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Isolation and characterization of antigenic proteins of *Cowdria ruminantium*

VAN KLEEF (M.), NEITZ (A.W.H.), DE WAAL (D.T.). Isolement et caractérisation de protéines antigéniques de *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 157-164

Deux protéines conservées antigéniquement, une immunodominante de 31 kilodalton et une mineure de 27 kDa, ont été caractérisées. Les protéines de 31 kDa et de 27 kDa sont des chaînes polypeptides simples. La protéine de 31 kDa ne contient pas de glycoconjugués et la séquence interne des acides aminés a été partiellement déterminée. La nature acide de cette protéine, déduite de sa composition en acides aminés, a été confirmée par la détermination du point isoélectrique (5,7). Des antisérums ont été préparés contre les protéines de 27 et de 31 kDa et les résultats indiquent qu'elles sont immunogènes et partagent des épitopes communs.

Mots clés : *Cowdria ruminantium* - Protéine antigénique - Isolement - Acide aminé - Point isoélectrique - Antisérum - Immunisation.

INTRODUCTION

Two antigenically conserved proteins, an immunodominant 31 kDa and a minor 27 kDa protein, were identified in our laboratories which are common amongst 9 stocks of *C. ruminantium*, differing in virulence, pathogenicity and origin (15). These proteins may be suitable for the development of nucleic acid probes, diagnostic assays and vaccines. In 1989, JONGEJAN and THIELEMANS identified a 32 kDa immunodominant, antigenically conserved *C. ruminantium* protein (7).

Amino acid analysis and partial sequencing of these proteins should make it possible to develop appropriate oligonucleotide probes either for screening *C. ruminantium* genomic libraries or in a diagnostic assay of the disease. Furthermore the development of a monospecific antisera against the 27 or 31 kDa proteins, would allow a means for identification of specific DNA clones coding for these proteins.

Determination of whether the 27 or 31 kDa are glycoproteins or not is of importance since prokaryotes are not capable of glycosylating proteins. Their presence would therefore imply that these organisms incorporate host cell material.

In this article the purification and certain characteristics of the 27 kDa and specifically the 31 kDa proteins of the *C. ruminantium* are described.

MATERIALS AND METHODS

In vitro cultivation of *C. ruminantium*

The Welgevonden stock (3) of *C. ruminantium* was cultured in a calf endothelial cell line (E_5 cell line) as described previously (1). Crude Welgevonden stock infected and uninfected extracts were prepared from cell cultures as previously described (15).

SDS-PAGE with or without reducing agent

Crude Welgevonden stock infected and uninfected cell cultures were dissolved in buffer containing 0.06 M Tris-HCl (pH 6.8), 16 % glycerol, 2 % sodium dodecyl sulphate (SDS) and 0.001 % bromophenol blue, with or without 2.5 % dithiothreitol (DTT), by heating at 100 °C for 10 min. The samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis SDS-PAGE and the gels either stained with Coomassie or immunoblotted with anti-Welgevonden stock bovine serum as described previously (15).

Production of monospecific anti-serum

Preparative SDS-PAGE was performed with crude Welgevonden stock infected culture extracts. An amount of 1900 µg crude protein was loaded per 1.5 x 120 x 160 mm gel, corresponding to approximately 133 µg of the 27 kDa protein and 114 µg of the 31 kDa protein. The amount of protein was estimated from a standard curve obtained after scanning a Coomassie stained SDS-PAGE gel of Welgevonden stock uninfected and infected crude cell cultures and known amounts of bovine serum albumin and chymotrypsinogen A. After preparative SDS-PAGE the gel was stained with 0.3 M $CuCl_2$ as described previously (8). The 27 and 31 kDa protein bands were excised and a volume of PBS added to the excised bands of one preparative gel, giving a final volume of 2.5 ml. The gel was then fragmented by passing back and forth

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between 2 syringes first connected by a 19 G needle followed by a 21 G needle, and stored at -70 °C until required for immunization.

Four rabbits, 2 goats and a sheep were immunized with either the 27 kDa protein or the 31 kDa protein according to table I. The serum collected at intervals indicated in table I were evaluated and titers determined by immunoblotting using crude Welgevonden stock infected and uninfected culture extracts as antigen in the western blots. Immunoblotting was performed as described earlier (15).

