr Thr Ser Lys Trp Pro
Asp Ser Ala Gly Ser Ile Phe Phe Ser Cys Lys Pro Asn Pro Pro Thr
Pro Pro Ser Ala Leu Arg Glu Ala Glu Asp Pro Glu Ser Thr Thr
Ser Ala Ala Asp Ala Cys Thr Val Arg Ile Gly Ile Arg Gly Lys Pro
Glu Lys Glu Ile Pro Ser Tyr Glu Cys Ser Thr Pro Thr Gly Glu Arg
Phe Phe Arg Val Asp Ser Gly Asp Ala Val Ser Phe Ser Cys Gly
 Ala Glu Met Ala Leu Glu Thr Gln Thr His Ala Tyr Gln Thr Ala Glu
Cys Thr Asp Leu Thr Pro Leu Thr Leu Leu Pro Ser Ala Ser Leu
Thr Gln Asp Thr Ser Gln Ser Gly Thr Leu Glu Asn Pro Leu Tyr Thr
Leu Thr Val Pro Gln Leu Pro Gly Glu Pro Ile Asn Gln Leu Cys Phe
Leu Cys Lys Ser Lys Glu Ser Ser Ser Thr Pro Asp Val Cys Lys
 Val Leu Ile Gly Phe Glu Thr Leu Pro Asn Asp

<210> SEQ ID NO 36
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Sarcocystis neurona
<400> SEQUENCES: 36
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Ile Ala Thr Pro Ala Asn Phe His Thr His Ala Cys Ala Gly Thr Gly
Ala Asn Cys Glu Asn Pro Glu Thr Tyr Ala Lys Leu Phe Pro Lys Ala
Ser Asn His Val Trp Val Ser Pro Ala Asp Ser Thr Ser Ala Thr His
Thr Trp Thr Ala Pro Ala Ala Asn Gln Leu Ser Gly Lys Thr Val Phe
Ser Val Gly Cys Thr Ser Thr Gly Asp Pro Ala Gly Ile Cys Ala Val
Asp Val Thr Val Ser Ser

<210> SEQ ID NO 37
What is claimed is:

1. A composition comprising an isolated nucleic acid set forth in the Sequence Listing as SEQ ID NO: 25, and sequences fully complementary thereto.

2. A vector comprising the nucleic acid of claim 1.

3. The vector of claim 2 in a host that expresses a polypeptide encoded by the nucleic acid.

4. The vector of claim 2 wherein the vector is selected from the group consisting of an Escherichia coli bacteria and an Alpha virus.

5. The composition of claim 1 wherein the isolated nucleic acid is capable of hybridizing under stringent conditions with a nucleic acid from Sarcozystis neurona.

6. The composition of claim 1, further comprising a pharmaceutically acceptable carrier.

7. A composition comprising an isolated nucleic acid capable of encoding an antigenic protein derived from Sarcozystis neurona, comprising a nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:25 and sequences fully complementary thereto.

8. A vector comprising the nucleic acid of claim 7.

9. The vector of claim 8 in a host that expresses the polypeptide encoded by the nucleic acid.

10. The vector of claim 8 wherein the vector is selected from the group consisting of an Escherichia coli bacteria and an Alpha virus.

11. The composition of claim 5 wherein the isolated nucleic acid is capable of hybridizing under conditions of low stringency with a nucleic acid from Sarcozystis neurona.

12. The composition of claim 5 wherein the isolated nucleic acid is capable of hybridizing under conditions of moderate stringency with a nucleic acid from Sarcozystis neurona.

13. The composition of claim 5 wherein the isolated nucleic acid is capable of hybridizing under conditions of high stringency with a nucleic acid from Sarcozystis neurona.

14. An isolated nucleic acid sequence comprising the nucleotide sequence of SEQ ID NO: 25, or a degenerate variant thereof, that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 26.

15. A vector comprising the nucleic acid of claim 14.

16. The vector of claim 15 in a host that expresses the polypeptide encoded by the nucleic acid.

17. A composition comprising (a) an isolated agent comprising a nucleotide sequence set forth in the Sequence Listing as SEQ ID NO: 25 or a degenerate variant thereof; that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 26 and (b) a pharmaceutically acceptable carrier.

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