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☐ **Brief Communication** ☐

# Specific bovine antibody response against a new recombinant *Cryptosporidium parvum* antigen containing 4 zinc-finger motifs

Dirk C. DE GRAAF[1]*, Hans DE CONINCK[1], Franz PETRY[2], Ilka B. EECKHOUT[1] and Johan E. PEETERS[1]

[1]*Veterinary and Agrochemical Research Centre, Groeselenberg 99, B-1180 Brussels, Belgium,*
[2]*Institute of Medical Microbiology and Hygiene, Johannes Gutenberg-University Mainz, D-55101 Mainz, Germany*

**Abstract:** A *Cryptosporidium parvum* sporozoite and oocyst λgt11 cDNA library was screened with a hyperimmune rabbit serum that was developed against insoluble fragments of ultrasonicated oocysts. A clone named Cp22.4.1 encoding a protein of 231 amino acids with 4 zinc-finger domains characterized by a Cys-X2-Cys-X4-His-X4-Cys motif was isolated and characterized. There was a complete match between the sequencing data of the coding region of Cp22.4.1 and the corresponding gene at chromosomal level. Cloning in a pBAD-TOPO-TA expression vector permitted to evaluate the antigenicity of the recombinant His-tagged antigen. This antigen was recognized by 2 out of 5 sera from *Cryptosporidium* immune calves and not by sera from parasite naive animals.

**Key words:** *Cryptosporidium*; antigen, gene cloning, zinc-finger, bovine

*Cryptosporidium parvum* is an important agent in the aetiology of the neonatal diarrhoea syndrome of calves, lambs and goat kids, causing considerable direct and indirect economic losses (de Graaf et al., 1999a). The infection is self-limiting and immunity is based on intestinal immunoglobulins and T-cell responses (de Graaf et al., 1999b). The *C. parvum* antigens dominantly recognized by antibodies from a broad range of immune animals have been described (Reperant et al., 1994). Screening of expression libraries with antibodies resulted in the cloning of 3 *Cryptosporidium* sporozoite surface antigens: CP15 (Jenkins and Fayer, 1995), CP15/60 (Jenkins et al., 1993) and P23 (Perryman et al., 1996). CP15 (Sagodira et al., 1999) and P23 (Perryman et al., 1999) have successfully been implemented in the development of a passive vaccine against cryptosporidiosis in ruminants. In such a passive vaccinal approach the newborns are protection against cryptosporidial infection by passive transfer of hyperimmune colostrum from their immunized dams. The oral administration of anti-CP15/60 IgA monoclonal antibodies to suckling mice also provided protection against infection (Tilley et al., 1991). Beside these sporozoite surface antigens, the micronemal proteins are likewise considered interesting

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*Corresponding author (e-mail: dideg@var.fgov.be.)

target molecules for immunoprophylaxis as they too are involved in parasite invasion into host cells (Prickett et al., 1994). This study was aimed to discover new *C. parvum* sporozoite surface or micronemal antigens and to test their antigenicity in relation to humoral immunity of the bovine host.

In order to select for membrane bound (surface) or vesicle enclosed (micronemal) antigens we developed a hyperimmune rabbit serum against insoluble fragments of ultrasonicated oocysts and used it for screening a *C. parvum* λgt11 cDNA library. *C. parvum* oocysts were isolated from faeces of diseased animals by biphasic diethyl ether/PBS extraction and differential centrifugation on Percol. Cytoplasmatic compounds were released by ultrasonication and removed after centrifugation. Insoluble fragments were resuspended in PBS and emulsified with complete Freund's adjuvant for a first s.c. immunisation of Minimum Disease Level rabbits, and with incomplete Freund's adjuvant for the 2 following i.m. booster injections given at 3 and 5 weeks intervals respectively. The collected hyperimmune rabbit serum (R3αCpUnsol) recognized a complex band pattern in Western blots of insoluble oocyst fragments that were boiled in Laemmli sample buffer (not shown).