Determination of whether the 31 kDa protein of *C. ruminantium* is protective towards infection with heartwater

Goats A and B and sheep A were immunized as described in table I. Goat C and sheep B were immunized once with Welgevonden stock infected blood stabilate (12) and treated as previously described (15). All these animals including a heartwater naive goat and sheep were challenged by inoculation with 5 ml Welgevonden stock blood stabilate as described in table II. Daily rectal temperatures of the goats and sheep were monitored. They were not treated when heartwater symptoms developed after challenge and the cause of death was determined by post mortem investigations.

Amino acid analysis

Welgevonden stock infected crude cell culture extracts were subjected to SDS-PAGE, blotted onto polyvinylidene difluoride (PVDF) membranes and stained with Coomassie as described earlier (15).

Amino acid analysis by HCl hydrolysis, standard phenylisothiocyanate derivatization and reverse phase high performance liquid chromatography (HPLC) analysis were performed according to the PICO-TAG method of Millipore. Tryptophan analysis by methane sulphonic acid (MSA) hydrolysis and cysteine analysis by performic acid oxidation were performed on duplicate samples according to the Millipore instruction manual. The data obtained was statistically analysed according to a Parameters of Varieties program written by Dr VAN ARK, Computer Section, OVI, Onderstepoort.

TABLE I Immunoblot titers obtained from monospecific anti-serum produced in rabbits, goats and a sheep. Only serum from one of the duplicate rabbits were tested. All inoculations were administered subcutaneously and intramuscularly.

Day	Inoculum bled	Reciprocal of immunoblot titer									
		Rabbit 27 kDa*		Rabbit 31 kDa#		Goat A		Goat B		Sheep A	
						31 kDa@		31 kDa@			
		27 kDa	31 kDa	27 kDa	31 kDa	27 kDa	31 kDa	27 kDa	31 kDa	27 kDa	31 kDa
0	Antigen/ FCA (1 : 1)	√		√		√		√		√	
14	Bled	neg	neg	neg	neg	1 000	125 000	200	5 000	1 000	2 500
28	Antigen/ FIA (1 : 1)	√		√		√		√		√	
42	Bled	neg	40	200	1 000	5 000	125 000	2 500	625 000	5 000	125 000
56	Antigen	√		√		√		nd		nd	
70	Bled	40	200	200	1 000	5 000	125 000	nd	nd	nd	nd
84	Antigen	√		√		√		nd		nd	
98	Bled	200	1 000	200	1 000	1 000	125 000	nd	nd	nd	nd

** inoculated with 133 µg of the 27 kDa protein/immunization.*
 # inoculated with 114 µg of the 31 kDa protein/immunization.
@ inoculated with 266 µg of the 31 kDa protein/immunization.
√ immunized.
nd not done.
FCA Freunds complete adjuvant.
FIA Freunds incomplete adjuvant.

TABLE II Determination of whether immunization with the 31 kDa protein of **C. ruminantium** *is protective towards infection with heartwater.*

Animal	Immunized	Reciprocal immunoblot titer prior to challenge	Week challenged# after first immunization	Outcome after challenge
Goat A	31 kDa*	< 500	48	febrile reaction, died
Goat B	31 kDa*	625 000	8	febrile reaction, died
Goat C	Welgevonden stock infected blood stabilate	< 500	45	no febrile reaction, survived
Goat D	nd	neg	0	febrile reaction, died
Sheep A	31 kDa*	125 000	8	febrile reaction, died
Sheep B	Welgevonden stock infected blood stabilate	neg	90	no febrile reaction, survived
Sheep C	nd	neg	0	febrile reaction, died

** immunized as described in Table I.*
 # inoculated with 5 ml of the Welgevonden infected blood stabilate.
nd : not done.
neg : negative.

