

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

APPLICATION FOR A STANDARD PATENT

Akzo N.V., incorporated in the Kingdom of The Netherlands, of Velperweg 76, 6824 BM Arnhem, THE NETHERLANDS, hereby apply for the grant of a standard patent for an invention entitled:

Eimeria Tenella Vaccine

which is described in the accompanying complete specification.

Details of basic application(s):-

<u>Basic Applic. No:</u>	<u>Country:</u>	<u>Application Date:</u>
89/4726	ZA	21 June 1989

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DATED this TWENTY EIGHTH day of DECEMBER 1989

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By:



Registered Patent Attorney

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DECLARATION IN SUPPORT OF A
CONVENTION APPLICATION FOR A PATENTAUSTRALIA
CONVENTION
STANDARD
& PETTY PATENT
DECLARATIONIn support of the Convention Application made for a
patent for an invention entitled:

Title of Invention

Eimeria Tenella Vaccine

-I/We Franciscus Guilielmus Maria Hermans and Cees Hoek,

Full name(s) and both
address(es) of
Declarant(s)

care

of Akzo N.V.,
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The Netherlands

do solemnly and sincerely declare as follows:-

Full name(s) of
Applicant(s)1. ~~I am/We are the applicant(s) for the patent~~

(or, in the case of an application by a body corporate)

1. ~~I am/We are authorised by~~ Akzo N.V.the applicant(s) for the patent to make this declaration on
its/~~their~~ behalf.2. The basic application(s) as defined by Section 141 of the
Act was/~~were~~ made

Basic Country(ies)

in Republic of South Africa

• Priority Date(s)

on 21 June 1989

• Basic Applicant(s)

by Akzo N.V.

• Full name(s) and
address(es) of
inventor(s)~~3. I am/We are the actual inventor(s) of the invention referred
to in the basic application(s)~~

(or where a person other than the inventor is the applicant)

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(respectively)

~~is/are~~ the actual inventor(s) of the invention and the facts upon
which the applicant(s) ~~is/are~~ entitled to make the application are
as follows:Akzo N.V. is entitled by Contract of Employment between
the inventors as employees and Akzo N.V. as employer, as a
person who would be entitled to have the patent assigned to
it if a patent were granted upon an application made by the
inventors.4. The basic application(s) referred to in paragraph 2 of this
Declaration was/~~were~~ the first application(s) made in a Convention
country in respect of the invention(s) the subject of the application.

Declared at Oss this 15th day of December 1989

F.G.M. Hermans

Proxy

C. Hoek

Proxy

Signature of Declarant(s)

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EIMERIA TENELLA VACCINE

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(57) The invention relates to a nucleic acid sequence encoding an Eimeria tenella polypeptide, a recombinant nucleic acid molecule comprising such a nucleic acid sequence, a vector or a host cell containing said nucleic acid sequence, a polypeptide of Eimeria tenella, vaccines against coccidiosis which are based on these products as well as an antibody or antiserum immuno-reactive with said polypeptide.

Coccidiosis is a disease which is caused by intra-cellular parasites, protozoa, of the subphylum Apicomplexa and the genus Eimeria. These parasites multiply in cells which form part of the gastrointestinal tract and digestive organs of their hosts.

Claim

1. A nucleic acid sequence substantially encoding at least an immunogenic part of a protein of Eimeria tenella, a portion of said protein being defined by the amino acid sequence represented in Figure 2.

8. A vaccine for the protection of poultry against coccidiosis comprising the expression product of a nucleic acid sequence according to claim 1 or claim 2, the expression product of a recombinant nucleic acid molecule according to claim 3, a vector virus according to claim 4, a host cell according to claim 5, or a polypeptide according to claim 6 or claim 7.

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COMPLETE SPECIFICATION

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Complete Specification Lodged:

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Complete Specification for the invention entitled:

Eimeria Tenella Vaccine

The following statement is a full description of this invention, including the
best method of performing it known to me/us

ABSTRACT

The invention is concerned with a polypeptide of Eimeria tenella which can be used for the immunization of chickens against coccidiosis.

