Use of Cloned Excretory/Secretory Low-Molecular-Weight Proteins of Cooperia oncophora in a Serological Assay

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The potential of Cooperia oncophora excretory/secretory (ES) proteins as antigens in a serological assay which aims to establish exposure levels in cattle was assessed. ES proteins were analyzed by one- and two-dimensional polyacrylamide gel electrophoresis and immunoblotting. The N-terminal domains of two ES proteins were sequenced, and the corresponding cDNAs were cloned. Two cDNAs, designated CoES14.0 and CoES14.2, were expressed in Escherichia coli. The recombinant proteins were tested in an indirect enzyme-linked immunosorbent assay (ELISA) in which crude worm antigen (CWA) was used as a reference standard. In total, 67 reference serum samples were used: 27 negative serum samples, 29 C. oncophora-specific serum samples, 7 Dictyocaulus viviparus-specific serum samples, and 4 Ostertagia ostertagi-specific serum samples. This showed respective sensitivities and specificities of 17 and 84%, 0 and 100%, and 100 and 100% by the ELISAs with the three different types of proteins (CWA, CoES14.0, and CoES14.2, respectively). Since the CoES14.2 ELISA had the best sensitivity and specificity with reference sera, its specificity was further validated in an antigen inhibition ELISA. In this assay CoES14.2 and CWA preparations of C. oncophora, Cooperia curticei, O. ostertagi, Nematodirus helvetianus, Fasciola hepatica, D. viviparus, Haemonchus placei, and Trichostrongylus colubriformis were used as competitor antigens. This experiment showed that only the homologous antigens C. oncophora CWA and CoES14.2 resulted in 100% inhibition. The CWA preparations of all other nematodes did not affect the ELISA, even if concentrations of 250 times the 50% inhibitory concentration of C. oncophora CWA were used. These results indicate that CoES14.2 does not share cross-reactive epitopes with heterologous CWAs. Finally, we tested the CoES14.2 ELISA with sequential serum samples from naturally infected groups of animals. The optical density values that were obtained correlated well with exposure levels based on cumulative egg excretion. Thus, the CoES14.2 ELISA seems to be a very sensitive tool for estimating exposure levels in cattle.

Gastrointestinal (GI) nematode diseases are major obstacles in the production of domestic ruminants (5, 18). This especially holds for Cooperia oncophora and Ostertagia ostertagi, the two most common nematodes in cattle in cool temperate regions (14), since they also have a negative economic impact at subclinical infection levels (22–25). Diagnosis of GI nematode infections or diseases in cattle largely rely on clinical signs, grazing history, and fecal egg counts (18). These parameters can be complemented with serum pepsinogen and gastrin levels (2, 3) and antibody detection (3, 13).

Infections with C. oncophora and O. ostertagi are acquired by all calves as soon as they are turned out to pasture. This implies that any diagnostic test used to monitor herds for these infections should enable quantification of exposure levels and correlation of these with production losses. Ploeger (22) demonstrated that serology with crude worm antigen (CWA) could be used for this purpose and that this technique was superior to techniques involving other parameters, such as fecal egg counts. The tests used in these studies are, however, not suitable for routine diagnosis during herd health monitoring. First, they lack specificity and sensitivity due to cross-reactivity with antibodies directed toward antigens of other helminth species or even microorganisms (8, 36). Second, there is a variation between the batches of antigen preparation used, hampering the standardization of the test (11).

The difficulties with cross-reactive antibodies can be overcome, as has been shown for lungworm disease in cattle, with an enzyme-linked immunosorbent assay (ELISA) developed on the basis of a recombinant Dictyocaulus viviparus antigen (31, 32). A similar strategy has been applied to improve the available diagnostic tests for O. ostertagi. Two antigens were recently cloned. This, however, did not result in a reliable ELISA based on a recombinant product (7).

A low-molecular-mass complex of 12- to 16-kDa antigens from adult worms of C. oncophora has been described to react specifically with sera obtained from C. oncophora-infected calves (6, 19, 20, 34, 35). Moreover, the combined data indicated two important points. First, the 12- to 16-kDa C. oncophora antigens do not cross-react with O. ostertagi-specific antibodies (6). Second, the staining intensity varies dramatically between individual animals (19, 35) and correlates with exposure levels (19, 26, 34).

