A.H.M. van Vliet [1]

F. Jongejan [2]

M. van Kleef [3]

B.A.M. van der Zeijst [1*]

Cloning and partial characterization of the Cr32 gene of *Cowdria ruminantium*

VAN VLIET (A.H.M.), JONGEJAN (F.), VAN KLEEF (M.), VAN DER ZEIJST (B.A.M.). Clonage et caractérisation partielle du gène codant pour la protéine Cr32 de *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 167-170

Les *Cowdria* ont été purifiées par centrifugation en gradient de densité. L'ADN a été utilisé pour la construction de banques génomiques d'expression. La protéine immunodominante Cr32 a été purifiée et la séquence N-terminale d'acides aminés déterminée. Les banques génomiques d'expression ont été criblées avec des anticorps monoclonaux spécifiques de la protéine Cr32, mais aucun n'a réagi. Une partie du gène codant pour la Cr32 a ensuite été amplifiée utilisant des amorces provenant de la séquence N-terminale et d'une autre séquence d'acides aminés interne. La séquence amplifiée a servi de sonde pour détecter le fragment d'ADN génomique codant pour la protéine Cr32. Ce fragment, provenant du stock Sénégal de *Cowdria ruminantium*, a alors été cloné. Une partie du gène, représentant les deux tiers de la longueur totale, a été exprimée dans le vecteur pGEX2T. Le produit d'expression obtenu est reconnu par les anticorps monoclonaux spécifiques de la Cr32.

Mots clés : *Cowdria ruminantium* - Gène - Protéine - Clonage moléculaire - ADN - Anticorps monoclonal.

INTRODUCTION

Molecular characterization of *Cowdria ruminantium*, the causative agent of heartwater (cowdriosis), depends on the availability of cloned genes. A problem in obtaining *Cowdria*-DNA is the contamination of the DNA-samples with bovine DNA originating from the cells used to cultivate the organism. With *Ehrlichia*- and *Rickettsia*-species this problem has been solved by purifying the rickettsiae using density gradient centrifugation (2, 12).

The Cr32 protein of *Cowdria ruminantium* is immunodominant, conserved in all *Cowdria* strains tested so far, and monoclonal antibodies directed at epitopes of this protein have been raised (3). One of these monoclonal antibodies is used in a competitive ELISA to detect antibodies against *Cowdria* in serum (3). The Cr32-antigen used in this system is derived from cultured *Cowdria*-organisms. Preparation of this antigen is time consuming and expensive. Each batch of new antigen must be standardized for optimal perforrnance. Recombinant Cr32-antigen would allow the preparation of large batches of optimized antigen, at low cost.

Therefore we directed our attention at cloning and expression of the gene coding for the Cr32-protein of *Cowdria*.

MATERIALS AND METHODS

Cowdria ruminantium (Senegal, Umm Banein and Welgevonden stocks) was cultivated in BUE9 cells and isolated from these cells as described previously (3). Unless specified otherwise, the Senegal stock was used. Rickettsial suspensions were treated with DNAse I (5 µg/ml) for 15 min at 37 °C to reduce the background of bovine DNA. Half of the DNAse-treated rickettsiae were purified by discontinuous Renografin density gradient centrifugation (2, 12). The band containing *Cowdria* organisms was collected, centrifuged, washed and resuspended in sucrose-phosphate-glutamate (SPG) buffer (1). A part (10 %) of the purified rickettsiae was centrifuged nd resuspended in Laemmli buffer, boiled for 5 min and subjected to sodium odecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (6). The proteins in this gel were blotted onto an Immobilon-filter, and the N-terminal amino acid sequence of the Cr32 protein was determined and interpreted at EuroSequence (Groningen, the Netherlands) as described previously (4). Sequencing of an internal part of the 31 kilodalton protein of the Welgevonden stock of *Cowdria ruminantium* is described elsewhere (10).