Amino acid sequencing

Automated amino acid sequencing was performed on the 31 kDa protein electroeluted from SDS-PAGE gels and a CNBr peptide fragment of this protein. The electrophoresis was performed with the pH 7.28 MZE 3328.IV buffer system as described previously (11). The gels were stained for 60 min with 0.1 % Coomassie, 10 % methanol and 0.5 % acetic acid and destained in 10 % methanol. The 31 kDa protein band was excised from the Coomassie stained SDS-PAGE gel, cut into small pieces and soaked in 0.5 % (w/v) N-cetyl-N,N,N,-trimethylammonium bromid (C-TAB) containing 10 % 2-merkapto-ethylammonium and 0.45 M acetic acid for 60 min at room temperature. The equilibrated gel piece was placed into the elution chamber of the Bio-Trap, containing C-TAB buffer. The buffer chamber of the Bio-Trap was filled with 0.45 M acetic acid and electroelution performed at 200 V for 120 min at room temperature. The eluted sample was freeze dried and a volume of 200 µl methanol added followed by 800 µl cold acetone. This solution was incubated at - 20 °C for 30 min and centrifuged at 11 000 x g for 6 min. The supernatant was removed and the pellet washed with 800 µl cold acetone and centrifuged at 10 000 x g for 6 min. The resulting pellet was dried under vacuum and its purity determined by SDS-PAGE before amino acid sequencing or CNBr cleavage.

CNBr cleavage was performed as described previously (10). A volume of 50 µl 70 % formic acid was added to the electroeluted and dried 31 kDa protein, thereafter a 600 molar excess of CNBr in 70 % formic acid was added. After incubating overnight at room temperature the samples were dried under N_2 gas. An aliquot was investigated by SDS-PAGE and the rest subjected to HPLC.

The sample was dissolved in 100 µl trifluoroacetic acid TFA and HPLC was performed, using a narrow-bore VIDAC C4 column 2.5 x 400 mm, with the following buffer system : 10 min isocratic with 0.1 % TFA in water and 60 min 0-100 % gradient of 0.08 % TFA in 70 % acetonitrile. The peptides were detected by monitoring at 229 nm. The peptide containing fractions were collected manually into Eppendorf tubes, freeze dried and stored at -20 °C.

Amino acid sequencing was performed on selected peptides in a gas phase sequencer constructed as previously outlined (6), and modified (2). The converted phenylthiohydantoin amino acids were identified by isocratic HPLC employing a 3 x 250 mm 3µ Lichrospher C_{18} (Bishoff) column as previously described (9).

Glycan assay

Welgevonden stock infected and uninfected crude cell cultures were subjected to SDS-PAGE, western blotted onto PVDF membranes and assayed for carbohydrates by an enzyme immunoassay according to the protocol described in the Glycan kit (Boehringer Mannheim). As reference the western blotted PVDF membranes were also stained for 10 min with Coomassie or immunostained with goat anti-Welgevonden stock serum as described earlier (15) .

Isoelectric focusing

The 31 kDa protein was eluted from electroblotted PVDF membranes as described previously (16). The protein was precipitated by adding 4 times the volume of acetone and incubating overnight at -20 °C. After centrifuging at 20 000 x g for 20 min the pellet was stored at -20 °C.

A portion of the pellet was checked for purity by SDS-PAGE. The gel was stained with Coomassie or western

blotted onto PVDF membranes and immunostained with goat anti-Welgevonden serum prepared as described earlier (15) .

Denaturing isoelectric focusing (IEF)

Crude Welgevonden stock infected and uninfected cell cultures, PVDF membrane eluted 31 kDa protein and standard proteins (Pharmacia) with known isoelectric points were dissolved 1:1 in a sample buffer containing 15 % (v/v) glycerol, 2 % (v/v) Triton X-100, 8 M urea, 15 mM DTT and 2.4 % (w/v) ampholyte pH 3-10 (Bio-Rad). Gels of dimensions 0.75 x 80 x 80 mm, containing 5.5 % (w/v) acrylamide, 0,15 % N,N-methylene bisacrylamide, 10 % (v/v) glycerol, 2 % (v/v) Triton X-100, 8 M urea, 2.4 % (w/v) ampholyte pH 3-10, 0.1 % (v/v) tetramethylenediamine (TEMED) and 0.04 % (w/v) ammonium persulphate were cast at 37 °C. Prefocusing was performed using 0.02 M NaOH at the cathode and 0.02 M CH_3COOH at the anode, at 200 V for 15 min followed by 300 V for 30 min and 400 V for 30 min, at 10 °C. Electrophoresis was carried out at 400 V for 16 h followed by 800 V for 1 h. The gel was either stained with Coomassie (5) or western blotted onto PVDF membranes using CAPS buffer, pH 9 and immunostained with goat anti-*C. ruminantium* 31 kDa protein monospecific serum diluted 1:5000 (15).