We screened a *C. parvum* sporozoite and oocyst λgt11 cDNA library (Petry et al., 1998) according to the immunological screening protocol of Sambrook et al. (1989). The 10 clones that were recognized by R3αCpUnsol and not by pre-immune rabbit serum, were isolated after several rounds of re-screening. The inserts of 4 clones (Cp18.2.1, Cp20.2.1, Cp21.2.1 and Cp22.4.1) were amplified by PCR using the 5′ and 3′ LD Amplimers of the λgt11 LD-Insert Screening Amplimer Set (Clontech Laboratories, Palo Alto, CA) and sequenced by Eurogentec s.a. (Seraing, Belgium) according to the Single Run service, meaning that their DNA sequence was read only once from one of the two λgt11 primers . The sequencing data revealed that all the 4 λgt11 clones were constructed with an analogous cDNA fragment, although in 3 of them this fragment was cloned in the reversed orientation (Cp18.2.1, Cp20.2.1 and Cp21.2.1). It is not clear to us how these 3 clones could have expressed their *Cryptosporidium* gene product properly. Only in the λgt11 clone Cp22.4.1 the fragment was cloned in same orientation as the λgt11 β-galactosidase gene in which it was inserted.

The amplicon of clone Cp22.4.1 was subcloned in pUC18 using Ready-To-Go T4 DNA ligase (Amersham Pharmacia Biotech Benelux, Roosendaal, Netherlands) and DH5α competent cells, and sequenced twice according to the Double Run service (Eurogentec s.a.), meaning that finally every base pair was read at least twice in each orientation. The insert of clone Cp22.4.1 had a total length of 1045 bp (excluding the flanking EcoRI adapters from the library construction) and its nucleotide sequence data are given in Fig. 1 (also GenBank™ acc. no. AY017370). The second frame showed an open reading frame of 1004 bp. However, since the translated aa sequence that preceded the first methionine did not show any homology with the known proteins (BLASTP, National Center for Biotechnology Information; Altschul et al., 1997), we assume that the coding region starts at this first ATG codon (assigned as position 1 in Fig. 1) and ends at position 696 (including the stop codon TAA). This was further supported by the fact that the start methionine lays in the consensus PuNNATGPu sequence (where Pu stands for a purine and N for any base). This coding region corresponds to a protein of 231 aa with 4 zinc-finger domains characterized by a Cys-X2-Cys-X4-His-X4-Cys motif (where X can be any residue). The CCHC motif has been found mainly in the nucleocapsid protein of retroviruses where it plays a role in the packaging of the viral genomic RNA (De Guzman et al., 1998), and also in cellular nucleic acid binding proteins which are involved in vertebrate embryogenesis (Flink et al., 1998). Among parasitic organisms, this zinc-finger domain has been described in *Leishmania major*, *Trypanosoma brucei brucei*, *Trypanosoma cruzi*, *Trypanosoma equiperdum* and *Crithidia fasciculata* (acc. no. Q04832, AJ132959, AF091396, AAB47542 and A54598 respectively). The role of two of these proteins has been reported: the HEXBP of *L. major*