Genomic DNA from purified and non-purified organisms was extracted using standard methods (11). Genomic expression libraries were constructed by ligating *Sau*3AI-digested genomic DNA with *Bam*HI digested, dephosphorylated pEX11, pEX12 and pEX13 vectors (5) using T4 DNA ligase. Transformation to *Escherichia coli* strain pop2136, growth and screening were performed as described previously (8). All other recombinant DNA techniques were performed as described previously (7).

1. Department of Bacteriology, Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, Pays-Bas.

2. Departments of Bacteriology and Parasitology, Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, Pays-Bas.

3. Department of Protozoology, Onderstepoort Veterinary Institute, Afrique du Sud.

* Auteur à contacter : Department of Bacteriology, Institute of Infectious Diseases and Immunology, P.O. Box 80.165, 3508 TD, Utrecht, Pays-Bas. Tél : (31) 30 534344 ; fax : (31) 30 540784.

A.H.M. van Vliet F. Jongejan M. van Kleef B.A.M. van der Zeijst

RESULTS

Genomic expression libraries were constructed in the vectors pEX11, 12 and 13, so that fusion products with ß-galactosidase could be formed in all three reading frames. Screening with four anti-Cr32 monoclonal antibodies (2) did not result in positive clones. However, a fifth monoclonal antibody (reacting with a *Cowdria*-protein of > 100 kDa) gave three positive clones. All inserts were *Cowdria*-specific, but the expression products did not react with polyclonal sera. Therefore these clones were not characterized further.

As this approach did not yield Cr32-related clones another approach was chosen. Using Cr32-protein derived from gradient purified rickettsiae the N-terminal amino acid sequence of the Cr32-protein was determined. The sequence is (in one-letter code) : (N-terminus) D V I Q E E N N P V G S V Y I S A K Y M P T... (C-terminus). An internal amino acid sequence was derived from the 31 kDa protein of the Welgevonden stock after cyanogen bromide cleavage (10). This 31 kDa protein is expected to be identical to the Cr32 protein. Oligonucleotide primers for use in the polymerase chain reaction (PCR) were deduced from both protein sequences. Using the PCR, a fragment of 100 basepairs was amplified from *Cowdria*-DNA as template, whereas this fragment was not seen when bovine DNA was used. The PCR-fragment was cloned and the nucleotide sequence was determined. Translation of the sequence between the primers yielded the rest of the N-terminal amino acid sequence. Using the PCR-fragment as a probe, a 1100 base pair fragment was detected in genomic DNA ; this fragment was cloned (pCRS18). The insert hybridized with genomic DNA of *Cowdria* but not with bovine DNA. The nucleotide sequence of this fragment has been determined and translation of this sequence showed an open reading frame (ORF) of 660 base pairs with no stop codon, which means that the rest of the ORF should be found on another, overlapping clone. A fragment containing the noncharacterized part of the Cr32-gene has been cloned from genomic DNA. It is indicated in figure 1 (pCRS23) ; figure 1 also shows the clones that were made and the position of the open reading frame. The insert of plasmid pCRS18 was used as a probe in a Southern hybridization experiment with a blot containing digestions of genomic DNA of three *Cowdria*-strains isolated in three different geographical regions (Senegal (Senegal), Umm Banein (Sudan) and Welgevonden (South Africa) stocks). This Southern blot is given in figure 2 : it shows this part of the gene is present in these three *Cowdria*-strains.

The part of the Cr32-gene found on plasmid pCRS18 has been amplified using PCR. It was cloned in the expression vector pGEX2T (8) as a fusion product with glutathion transferase, a protein with molecular weight of 27 kDa. The fusion product, with a size of 49 kDa was