Native IEF

The PVDF membrane eluted 31 kDa protein as well as protein standards with known isoelectric points were dissolved in distilled water and applied at the anodal, cathodal or middle position. IEF and Coomassie staining was performed in a PhastSystem™ using PhastGel IEF 3-9, as described by the Pharmacia instruction manual (Pharmacia).

RESULTS

SDS-PAGE with or without reducing agent

The 27 kDa and 31 kDa proteins of *C. ruminantium* were detected in the Coomassie stained gel and immunoblots irrespective of whether DTT was present or not (fig. 1).

Production of monospecific anti-serum

Serum from one of two rabbits inoculated with the 27 kDa protein remained negative even after three inoculations and only recognized the 31 kDa (and not the 27 kDa) protein after the fourth immunization (results not shown). Serum from the second rabbit identified the 31 kDa (and not the 27 kDa) protein of *C. ruminantium* after the second administration. After the third immunization the 27 kDa and the 31 kDa proteins were recognized (table I, fig. 2).

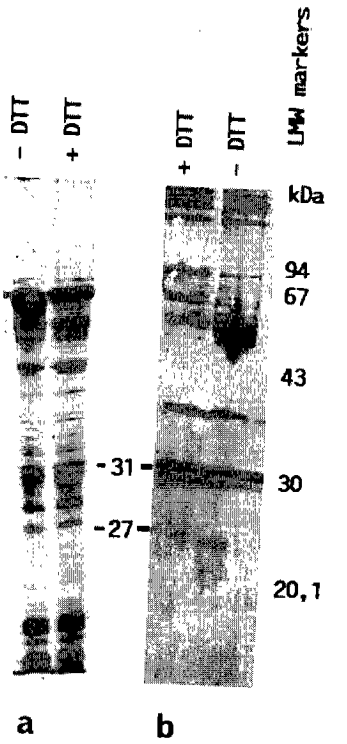

Figure 1 : SDS-PAGE protein patterns of Welgevonden stock infected crude culture extracts with or without reducing agent. a) Coomassie stained gel and b) western blot probed with bovine anti-Welgevonden serum.

Serum obtained from two rabbits inoculated with the 31 kDa protein recognized the 27 and the 31 kDa proteins after the second, third and fourth inoculation (table I, fig. 2).

The serum of goat A inoculated with the 31 kDa protein recognized the 31 kDa protein and cross reacted with the 27 kDa protein at low serum dilutions after the first, second, third and fourth inoculation (table I, fig. 3). Other *C. ruminantium* proteins were also detected by the serum and an E_5 protein in the region just above 31 kDa was recognized at a dilution of ≤1:200.

Determination of whether the 31 kDa protein of *C. ruminantium* is protective towards infection with heartwater

The goats and sheep, immunized with the 31 kDa protein of *C. ruminantium* as well as the naive controls all developed a febrile response after challenge which lasted bet-

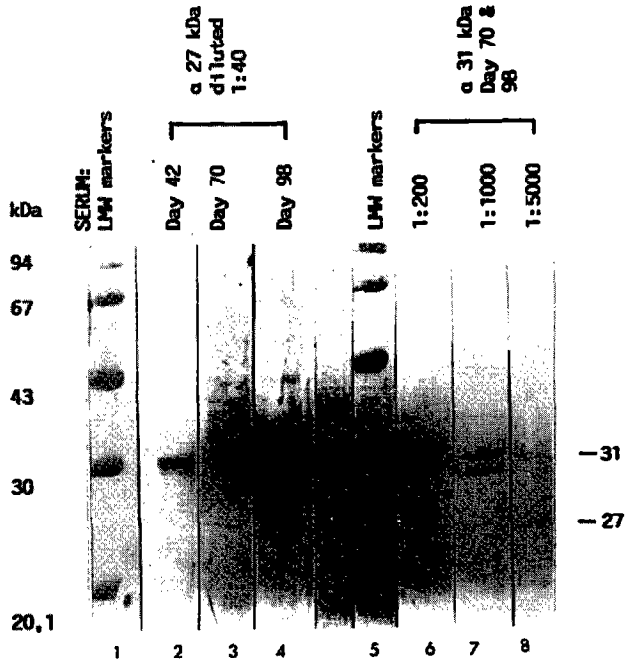

Figure 2 : Western blot analysis of crude Welgevonden stock infected culture extracts probed with rabbit anti-serum raised against the 27 kDa or 31 kDa proteins of C. ruminantium. *Lanes 6, 7 and 8, representative of days 70 and 98 anti-31 kDa serum.*