The invention also relates to a nucleic acid sequence encoding such a polypeptide. Said nucleic acid sequence is especially useful for the preparation of vector vaccines.

Eimeria tenella vaccine

The invention relates to a nucleic acid sequence encoding an Eimeria tenella polypeptide, a recombinant nucleic acid molecule comprising such a nucleic acid sequence, a vector or a host cell containing said nucleic acid sequence, a polypeptide of Eimeria tenella, vaccines against coccidiosis which are based on these products as well as an antibody or antiserum immuno-reactive with said polypeptide.

Coccidiosis is a disease which is caused by intracellular parasites, protozoa, of the subphylum Apicomplexa and the genus Eimeria. These parasites multiply in cells which form part of the gastrointestinal tract and digestive organs of their hosts.

Due to the increasing intensive production, the damage which is caused by these parasites in the poultry industry has risen alarmingly in recent decades. The losses which poultry farmers in the Netherlands suffer every year run into millions of guilders; the loss in 1986 was about 13 million guilders. In the same year a loss of U.S. \$ 300 million was suffered in the U.S., despite the use of coccidiostats.

The pathogens of coccidiosis in chickens can be subdivided into nine different types, i.e. Eimeria acervulina, E. maxima, E. tenella, E. necatrix, E. brunetti, E. mitis, E. praecox, E. mivati and E. hagani. However, some people doubt the existence of the last two types. All of these types have only the chicken as host and display a high degree of tissue specificity. The life cycles of the said types are, however, similar.

During the life cycle, the Eimeria parasites pass through a number of stages. The infectious stage (the sporulating oocyst) is taken in orally and passes into the stomach of the chicken, where the shell of the cyst bursts open as a result of the grinding action. The four sporocysts, which this oocyst contains, are released and pass into the duodenum, whereby they are exposed to bile and digestive enzymes. As a result, an opening is made in the sporocyst wall and the sporozoites present in the sporocyst are released. These sporozoites are mobile and search for suitable host cells, for example epithelium cells, in order to penetrate and to reproduce. Depending on the type, this first reproduction phase lasts 20 to 48 hours and several tens to hundreds of merozoites are formed, which each again penetrate a new host cell and reproduce. After two to sometimes five of these asexual reproduction cycles, the intracellular merozoites grow into sexual forms, the male and female gametocytes. After fertilization of the female by a male gamete, a zygote is formed which creates a cyst wall around itself. This oocyst leaves the host cell and is driven out with the faeces. If the temperature and humidity outside the chicken are relatively high and, at the same time, there is sufficient oxygen in the air, the oocyst can sporulate to the infectious stage.

Thus, no intermediate host is needed for transfer of the parasite from chicken to chicken. It is therefore conceivable that with a high degree of occupation of the available surface area the infection pressure in a chicken farm rapidly increases.

The parasite can be combatted in various ways.

In addition to using good management, coccidiosis can be combatted by using combatting agents which frequently are mixed in the feed or drinking water. However, these agents have suffered a drop in effectiveness in recent years, partly because of the high genetic capacity of the parasite to develop a resistance towards various combatting agents. In addition, a number of these agents leave residues in the meat which can give rise to problems on consumption.

Immunological prophylaxis would, therefore, constitute a much better combatting method. It is known that chickens which have lived through a sufficiently high infection are able to resist a subsequent contact with the same type of Eimeria. Resistance towards Eimeria can also be induced by infecting the birds several times with low doses of oocysts or with oocysts of weakened (non-pathogenic) strains. However, controlled administration to, specifically, large numbers of chickens for slaughter is a virtually insurmountable problem in this case. Inactivated vaccines therefore appear to be the only remaining solution.

An inactivated vaccine can consist of an antigen originating from the parasite, possibly with an adjuvant.

As an alternative for an antigen isolated from parasites, it is possible to use a product prepared with the aid of recombinant DNA technology, a technique which can be carried out according to known methods.