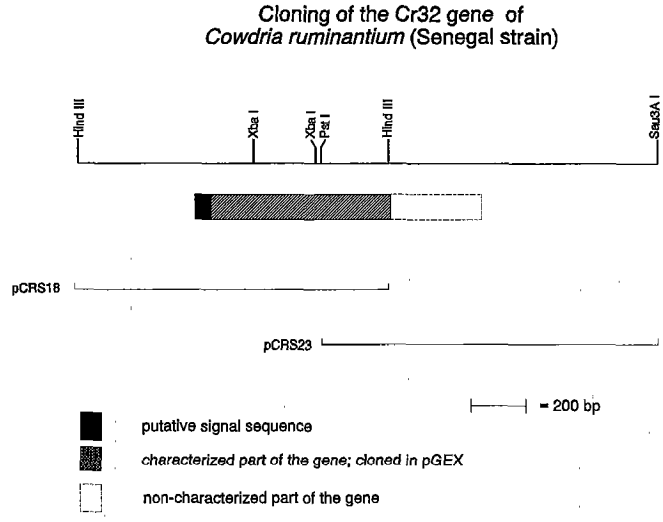

Figure 1 : Genomic restriction map of the region containing the Cr32-gene of Cowdria ruminantium *(Senegal stock). The position of the Cr32-gene and constructed clones are indicated.*

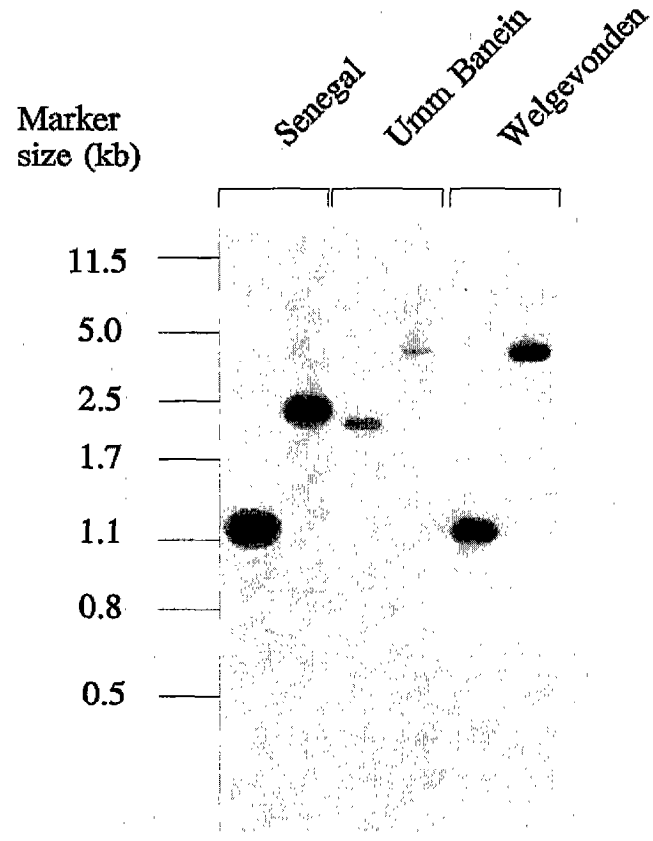

Figure 2 : Southern blot of HindIII and Sau3AI digested genomic DNA derived from Cowdria *stocks isolated in different geographical areas: Senegal (Senegal), Umm Banein (Sudan) and Welgevonden (South Africa). The probe used was the insert of plasmid pCRS18 (see fig. 1).*

subjected to SDS-PAGE, transferred to nitrocellulose and incubated with a mixture of the four Cr32-specific monoclonal antibodies. This Western blot is shown in figure 3 : positive signals were obtained with *Cowdria* antigen and the fusion protein, though not with bovine antigen and the pGEX protein. The additional bands under the 49 kDa fusion protein are degradation products of the fusion protein.