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-211                                                    GAAAGTATCAG
-200  AAATTTTAAATGAGCCAGTAAATATTGAAAAGACTCTTGAAAAAAAGAAA
-150  AAATCCAAGTCAAAACAATTAGATGAAAATCATGAAGAAAAAAATATTTG
-100  TAGTGACAAACAGGCTAAAGAAGAGGATCTGAAGGTGAAAAAAAAATCTA
 -50  AAGGATCAAAAAAGGCGAAGAAAATTAAAAGCGGATGTTGGACAACTGAT
   1  ATGATAAATGAAAGCATTCAAAATTTGGAAAGTATGTTAAATGATGAATC
   1  M  I  N  E  S  I  Q  N  L  E  S  M  L  N  D  E  S
  51  TGGAAAATTATCAAAATCCAAAAAGCGAAGAATAATAATGAGACTCTCAA
  18   G  K  L  S  K  S  K  K  R  R  I  I  M  R  L  S  K
 101  AACTAAAAAAGGGTCTTAGAGGAGAAGTTGAAATTGGAGGACAAATCCAA
  35     L  K  K  G  L  R  G  E  V  E  I  G  G  Q  I  Q
 151  AAAAGTATTAAAACAATTAAGAGGGAAATGAAACAAAAAAGTATGAATTC
  51  K  S  I  K  T  I  K  R  E  M  K  Q  K  S  M  N  S
 201  AAGTATATCACTTAGGAAAAATTCAAATGTAGTTTGTTTATGTTGTAGAA
  68   S  I  S  L  R  K  N  S  N  V  V  C  L  C  C  R  K
 251  AGAAAGGTCATCAGATGTCTGATTGTAGGTATTATAAACAAACAAATGAA
  85     K  G  H  Q  M  S  D  C  R  Y  Y  K  Q  T  N  E
 301  GAAGCTGAAAATGGAGATAATGAAATAAATAGTATTAGTGAAAGAAATGC
 101  E  A  E  N  G  D  N  E  I  N  S  I  S  E  R  N  A
 351  ATCAGGAAAAGAAGTATTTAAATGCTTTTTATGCGGAGAATTAGGACATA
 118   S  G  K  E  V  F  K  C  F  L  C  G  E  L  G  H  T
 401  CTCTTAAAGACTGTAAAAAACCAAGAAACGATAACTCTGTATTACCATTT
 135     L  K  D  C  K  K  P  R  N  D  N  S  V  L  P  F
 451  GCTAGCTGTTTTAGATGTGGGAAAACTGGACATATAGTCGCTTTTTGTCC
 151  A  S  C  F  R  C  G  K  T  G  H  I  V  A  F  C  P
 501  AAATAACGAAACAGGATCTATATATCCAAGAGGAGGAAGCTGTAATATAT
 168   N  N  E  T  G  S  I  Y  P  R  G  G  S  C  N  I  C
 551  GTGGGTCAGTTAAACATCTAGCTAGAAACTGTGATCAACAAATATCAAAA
 185     G  S  V  K  H  L  A  R  N  C  D  Q  Q  I  S  K
 601  ACTAACAAAAACAAGAAATCTATTGAAGGTAAAAATAAAGAAAAGATGAA
 201  T  N  K  N  K  K  S  I  E  G  K  N  K  E  K  M  N
 651  TATTGGAATGGATGATCCAGATTTAATGTGGAATGAAGCAATGTAATGAA
 218   I  G  M  D  D  P  D  L  M  W  N  E  A  M  Stop
 701  TAAGTGAATTATTGCTTTAAGATATTAGAAGAAAGAATTGATTAAAATTA
 751  AATAACTTAGATACAATTTAATTAGATTCTAGAATGATTAATTAACTTAG
 801  TTAAATTCTATATATATAAAAAAAAAAAAAAAAA
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**Fig. 1.** Complete nucleotide sequence data of the λgt11 clone Cp22.4.1 ending with a 17-mer poly-A tail. The deduced aa sequence is given from the first ATG-codon (in bold and underlined) to the first TAA-stop codon at position 694-696 (also in bold and underlined). The zinc-finger domains characterized by a Cys-X2-Cys-X4-His-X4-Cys motif are marked in gray.

binds to single stranded DNA containing a hexanucleotide repeat (Webb and McMaster, 1993) and the universal minicircle sequence binding protein of *C. fasciculata* binds to the conserved universal sequence of kinetoplast DNA minicircles (Abeliovich et al., 1993). It should be noted that another *C. parvum* zinc-finger sequence has been described (acc. no. U48717). Further, the EST and GST projects have yielded at least 5 putative zinc-finger sequences: CpEST.387, CpEST.088, CpEST.197, CpG0020B and CpG0306A (more information on the world wide web http://www.ebi.ac.uk/parasites/cparv.html).

In order to study the antigenicity of the Cp22.4.1 protein, the coding region of the clone (stop codon excluded) was subcloned in a pBAD-TOPO-TA expression vector (Invitrogen, Carlsbad, CA). We designed two primers for the amplification of the corresponding fragment by PCR: forward primer 5′-ATGATAAATGAAAGCATTCAAAA-3′ and reverse primer 5′-CATTGCTTCATTCCAC ATTAA-3′. We have chosen to use sporozoite DNA as template for this PCR reaction, as it would allow us to confirm the origin of the cloned gene (= *C. parvum*) and to compare its DNA sequences at the pre-transcriptional (chromosomal DNA) and post-transcriptional (cDNA) level. Cloning of the PCR-product in pBAD-TOPO-TA was according to manufacturer's instructions and the resulting pBAD clone was proven to have an insert that was 100% identical to that of the Cp22.4.1 λgt11 clone. Thus, the chromosomal organisation of this gene lacks introns. This sequencing result permitted us to use the name Cp22.4.1 also for the pBAD clone and its recombinant