Moreover, vaccination can be carried out by administering a live host organism such as a bacterium, a fungus or a virus in which a gene coding the antigen has been incorporated. This organism then ensures adequate long-term synthesis of antigen so that the immune system of the chicken is adequately stimulated.

At the same time it is possible synthetically to reproduce the antigen or parts thereof and to administer this to the birds in an immunologically recognizable and stimulating form, for example bonded to a carrier protein in the presence of an adjuvant.

5 According to a first embodiment of this invention, there is provided a nucleic acid sequence substantially encoding at least an immunogenic part of a protein of Eimeria tenella, a portion of said protein being defined by the amino acid sequence represented in Figure 2.

According to a second embodiment of this invention, there is
10 provided a recombinant nucleic acid molecule comprising a nucleic acid sequence according to the first embodiment, operably linked to control sequences enabling expression of said nucleic acid sequence.

According to a third embodiment of this invention, there is provided a vector virus containing a recombinant nucleic acid molecule
15 according to the second embodiment.

According to a fourth embodiment of this invention, there is provided a host cell containing a nucleic acid sequence according to the first embodiment, a recombinant nucleic acid molecule according to the second embodiment or a vector virus according to the third embodiment.

20 According to a fifth embodiment of this invention, there is provided a polypeptide with an amino acid sequence of which at least a part is coded by a nucleic acid sequence according to the first embodiment.

According to a sixth embodiment of this invention, there is
25 provided a polypeptide comprising at least an immunogenic part of the amino acid sequence represented in Figure 2.

According to a seventh embodiment of this invention, there is provided a vaccine for the protection of poultry against coccidiosis comprising the expression product of a nucleic acid sequence according to
30 the first embodiment, the expression product of a recombinant nucleic acid molecule according to the second embodiment, a vector virus according to the third embodiment, a host cell according to the fourth embodiment, or a polypeptide according to the fifth and sixth embodiments.

According to an eighth embodiment of this invention, there is
35 provided an antibody or antiserum immuno-reactive with a polypeptide according to the fifth or sixth embodiments.



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According to the present invention a nucleic acid sequence substantially encoding at least a part of a protein of Eimeria tenella, a portion of said protein being defined by the amino acid sequence represented in Figure 2, can be applied for the preparation of a vaccine for the immunization of poultry against coccidiosis.

"Nucleic acid sequence" as used herein refers to a polymeric form of nucleotides of any length, both to ribonucleic acid sequence and to deoxyribonucleic acid sequences. In principle, this term refers to the primary structure of the molecule. Thus, this term includes double- and single stranded DNA, as well as double- and single stranded RNA and modifications thereof.

A nucleic acid sequence encoding a portion of an Eimeria tenella protein, said portion having an amino acid sequence shown in Figure 2 (named Et1A1) is present in phage λ gt10Et1A1. Escherichia coli strain K12 BNN 102 λ gt10Et1A1 was deposited with the Central Bureau voor Schimmelcultures, Baarn (The Netherlands) on 21 June 1989 under no. CBS 286.89.

The phage λ gt10Et1A1 is prepared by first constructing a cDNA library from sporulated E. tenella oocysts. Screening of this cDNA library has been carried out with a labeled 296 bp EcoRI probe present in plasmid pEa1A. Escherichia coli strain K12Ja221 containing plasmid pEa1A was deposited with the Central Bureau voor Schimmelcultures, Baarn (The Netherlands) on 12 February 1988 under no. CBS 143.88.

Subsequently, the E. tenella clone comprising a nucleic acid sequence according to the invention was plaque-purified. The DNA sequence inserted into λ gt10Et1A1 can be isolated from this phage clone. A restriction enzyme map of Et1A1 is prepared (Figure 1).

The nucleotide sequence which is determined for the cDNA section of abovementioned insertion is given in Figure 2, likewise the amino acid sequence derived herefrom.

Said sequences only represent a portion of a protein of Eimeria tenella. With respect to the nucleotide sequence, the 5'-end of the gene encoding the E. tenella protein is not present, the N-terminus probably being absent from the amino acid sequence of the protein of E. tenella.