Characterization of individual proteins of the low-molecular-mass complex of 12- to 16-kDa antigens of C. oncophora is thus an important factor for the development of a reliable ELISA. In the present study, we first demonstrated the presence of these immunodominant products in immunoblots of excretory/secretory (ES) products of C. oncophora. These proteins were subsequently separated by two-dimensional gel electrophoresis. Next, the N-terminal domains of two of these ES proteins were sequenced, and the corresponding cDNAs were cloned. Two cDNAs, designated CoES14.0 and CoES14.2, were expressed in Escherichia coli. The recombinant proteins were tested in an indirect enzyme-linked immunosorbent assay (ELISA) in which crude worm antigen (CWA) was used as a reference standard. In total, 67 reference serum samples were used: 27 negative serum samples, 29 C. oncophora-specific serum samples, 7 Dictyocaulus viviparus-specific serum samples, and 4 Ostertagia ostertagi-specific serum samples. This showed respective sensitivities and specificities of 17 and 84%, 0 and 100%, and 100 and 100% by the ELISAs with the three different types of proteins (CWA, CoES14.0, and CoES14.2, respectively). Since the CoES14.2 ELISA had the best sensitivity and specificity with reference sera, its specificity was further validated in an antigen inhibition ELISA. In this assay CoES14.2 and CWA preparations of C. oncophora, Cooperia curticei, O. ostertagi, Nematodirus helvetianus, Fasciola hepatica, D. viviparus, Haemonchus placei, and Trichostrongylus colubriformis were used as competitor antigens. This experiment showed that only the homologous antigens C. oncophora CWA and CoES14.2 resulted in 100% inhibition. The CWA preparations of all other nematodes did not affect the ELISA, even if concentrations of 250 times the 50% inhibitory concentration of C. oncophora CWA were used. These results indicate that CoES14.2 does not share cross-reactive epitopes with heterologous CWAs. Finally, we tested the CoES14.2 ELISA with sequential serum samples from naturally infected groups of animals. The optical density values that were obtained correlated well with exposure levels based on cumulative egg excretion. Thus, the CoES14.2 ELISA seems to be a very sensitive tool for estimating exposure levels in cattle.

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proteins were sequenced and the corresponding cDNAs were cloned. In addition, the application of recombinant ES proteins for serodiagnosis was evaluated. Two cDNAs, designated CoES14.0 and CoES14.2, were expressed in *Escherichia coli*, and the sensitivities of the recombinant proteins were tested in an ELISA with sera from cattle monoinfected with *C. oncophora*. On basis of the outcome of these experiments, an ELISA was developed by using the recombinant CoES14.2 product as the test antigen.

**MATERIALS AND METHODS**

Isolation and cloning of ES products. Calves (age 4 to 10 months) were infected with 100,000 infective larvae of *C. oncophora*. This isolate was obtained from the Department of Animal Husbandry, Agricultural University, Wageningen, The Netherlands. Three groups of calves of six animals each with three markedly different exposure levels were collected postinfection, and adult worms were collected by the agar gel method as described elsewhere (37). ES material from adult worms was obtained as described by our laboratory for *Haemonchus contortus* (29). The proteins obtained were fractionated by one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1-DGE and 2-DGE, respectively) and analyzed by immunoblotting (see below). For amino acid sequence analysis, proteins were separated by 2-DGE (15) and were transferred to a ProBlott positive charge dioctyl membrane (Applied Biosystems Inc.) by using 100 mm 3-cyclonexylamino-1-propane-sulfonic acid (pH 11.0) in 10% methanol essentially as described previously (33). Next, the blot was stained with 0.1% Coomassie blue R-250 in 1% acetic acid–40% methanol, and after destaining with 50% methanol, the bands of interest were excised. The N-terminal amino acid sequence of the 14.0- and 14.2-kDa ES products was performed on an Applied Biosystems protein sequencer (model 473A). The obtained N-terminal amino acid sequences were NRPVTYARKEEY (s) and NYPDALAKKIT YRPV, respectively. The one-letter code was used, and the residues in parentheses were not identified with certainty. Specific oligonucleotides deduced from the N-terminal amino acid sequence data were synthesized at Pharmacia Biotech, Roosendaal, The Netherlands. Insone was substituted in positions of high ambiguity in an effort to decrease oligonucleotide redundancy. The synthesized 5′ primers both contained additional BamHI sites, which are underlined: 5′-CGCCGATCT AATCC GAI CGI CCI ACI CAT/C ACI GC-3′ (ES 14.0; primer 1) and 5′-CGCCGATCT AATCC GAI CGI CCI CAT/C GCI CTT/C GC-3′ (ES 14.2; primer 2). These primers were used in combination with an oligo(DT) primer containing a NotI restriction site (primer 3) to amplify the appropriate cDNAs by PCR. Total RNA was isolated from adult *C. oncophora* by extraction with RNAzol (Campro Benelex, Elst, The Netherlands). RNA was reversed transcribed with an oligo(DT) primer by using reverse transcriptase (Superscript RNAase H Reverse Transcriptase; Gibco BRL) to synthesize cDNA (27). PCR products were cloned into a pGEM vector (Qiagen vector; Qiagen, Chatsworth Calif.) by standard techniques (27). The expression construct was transformed into *E. coli* JM109, and recombinant expression was induced by using isopropyl-β-D-thiogalactopyranoside (SIGMA) at a final concentration of 1 mm. After amplification, the sizes of the PCR products that were obtained were assessed on a 1.5% agarose gel. PCR generated in both cases a single product of approximately 400 bp (data not shown). Next, the amplification products were cloned into pUC BM20 by standard techniques (27), generating clones pCoES14.0 and pCoES14.2.