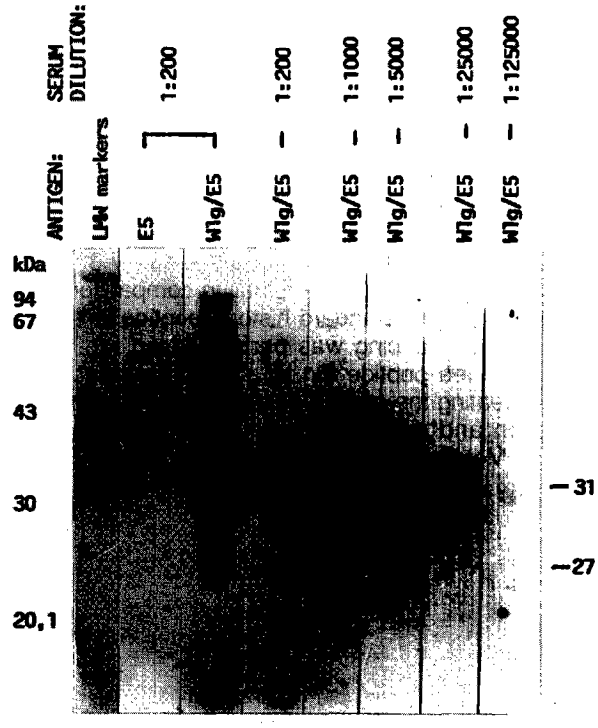

Figure 3 : Western blot analysis of crude Welgevonden stock infected and uninfected culture extracts probed with goat anti-serum raised against the 31 kDa protein of C. ruminantium.

ween 5 - 6 days before death (table II). Post mortem examinations confirmed heartwater as the cause of death. The goat and sheep previously immunized with Welgevonden stock blood stabilate did not develop a febrile response after challenge and were considered immune to heartwater (table II).

Amino acid analysis

The amino acid composition of western blotted 31 kDa protein of *C. ruminantium* is shown in table III. From this it was calculated that the 31 kDa protein contains 23 % acidic and 12 % basic amino acids. It appears that the 31 kDa protein does not contain tryptophan or cysteine since these amino acids were absent following MSA hydrolysis and performic acid oxidation, respectively.

Amino acid sequencing

Automated amino acid sequencing was done on approximately 2000 pmol of the 31 kDa protein, eluted from Coomassie stained SDS-PAGE gels. No clear amino acid sequence could be obtained, therefore it was concluded that the protein was N-terminally blocked.

TABLE III Amino acid composition (residues per mole) of the 31 kDa protein of* C. ruminantium *western blotted onto PVDF membranes.

Amino acid component	Average no of residues ± SD n = 5
asp	41 ± 4
glu	28 ± 3
ser	33 ± 3
gly	29 ± 2
his	7 ± 1
arg	11 ± 2
thr	22 ± 2
ala	24 ± 1
pro	9 ± 3
tyr	15 ± 1
val	14 ± 1
met	5 ± 1
ile	17 ± 3
leu	17 ± 1
phe	13 ± 2
lys	19 ± 1
cys	0
trp	0

SD: standard deviation.
n : number of variables.

Amino acid analysis revealed that there are 5 methionine residues per mole of the 31 kDa protein. Therefore the 31 kDa protein was cleaved with CNBr to yield peptide fragments not N-terminally blocked. Approximately 1600 pmol of the 31 kDa protein was eluted from SDS-PAGE gels. Re-electrophoresis of the electroeluted protein showed a single polypeptide in the region of 31 kDa. Purification of the resultant CNBr peptides by HPLC showed several peaks (fig. 4). The amount of material cleaved with CNBr appeared to be in the region of approximately 3 µg as the size of the HPLC peaks were small in comparison to peak heights of 10 µg for standard histone peptides. Automated amino acid sequencing was performed on the indicated selected purified peptide (fig. 4). The sequence was verified by repeating the protein purification, cleavage, peptide purification and analysis. The following sequence was obtained : Met-Pro-Ile-Ala-Glu-Asp-Phe-Gly-Asp-Thr.