The present invention comprises a nucleic acid sequence encoding a polypeptide having an amino acid sequence shown in Figure 2 or antigenic fragments thereof as well as a nucleic acid sequence encoding the whole protein of E. tenella a portion of which is defined by said amino acid sequence, or antigenic fragments thereof.

5' Nucleic acid sequences of the E. tenella protein not present in Figure 2 can be obtained by standard molecular biology techniques, for example by isolating RNA of E. tenella, hybridizing said RNA with a primer derived from the 5' side of the present nucleic acid sequence and extending this primer with, e.g. reverse transcriptase. Thereafter, the newly synthesized DNA can be converted to its double-stranded form using standard molecular biology techniques cloned, in a suitable vector and sequenced subsequently.

Another possibility is screening the cDNA library mentioned above with a restriction fragment derived from the 5' side of the present nucleic acid sequence and isolating clones comprising DNA sequences which overlap with the nucleic acid sequence shown in Figure 2 but comprise additional 5' sequences.

A deoxynucleic acid sequence encoding a portion of an E. tenella protein is shown in Figure 2. This cDNA sequence is 1970 nucleotides in length (including the stop codon). A nucleic acid sequence substantially comprising said cDNA sequence or a fragment thereof as well as a nucleic acid sequence comprising beside said cDNA or fragments thereof additional nucleotides corresponding to the E. tenella protein, e.g. the complete gene encoding the E. tenella protein, form part of the present invention.

As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in an other codon but still coding for the same amino acid, e.g. the codon for the amino acid glutamic acid is both GAT and GAA. Consequently, it is clear that for the expression of a polypeptide with the amino acid sequence shown in Figure 2, or an antigenic fragment thereof use can be made of a nucleic acid sequence with such an alternative codon composition different from the nucleic acid sequence shown in Figure 2.

Also included within the scope of the invention is a nucleic acid sequence which for at least a part displays a substantial homology with a nucleic acid sequence shown in Figure 2, or with a fragment thereof but may comprise nucleotide substitutions, mutations, insertions, deletions, inversions etc. and encodes a protein or polypeptide which is functionally equivalent to the polypeptide shown in Figure 2 or a fragment thereof.

The present invention comprises also a polypeptide which is encoded by a nucleic acid sequence mentioned above and which can be used for the immunization of poultry against coccidiosis.

Furthermore, a polypeptide substantially comprising at least part of the amino acid sequence represented in Figure 2 is included in the present invention.

The term polypeptide refers to a molecular chain of amino acids and does not refer to a specific length of the product; thus, inter alia peptides oligopeptides and proteins are included within the definition of polypeptide.

It will be understood that for the particular polypeptide shown in Figure 2, embraced herein, natural variations can exist. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said polypeptide.

Moreover, the potential exists in the use of recombinant DNA technology for the preparation of various derivatives of the polypeptide shown in Figure 2, variously modified by resultant single or multiple amino acid substitutions, deletions, additions or replacements. All abovementioned modifications resulting in derivatives of the polypeptide shown in Figure 2 or fragments thereof as well as a polypeptide comprising such a derivative or fragment thereof are included within the scope of this invention so long as the essential, characteristic activity of the polypeptide shown in Figure 2 or an antigenic fragment thereof, remains unaffected in essence.

In addition, fragments of these polypeptides, which can be used for immunization of poultry against coccidiosis, also form part of the invention. Various methods are known for detecting such usable polypeptide fragments (termed epitopes) within a known or unknown amino acid sequence. On the basis of a known amino acid sequence, these epitopes can, for example, be determined experimentally with the aid of the screening techniques described in patent publications WO 84/03564 and WO 86/06487.

In addition, a number of regions of the polypeptide, can be designated epitopes on the basis of theoretical considerations and structural agreement with epitopes which are now known. The determination of these regions was based on a combination of the hydrophilicity criteria according to J.P. Hopp and K.R. Woods (ref. 5) and the secondary structure aspects according to P.Y. Chou and G.D. Fasman (ref. 6).