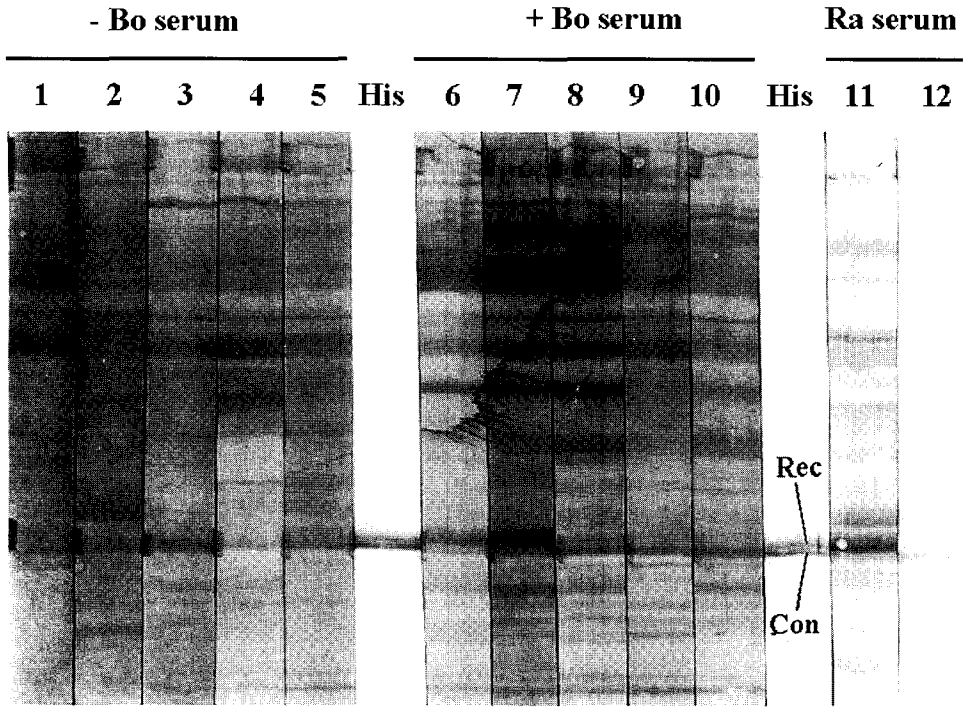


**Fig. 2.** Bovine immune response against the Cp22.4.1 protein. Western blots of the bacterial lysate of the pBAD clone that expressed the His-tagged *Cryptosporidium* protein. An anti-His (C-terminal)-HRP antibody (His), together with immune (= R3αCpUnsol; lane 11) and pre-immune (lane 12) rabbit serum (Ra serum), served to localize the Cp22.4.1 protein on the blots (Rec = recombinant Cp22.4.1 protein; Con = immuno-recognized contaminant). At the same altitude as Cp22.4.1 (approximately 35 kDa; molecular mass was determined by introducing markers on an other - not shown - blot) a band was visualized by 2 (lanes 6 and 7) out of 5 sera from *Cryptosporidium* immune animals (+ Bo serum; lanes 6-10) and not using sera from parasite naive animals (- Bo serum; lanes 1-5). The secondary antibody was an anti-bovine IgG (whole molecule) peroxidase conjugate.

expression product (= His-tagged Cp22.4.1 protein). Optimal expression was obtained by stimulating liquid cultures at $OD_{600}$ = 0.5 with 0.002% L-arabinose (or more) and by harvesting cells after 4 h incubation at 37°C. These cells boiled in Laemmli sample buffer were used to test bovine humoral recognition of the His-tagged Cp22.4.1 protein on Western blots. An anti-His (C-terminal)-HRP antibody served to localize Cp22.4.1 protein on the blots, but revealed a double band of approximately 35 kDa (Fig. 2). Comparison with bacterial lysates from other His-tagged *Cryptosporidium* antigens (not shown), and staining with the rabbit serum R3$\alpha$CpUnsol (Fig. 2), revealed that only the upper band corresponded with the His-tagged Cp22.4.1 protein. At the same altitude a band was recognized by 2 out of 5 sera from calves that recovered from natural *Cryptosporidium* infection. This band was not visualized using sera from neonatal animals (aged between 8 and 10 days) that were kept free of infection by Halocur (Intervet Belgium) treatment (Fig. 2).

In conclusion, our efforts to clone new surface and micronemal antigens resulted in the finding of a $\lambda$gt11 clone corresponding to a protein with 4 zinc-finger domains that was recognized by serum antibodies from immune neonatal calves in a specific way. It would be interesting to study the cellular localization (most probably nuclear) and function (most probably in gene regulation) of this protein in further projects.

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