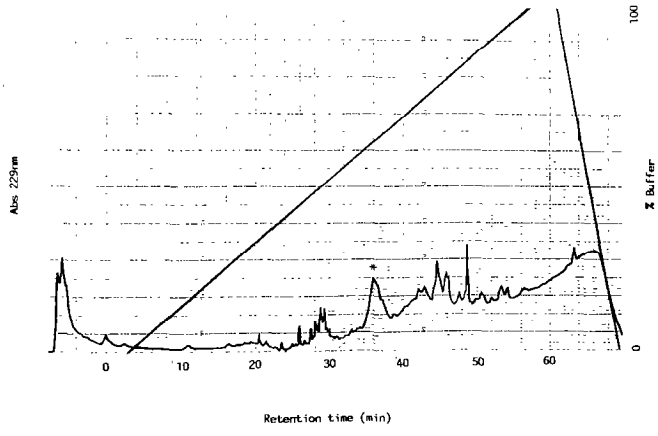

Figure 4 : HPLC chromatogram of peptides released after CNBr cleavage of the 31 kDa protein of C. ruminantium. ** - peptide sequenced. Buffer = 0,08 % TFA in 70 % acetonitrile.*

Glycan assay

The glycan enzyme immunoassay revealed that the 29 and 31 kDa proteins of *C. ruminantium* are not glycoconjugated proteins (fig. 5). No conclusion could be made as to whether the 27 kDa or other proteins of *C. ruminantium* are glycoproteins or not as the patterns of infected and uninfected cell cultures were identical.

Isoelectric focusing

The isoelectric point of the 31 kDa protein was determined to be 5.7 under denaturing conditions (fig. 6).

The pI could not be determined under native conditions because the protein precipitated regardless of the position of application.

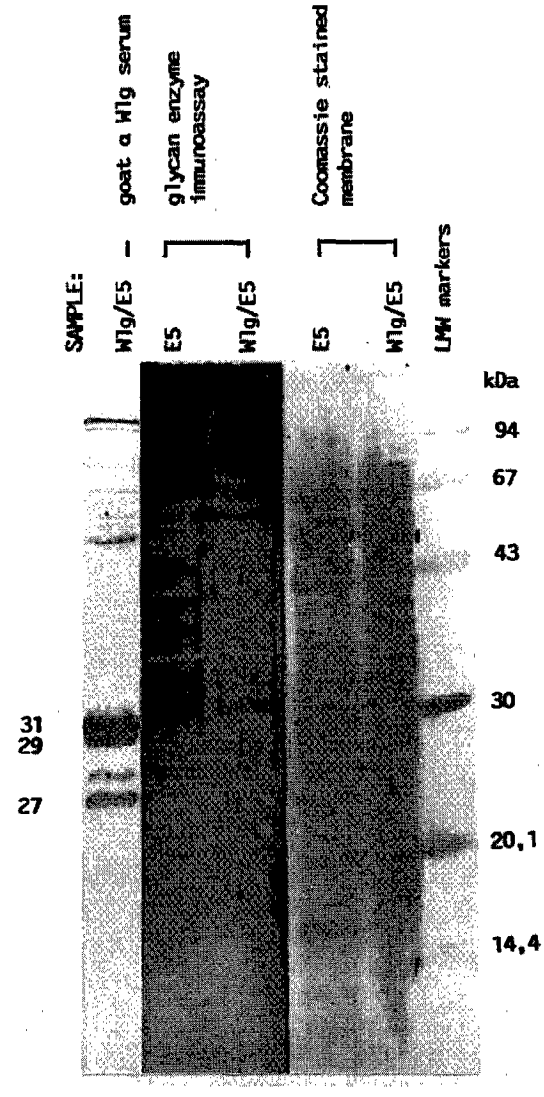

Figure 5 : Glycan enzyme immunoassay, immunostaining and Coomassie staining of crude, Welgevonden stock infected and uninfected cell cultures.

DISCUSSION

Although antibodies have become useful reagents for identification, localization and purification of proteins, their usefulness depends on their specificity. The results obtained when monospecific antisera were prepared against the 27 and 31 kDa proteins, suggest that the 27 and 31 kDa proteins share common epitopes and that the epitopes on the 31 kDa protein are immunologically and antigenically dominant in comparison to the 27 kDa protein. The antibodies that are produced and directed towards these epitopes are therefore termed heteroclitic antibodies (13). Although the isolated 27 and 31 kDa proteins were in a denatured state they retained their immunogenicity which was also unaltered by the staining procedures.