T-cell epitopes which may be necessary can likewise be derived on theoretical grounds with the aid of Berzofsky's amphiphilicity criterion (ref. 7).

For immunization against coccidiosis infection in accordance with the present invention it is also possible to use, for example, anti-idiotypic antibodies or antigen-binding fragments thereof. Such antibodies are directed against the idiotype of antibodies, which, in turn, are directed against the polypeptide according to the invention. The immunogenic equivalents of the polypeptide according to the invention which have been indicated above are understood to mean, inter alia, anti-idiotypic antibodies of this type.

A nucleic acid sequence according to the present invention can be ligated to various expression effecting DNA sequences, optionally containing portions of polypeptide encoding sequences such as β -galactosidase, resulting in a so called recombinant nucleic acid molecule which can be used for the transformation of a suitable host. Such hybrid DNA molecules, are preferably derived from, for example plasmids, or from nucleic acid sequences present in bacteriophages or viruses. "Transformation", as used herein, refers to the introduction of a heterologous nucleic acid sequence into a host cell, irrespective of the method used, for example direct uptake or transduction. The heterologous nucleic acid sequence may be maintained through autonomous replication or alternatively, may be integrated into the host genome. The recombinant DNA molecules preferably are

provided with appropriate control sequences compatible with the designated host which can regulate the expression of the inserted nucleic acid sequence.

A suitable host cell is a cell which can be transformed by a nucleic acid sequence encoding a polypeptide or by a recombinant nucleic acid molecule comprising such a nucleic acid sequence and which can be used to express said polypeptide coded by said nucleic acid sequence. The host cell can be of procaryotic origin, e.g. bacteria such as *E. coli*, *B. subtilis* and *Pseudomonas* species; or of eucaryotic origin such as yeasts, e.g. *Saccharomyces cerevisiae* or higher eucaryotic cells such as insect, plant or mammalian cells, including HeLa cells and Chinese hamster ovary (CHO) cells.

The intended immunization can, for example, be effected by administering the present polypeptide, or an immunogenic section or equivalent thereof, as such to the birds, or by administering to the birds to be immunized a microorganism which has been genetically modified by a recombinant DNA and which is able to produce the polypeptide, or an immunogenic section or equivalent thereof, in situ.

For immunization of poultry against coccidiosis in accordance with the present invention, it is possible, on the one hand, to administer the present polypeptides, fragments or immunogenic equivalents as such to the birds or, on the other hand, if desired to administer microorganisms which by genetic manipulation have acquired the ability to produce the present polypeptides etc. "Subunit vaccines" is a frequently used term for the first case and the term "vector vaccines" is usually used for the second case - we will also adopt this nomenclature here.

The subunit vaccines according to the invention in general contain the polypeptides in purified form, optionally in the presence of a pharmaceutically acceptable excipient. The polypeptide can optionally be covalently bonded to a non-related protein, which, for example, can be of advantage in the purification of the fusion product. Examples are β -galactosidase, protein A, prochymosine, blood clotting factor Xa, etc.

The polypeptides for such applications can be prepared with the aid of known methods, such as by isolation from E.tenella, by means of recombinant DNA techniques or by peptide synthesis.

If desired, the polypeptides can also be modified in vivo or in vitro by, for example, glycosylation, amidation, carboxylation or phosphorylation.

In vector vaccines, the polypeptide product according to the invention is made up by a genetically manipulated organism which is itself administered to the individual to be immunized and which maintains itself for some time, or even reproduces, in the body. Diverse organisms can be used as the host for this purpose, such as, for example, bacteria such as Escherichia coli, Bacillus, or Salmonella, or viruses such as cowpox or avian pox virus. With host organisms of this type, the polypeptide can express itself as a surface antigen. In this context fusion of the said polypeptide with OMP proteins or pilus proteins of Escherichia coli or synthetic provision of signal and anchor sequences which are recognized by the organism are conceivable. It is also possible that the said immunogenic polypeptide, if desired as part of a larger whole, is released inside the animal to be immunized. In all of these cases it is also possible that one or more immunogenic products will find expression which generate protection against various pathogens and/or against various antigens of a given pathogen.