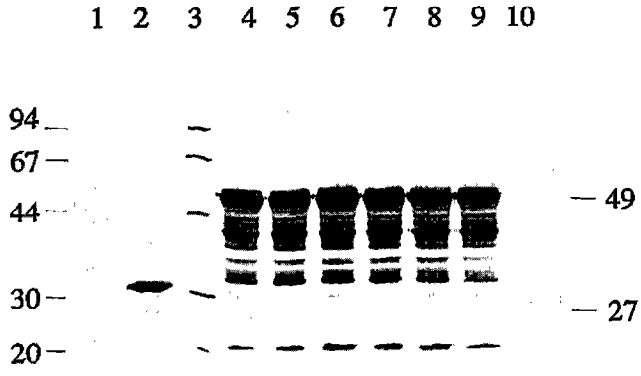

Figure 3 : Western blot of pGEX-fusion proteins expressing a part of the Cr32-gene (pGEX-Cr32(S)) reacted with Cr32-specific monoclonal antibodies. Lane 1 : BUE9 antigen, lane 2 : Cowdria ruminantium *antigen, lane 3 : the molecular weight marker, lane 4-9 : pGEX-Cr32(S) clones and lane 10 : pGEX-protein encoded by the empty vector. Marker sizes are indicated on the left, the sizes of fusion protein (49 kDa) and pGEX-protein (27 kDa) are indicated on the right.*

DISCUSSION

The gene coding for the Cr32 protein of *Cowdria ruminantium* has been cloned from the Senegal stock and has partially been characterized. Clones to complete the sequence of the Cr32-gene have been obtained. Cloning of the first 21 kDa of the mature protein in the pGEX vector yielded a fusion protein which reacted with Cr32-specific monoclonal antibodies. After completion of the sequence, the search for *Cowdria*-specific epitopes can start. A specific epitope will exclude cross-reactivity with *Ehrlichia*-species, which hampers serological tests at present.

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VAN VLIET (A.H.M.), JONGEJAN (F.), VAN KLEEF (M.), VAN DER ZEIJST (B.A.M.). Cloning and partial characterization of the Cr32 gene of *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 167-170

Cowdria **organisms were purified by density gradient centrifugation. The DNA was used to construct expression libraries. The immunodominant Cr32 protein was purified and its N-terminal amino acid sequence was determined. The expression libraries were screened with Cr32-specific monoclonal antibodies, but did not yield Cr32-positive clones. Therefore a part of the Cr32-gene was amplified using primers derived from the N-terminal and an internal amino acid sequence. This DNA was used as a probe to detect the genomic DNA fragment encoding the Cr32 protein. This fragment was cloned, using genomic DNA of the Senegal strain of *Cowdria ruminantium*. A part of the gene comprising two third of its total length has been expressed in vector pGEX2T. This expression product is recognized by Cr32-specific monoclonal antibodies.**

Key words : *Cowdria ruminantium* - Gene - Protein - Molecular cloning - DNA - Monoclonal antibody.

VAN VLIET (A.H.M.), JONGEJAN (F.), VAN KLEEF (M.), VAN DER ZEIJST (B.A.M.). Caracterización parcial y clonaje del gen Cr32 de *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 167-170

Se purificaron organismos de *Cowdria* mediante centrifugación de gradiente de densidad. Para la construcción de los libreros de expresión se uso ADN. La proteína inmunodominante Cr32 se purificó y se determinó la secuencia del amino ácido N terminal. La expresión de los libreros se monitoreó con anticuerpos monoclonales específicos para Cr32, pero no se produjeron clones Cr32-positivos. Sin embargo, una parte del gen Cr32 se amplificó, gracias al uso de "primers" derivados de la secuencia del amino ácido N terminal y del amino ácido interno. Este ADN se utilizó como agente probador para la detección del fragmento codificador de ADN del genoma de la proteína Cr32. Este fragmento se sometió a un clonaje, mediante el uso de ADN genómico de la cepa Senegal de *Cowdria ruminantium*. Una parte del gen, que comprende dos terceras partes de su longitud total se exprimió en el vector pGEX2T. Este producto es reconocido mediante anticuerpos monoclonales específicos para Cr32.

Palabras claves : Cowdria ruminantium - Gene - Proteína - Clonación molecular - ADN - Anticuerpo monoclonal.