Nucleotide sequence analysis and database searches. DNA sequence analysis of both cDNAs was performed by the dideoxy chain termination method (28) with a T7 DNA polymerase sequencing kit (Pharmacia Biotech). Homology searches in protein and nucleotide sequence databases (GenBank, EMBL, and NCBI databases) were carried out with the BLAST and FASTA programs (21), as supplied by the CAOS/CAMM Centre (Nijmegen, The Netherlands). Nucleotide sequence analysis and database searches were carried out with the BLAST and FASTA programs (21) and have been given accession numbers U66187 and U66188, respectively.

**RESULTS**

The 12- to 16-kDa complex is present in ES proteins of adult worms. The isolated ES material was first analyzed by 1-DGE and Western blotting. The ES material showed a simple profile compared with that for soluble- and nonsoluble CWA (Fig. 1A). The Western blot analysis showed that the immunogenicity of the ES products of adult worms reacted predominantly with the immunodominant 12- to 16-kDa complex, a fragment of 27 kDa, and a number of polypeptides with a high molecular mass (Fig. 1A).

In the remainder of this study we focused on the 12- to 16-kDa complex of the ES material. This complex appeared as a discrete band. By 2-DGE we showed that the complex of 12- to 16-kDa ES proteins consists of approximately 20 polypeptides (Fig. 1B). The COON complex was defined as the 12- to 16-kDa fraction.
activity in Western blots, and (iii) the fact that they were free from neighboring spots, thus avoiding contamination with other ES proteins. We selected two spots with molecular sizes of approximately 14.0 and 14.2 kDa (Fig. 1C), transferred them to nitrocellulose, excised the spots, and determined the N-terminal amino acid sequences of these proteins (Fig. 2).

Cloning and characterization of two cDNAs encoding ES proteins. The DNA sequences of the PCR products and the deduced protein sequences that were obtained are presented in Fig. 2. The nucleotide sequence of clone pCoES14.0 consisted of an uninterrupted reading frame which extended for 384 nucleotides and encoded a protein of 128 amino acids (approximately 14.5 kDa). The N-terminal amino acid sequence differed at four positions compared to the sequence obtained by Edman degradation (Fig. 2A). We cannot explain this difference, but it suggests that pCoES14.0 could belong to a gene family, as we demonstrated previously for similar ES products of H. contortus (30). A putative polyadenylation signal sequence (AATAA) was present at 23 nucleotides 3' to the stop codon. The deduced amino acid sequence contains a potential N-linked glycosylation site at asparagine 78 (16). The nucleotide sequence of pCoES14.2 that was obtained consisted of an uninterrupted reading frame which extended for 351 nucleotides and encoded a protein of 117 amino acids (approximately 13.2 kDa). The N-terminal amino acid sequence differed at a single position; the second lysine is replaced by a cysteine residue (Fig. 2B). This is due to the Edman degradation. This technique cannot identify cysteine residues, but reads the preceding residue instead, thus, in this case, a lysine. A putative polyadenylation signal was present 41 nucleotides 3' to the stop codon.

Homology searches in protein and nucleotide sequence databases (1, 21) revealed that clones pCoES14.0 and pCoES14.2 did not share significant homologies to other known sequences.

Heterologous expression. The cDNAs of pCoES14.0 and pCoES14.2 were subcloned into a pQE9 vector by standard techniques, thus generating constructs pQCoES14.0 and pQCoES14.2. Four randomly picked clones were selected, expression was induced, and the recombinant fusion proteins were subsequently affinity purified over a nickel agarose column. The purification process was monitored by SDS-PAGE, and recombinant fusion proteins were characterized in Western blots. Antisera of C. oncophora-infected calves recognized both recombinant proteins. Antisera of D. viviparus-, F. hepatica-, H. placei-, N. helvetianus-, and O. ostertagi-infected calves did not react with the CoES14.2 recombinant protein on Western blots, but cross-reacted with the CoES14.0 recombinant protein (data not shown). This strongly indicates that CoES14.2 is species specific, whereas CoES14.0 encodes cross-reacting epitopes.