Regarding the substantial homology between the nucleic acid sequence according to the invention and a DNA fragment, encoding a protective polypeptide, derived from *E. acervulina* (Example 2) it is anticipated that said nucleic acid sequence or a corresponding polypeptide can be used to vaccinate poultry against other species of *Eimeria*, in particular *E. acervulina*.

It goes without saying that birds already infected by *E. tenella* can be treated with antibodies directed against said *E. tenella*. Antiserum or antibodies characteristic for a polypeptide according to the invention can be used for the therapeutic treatment of coccidiosis. Said antiserum or antibodies may be obtained by immunizing an animal with said polypeptide and isolating the antiserum therefrom.

Monoclonal antibodies directed against a polypeptide according to the invention can also be used for the therapy of birds infected with *E. tenella*. Said monoclonal antibodies can be produced by methods known in the art for this purpose, e.g. by immunizing mice with said polypeptide, immortalizing mouse spleen cells and selecting hybridomas producing useful antibodies. Immortal antibody-producing cell lines can also be created by direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus.

Monoclonal antibodies, in particular, may be used to raise anti-idiotypic antibodies by methods known in the art. These anti-idiotypic antibodies may also be useful for prevention of coccidiosis in birds.

Abovementioned antiserum and monoclonal antibodies can also be used for the immunological diagnosis of birds infected with *E. tenella*.

Example 1Sporulation of *E. tenella* oocysts

A suspension of 5×10^8 *E. tenella* oocysts in 60 ml 10^{-4} M sodium dithionite was centrifuged, after which the pellet was washed once with 100 ml sterile water. The cells were resuspended in 500 ml 2% potassium bichromate and then incubated under the influence of strong aeration for 7 hours at 30 °C. The oocysts were then collected by centrifuging and washed three times with 200 ml sterile water.

Isolation of RNA

For the isolation of RNA (ref. 1) the cell pellet was taken up into 2.8 ml of buffer containing 10 mM Tris acetate (pH 7.6), 75 mM sodium acetate, 1% SDS, 2 mM EDTA, 0.2 mg/ml proteinase K and 10 mM vanadyl ribonucleoside complexes. The oocysts were destroyed by vortexing for 60 seconds (max) in the presence of 13 g glass beads (ϕ 0.5 mm). 5 ml of phenol was added to the total extract and the mixture was vortexed for a further 60 seconds. After centrifuging, the supernatant liquor was pipetted off and again extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). RNA was precipitated after adding 2.5 volume ethanol and the resulting precipitate was dissolved in 800 μ l Tris 10 mM, EDTA 0.1 mM pH 7.6 ($T_{10}E_{0.1}$), after which the product was extracted a further twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform/isoamyl alcohol (24:1) and then precipitated with ethanol. PolyA⁺-RNA was isolated by means of oligo(dT)-cellulose chromatography (ref. 2). Approximately 100 μ g polyA⁺-RNA was isolated from 5×10^8 oocysts.

cDNA synthesis

PolyA⁺-RNA was converted to cDNA by means of the enzyme MMLV reverse transcriptase. For this purpose 25 µg polyA⁺-RNA was dissolved in 90 µl of water and denatured for 5 minutes at 20 °C by adding mercury methyl hydroxide to 10 mM, after which β-mercaptoethanol was added to 45 mM and the mixture incubated for a further 3 minutes at 20 °C. The enzyme reaction was carried out in 190 µl buffer containing 4 µg oligo(dT)₁₅, 150 U RNAsin^(R), 20 mM Tris (pH 7.6), 30 mM KCl, 4 mM dithiothreitol (DTT), 2 mM MgCl₂, 1 mM of each dNTP and 3000 U MMLV reverse transcriptase. The reaction was stopped after 1 hour's incubation at 37 °C by adding 10 µl 0.5 M EDTA. After extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), the RNA/DNA hybrid was precipitated by adding ammonium acetate to 2 M and 2.5 volumes ethanol. The combined action of the enzymes DNA-polymerase I and RNase H (ref. 3) results in the synthesis of the second string. The pellet was dissolved in 960 µl of buffer containing 20 mM Tris (pH 7.6), 5 mM MgCl₂, 100 mM (NH₄)₂SO₄, 0.6 mM β-NAD, 16 U RNase H, 200 U DNA-polymerase I and 20 U DNA-ligase (E.coli). The incubation time was 1 hour at 12 °C and then 1 hour at 22 °C, after which the reaction was stopped by adding an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitating with ethanol.

