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

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Abstract: A *Cryptosporidium parvum* sporozoite and oocyst λ gt11 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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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* λ gt11 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 (R3 α CpUns0l) 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 λ gt11 cDNA library (Petry et al., 1998) according to the immunological screening protocol of Sambrook et al. (1989). The 10 clones that were recognized by R3 α CpUns0l 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 λ gt11 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 λ gt11 primers. The sequencing data revealed that all the 4 λ gt11 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 λ gt11 clone Cp22.4.1 the fragment was cloned in same orientation as the λ gt11 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 DH5 α 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 GenBankTM 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*, *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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-411                                     GAAAGGATTCAG
-200  EAAATTTCTTAAATTTACGCGACGCAAGCTGCTTCAGAGGCTCTCTTGAAGAGAGAGAG
-180  AAGTTCGAAAGCTCAAGACGATTAATATCAAGACGATCGAGGAGAAAGAGATATTTTC
-160  TATTTTACCAATCAAGCTTAAATTAAGATGATTTTGAAGGCTTAAAGAGAGATCTTA
-80  AATCAATTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  1  ATGAGATCAATCAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  1  A  M  T  M  H  S  I  Q  N  L  E  H  M  L  H  D  M  E
  51  TGGGAGAGATTATTAAGATCTCAAGAGAGAGAGAGAGAGAGATTAATAGATCAAGATCTCTCA
  15  G  A  L  N  K  D  S  K  R  K  L  L  M  R  L  S  K
  101  AACTTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  35  I  E  K  G  L  R  G  E  V  E  I  D  G  Q  X  Q
  151  AAGAGATTAATTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  71  K  N  L  F  I  L  K  M  M  K  Q  X  S  M  M  D
  201  AAGATTAATTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  45  S  I  K  L  R  F  M  K  R  V  V  GAGTCTCTCAAGAGAGAGAGAGAGAGAGAGAG
  241  AAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  65  ATGAGATCAATCAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  301  AAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  141  S  A  E  N  G  D  N  M  L  M  C  I  S  D  R  N  A
  341  AATTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  110  S  G  K  R  V  F  E  GAGTCTCTCAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  401  CTCTTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  125  AAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  441  CTCTTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  151  A  G  GAGTCTCTCAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  341  AAGATTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  170  N  N  E  T  G  E  I  Y  P  R  G  G  S  GAGTCTCTCAAGAGAGAGAGAGAGAGAGAG
  351  CTCTTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  185  AAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  401  AACTTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  381  T  N  K  M  E  E  E  L  K  G  K  R  E  E  M  W
  441  TATTGAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  310  I  G  M  D  D  F  D  L  E  M  E  E  E  M  SDD
  461  TAAATTAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  481  AAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
  491  TTAAGATCTCAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG

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Fig. 1. Complete nucleotide sequence data of the *lgt11* 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.

expression product (= His-tagged Cp22.4.1 protein). Optimal expression was obtained by stimulating liquid cultures at OD₆₀₀ = 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 R3αCpUnsol (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 λgt11 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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