Development of a CoES14.2 ELISA. Twenty-seven serum samples from noninfected calves, 29 serum samples from calves monoinfected with C. oncophora, 7 serum samples from calves monoinfected with D. viviparus, and 4 serum samples from calves monoinfected with O. ostertagi were subjected to three ELISAs in which CoES14.0, CoES14.2, and CWA proteins were used, respectively. The cutoff values for these preparations were 0.524, 0.486, and 0.710, respectively. Figure 3 shows the ODs obtained with these sera for each ELISA. The respective sensitivities and specificities of these ELISAs with reference sera were 17 and 84% (CWA ELISA), 0 and 100% (CoES14.0 ELISA), and 100 and 100% (CoES14.2 ELISA).

FIG. 1. (A) SDS-PAGE under reducing conditions (A) of soluble CWA (lane 2), nonsoluble CWA (lane 3), and ES material (lane 4) from adult C. oncophora. Lane 1, molecular size standards (in kilodaltons); lane 5, Western blot of ES material from C. oncophora probed with a representative serum sample from an immune calf. (B) 2-DGE after silver staining of C. oncophora ES proteins. (C) Western blot of C. oncophora ES proteins. The molecular size markers are given on the left (in kilodaltons). The spots sequenced by Edman degradation are indicated by arrows.

FIG. 2. Nucleotide sequence and predicted amino acid sequence of the CoES14.0 (A) and CoES14.2 (B) cDNAs. The deduced amino acid sequence of the cDNAs are shown as a single-letter code below the DNA sequence. The letters in boldface indicate the amino acids determined by Edman degradation (see Materials and Methods). The asterisks indicate the stop codon, and the putative polyadenylation signal sequences are underlined. A potential N-linked glycosylation site at asparagine 78 (16) in CoES14.0 is marked by a double line.
level at all. In contrast, the cumulative epg, a much better indicator of the level of exposure than the epg, corresponds well with the OD values for group H (Fig. 5B). For group L, an increase in OD values was not observed before day 120. Despite the very low epg, the presence of *C. oncophora* infections was demonstrated from day 28 onward. This increase in OD values represented a slight increase in pasture infectivity at the end of the experiment, which was also reflected by increases in the mean cumulative epg and the mean epg for group L (Fig. 5A and B).

**DISCUSSION**

The current serological tests available for the diagnosis of *C. oncophora* infection are based on CWA. This reduces both the sensitivities and specificities of these tests, particularly in correlating exposure levels in animals with subclinical infections (3, 13, 22–25). Moreover, other conventional diagnostic tests, such as fecal egg count (18) and serum pepsinogen and gastrin level determinations (2, 3), are labor intensive. Therefore, we used molecular biology-based techniques to identify a suitable protein with acceptable sensitivity and specificity enabling its use in recombinant form as a purified test antigen. The latter aspect is an intrinsic problem of the current serological tests (8, 36). In addition, the test should show a quantitative relationship with exposure levels and thus be suitable for routine herd health-monitoring schemes (26). The latter implies that the test will not be used to diagnose infections in the individual animal, but it will be used to monitor groups of animals.

It was recently demonstrated that a low-molecular-mass complex of 12- to 16-kDa antigens from adult worms of *C. oncophora* specifically reacts with sera obtained from *C. oncophora*-infected calves (6, 19, 20, 26, 34, 35). In addition, these proteins did not cross-react with *O. ostertagi*-specific antibodies (6). Furthermore, the staining intensity varied between individual animals (19, 35) and correlated with exposure levels in individual animals (19, 34).

Our results indicate that these antigens are present in low-molecular-mass ES material of *C. oncophora*. The 2-DGE analyses subsequently demonstrated that this material consisted of approximately 20 proteins (Fig. 1). The cDNAs of two
of these proteins, designated CoEs14.0 and CoEs14.2, were cloned and sequenced. The sequence analyses demonstrated that the two ES proteins were not related to each other (Fig. 2). This supports the hypothesis that the complex analyzed consists of unrelated proteins with different biological functions.

Our series of tests with CoEs14.0, CoEs14.2, and CWA showed that the CoEs14.2 recombinant protein is a reliable tool for the serodiagnosis of *C. oncophora*. This is based on three criteria. First, comparisons of the sensitivities and specificities of the CWA, CoES14.0, and CoES14.2 with 67 reference serum samples (Fig. 3) showed that the CoES14.2 antigen, with a sensitivity and specificity of 100 and 100%, respectively, should be considered the standard for serodiagnosis (5). Our data demonstrate that the CoES14.2 ELISA developed in the present study is a suitable tool for determining *C. oncophora* exposure levels in cattle. The value of this test for routine incorporation in a herd health scheme needs to be evaluated at the farm level. These experiments are performed in our laboratory.

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