Before the cDNA was cloned in a vector suitable for this purpose it was first modified. cDNA (5 µg) was dissolved in 100 µl of buffer containing 30 mM sodium acetate (pH 5.6), 50 mM NaCl, 1 mM ZnSO₄ and 21 U Mung Bean Nuclease. After incubation for 30 minutes at 37 °C the reaction was stopped by adding EDTA to 10 mM and Tris to 25 mM. After extraction with phenol/chloroform/isoamyl alcohol (25:24:1) the mixture was desalinated over a Sephadex G50 column.

The following were added to the eluate (125 μ l): Tris pH 7.6 to 50 mM, EDTA to 2.5 mM, DTT to 5 mM, S'-adenosylmethionine to 0.5 μ M and 100 U EcoRI-methylase. After incubation for 30 minutes at 37 $^{\circ}$ C, the reaction was stopped by heating for 15 minutes at 65 $^{\circ}$ C, after which 1/10 volume of a solution containing Tris-HCl 100 mM, $MgCl_2$ 100 mM and NaCl 500 mM (pH 7.5) was added, and, at the same time, each dNTP to 1 mM and 12.5 U Klenow DNA-polymerase. The reaction was stopped by adding an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) after incubating for 60 minutes at 22 $^{\circ}$ C. The supernatant liquor was precipitated after adding 350 μ l H_2O and 50 μ l 3 M sodium acetate (pH 5.6) with 500 μ l isopropanol. After dissolving in 100 μ l H_2O , the pellet was desalinated over Sephadex G50 and the eluate precipitated with ethanol.

After dissolving the pellet in 24 μ l H_2O , ligation was carried out in 50 μ l by adding 2 μ g EcoRI linker, Tris-HCl (pH 8.0) to 30 mM, $MgCl_2$ to 10 mM, dithiothreitol to 10 mM, ATP to 1 mM, gelatin to 0.1 mg/ml and 10 U T_4 DNA-ligase. The reaction was stopped after 16 hours' incubation at 4 $^{\circ}$ C by heating (for 15 minutes at 70 $^{\circ}$ C) after which cutting was carried out with restriction endonuclease EcoRI in 210 μ l buffer containing 100 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$, 2.5 mM DTT and 500 U EcoRI. After 90 minutes' incubation at 37 $^{\circ}$ C, the reaction was stopped by means of extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant liquor was precipitated with 2.5 volume ethanol after adding sodium acetate (pH 5.6) to 300 mM cDNA and linkers were separated by means of a Biogel A15m column. The cDNA was precipitated with ethanol, after which the precipitate was dissolved in Tris-HCl 10 mM, EDTA 0.1 mM (pH 7.6). The cDNA molecules were then cloned in phage λ gt10 (4).

Example 2Screening of the *E. tenella* cDNA library with
E. acervulina DNA

The 296 bp EcoRI fragment from pUC18/EalA was labeled with digoxigenin-dUTP by random priming, exactly following the protocol going with the "DNA labeling and detection kit, non-radioactive" from Boehringer, Mannheim (Cat. No. 1093657).

Filters containing immobilized DNA from the *E. tenella* cDNA library described above were prepared as described by Maniatis et al. (2) and probed by the freshly denatured (10 min. 95 °C), labeled *E. acervulina* fragment for 16 hours at 42 °C according to the manufacturer's instructions. Filters were washed as follows: twice for fifteen minutes with 2 x SSC, 0.1% (w/v) SDS (1 x SSC is 0.015 mol/l sodium citrate pH 7.0 plus 0.15 mol/l NaCl) at room temperature, twice for fifteen minutes with 1 x SSC, 0.1% (w/v) SDS at 68 °C, twice for thirty and once for fifteen minutes with 0.1 x SSC, 0.1% (w/v) SDS at 68 °C and twice with PBS-tween (7.65 g/l NaCl, 0.91 g/l Na₂HPO₄·2H₂O, 0.21 g/l KH₂PO₄, 0.05% (v/v) Tween 80, pH 7.3) for 15 minutes at room temperature.

The filters were then reacted with a 1:5000 dilution in PBS-tween of polyclonal sheep anti-digoxigenin Fab-fragments, conjugated to alkaline phosphatase, for thirty minutes at room temperature. After washing the filters for four times fifteen minutes with PBS-tween at room temperature and once for fifteen minutes with 0.01 M Tris-HCl pH 8.0, 0.15 M NaCl, binding of the alkaline phosphatase to the filters was detected upon incubation with a solution of 0.33 g/l Nitroblue tetrazolium and 0.17 g/l 5-bromo-4-chloro-3-indolyl-phosphate in 0.1 M Tris-HCl pH 9.6, 0.1 M NaCl, 0.01 M MgCl₂. One out of every 400 λgt10 *E. tenella* clones reacted with the *E. acervulina* probe; ten of these, called *E. tenella*1A1

to 10 (λ gt10Et1A1 to 10) were plaque-purified. λ gt10Et1A1 together with the *Escherichia coli* strain BNN102 have been deposited with the Centraal Bureau voor Schimmelcultures, Baarn (The Netherlands) under no. CBS 286.89. From subclones of this cDNA insert, prepared either in plasmid pGEM4Z or bacteriophage M13 (2), the complete nucleotide sequence was determined according to the manufacturer's instructions (USB sequence kit).

References

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Restriction enzyme map of a DNA fragment, Et1A1, inserted into phage λ gt10Et1A1.

Nucleotide sequence of the DNA inserted into phage λ gt10Et1A1 and the deduced amino acid sequence of Et1A1.

The claims defining the invention are as follows:

1. A nucleic acid sequence substantially encoding at least an immunogenic part of a protein of Eimeria tenella, a portion of said protein being defined by the amino acid sequence represented in Figure 2.
- 5 2. A nucleic acid sequence according to claim 1, characterized in that a portion of said protein is encoded by the deoxynucleic acid sequence represented in Figure 2.
3. A recombinant nucleic acid molecule comprising a nucleic acid sequence according to claim 1 or claim 2, operably linked to control
10 sequences enabling expression of said nucleic acid sequence.
4. A vector virus containing a recombinant nucleic acid molecule according to claim 3.
5. A host cell containing a nucleic acid sequence according to claim 1 or claim 2, a recombinant nucleic acid molecule according to
15 claim 3 or a vector virus according to claim 4.
6. A polypeptide with an amino acid sequence of which at least a part is encoded by a nucleic acid sequence according to claim 1 or claim 2.
7. A polypeptide comprising at least an immunogenic part of the
20 amino acid sequence represented in Figure 2.
8. A vaccine for the protection of poultry against coccidiosis comprising the expression product of a nucleic acid sequence according to claim 1 or claim 2, the expression product of a recombinant nucleic acid molecule according to claim 3, a vector virus according to claim 4, a
25 host cell according to claim 5, or a polypeptide according to claim 6 or claim 7.
9. An antibody or antiserum immuno-reactive with a polypeptide according to claim 6 or claim 7.
10. A process for preparing a nucleic acid sequence substantially
30 encoding at least an immunogenic part of a protein of Eimeria tenella, which process is substantially as hereinbefore described with reference to Example 2.

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Figure 1