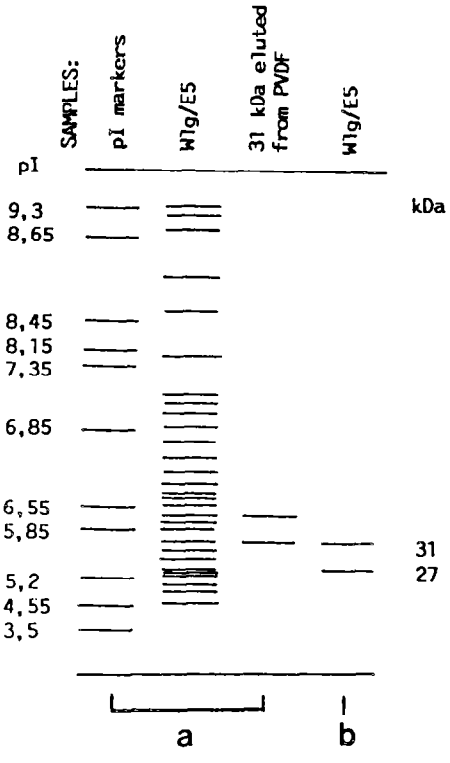

Figure 6 : Representation of analytical denaturing isoelectric focusing of the 31 kDa protein of C. ruminantium. *a) Coomassie stained gel and b) Western blot probed with goat anti-31 kDa protein serum.*

The monospecific anti-serum prepared against the 31 kDa protein appears unspecific at low serum dilutions in the immunoblot and the specificity increased as the dilution of the serum increased. However, the anti-27 kDa and anti-31 kDa sera do not react with cell culture proteins at high serum dilutions and are therefore specific for *C. ruminantium* proteins.

The acidic nature of the 31 kDa protein as determined from the amino acid composition correlates with the pI of 5.7 that was obtained by IEF. Knowledge of the pI of a protein is important for the proper use of several purification techniques such as disc electrophoresis, isotachophoresis, IEF, ion-exchange chromatography and even ammonium sulphate fractionation (14).

Results of amino acid analysis must be evaluated carefully and critically due to the fact that the quantification of several amino acids is biased because of artefacts caused by contamination (affecting gly, glu, ser), the hydrolysis procedure (affecting cys, ser, thr, trp, met, tyr), the derivatization procedure (affecting lys) or chromatography (affecting his) (4).

Considering that complete, partial or no cross-protection is observed between various stocks of *C. ruminantium* in vitro, it seemed unlikely that the common 27 and/or the 31 kDa proteins play a role in cross-protection. The animals that were immunized with the 31 kDa protein failed to survive a challenge with heartwater infective blood. This should be further investigated with respect to the titer at the time of challenge, the dose of antigen and immunization strategy before any conclusions may be made regarding the protection by this immunogen towards heartwater infection.

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VAN KLEEF (M.), NEITZ (A.W.H.), DE WAAL (D.T.). Isolation and characterization of antigenic proteins of *Cowdria ruminantium*. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 157-164

Two antigenically conserved *Cowdria ruminantium* proteins, an immunodominant 31 kDa and a minor 27 kDa protein, were characterized. The 31 kDa and 27 kDa proteins are single polypeptide chains. The 31 kDa protein contains no glycoconjugates and the partial, internal amino acid sequence was determined. The acidic nature of this protein, deduced from the amino acid composition, was confirmed by IEF (pI 5.7). Monospecific antiserum was prepared against the 27 and 31 kDa proteins and results indicate that they are immunogenic and share common epitopes.

Key words : *Cowdria ruminantium* - Antigenic protein - Isolation - Amino acid - Isoelectric point - Antiserum - Immunization.

VAN KLEEF (M.), NEITZ (A.W.H.), DE WAAL (D.T.). Aislamiento y caracterización de las proteínas antigénicas de *Cowdria ruminantium*.. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 157-164

Se caracterizaron dos proteínas conservadas antigenicamente para *Cowdria ruminantium*, una inmunodominante de 31 kilodalton y una menor de 27 kDa. Ambas estructuras son cadenas simples de polipéptidos. La proteína de 31 kDa no contiene glicoconjugados y se determinó la secuencia parcial del amino ácido interno. La naturaleza ácida de esta proteína, deducida a partir de la composición del amino ácido, se confirmó mediante IEF (pI 5,7). Se preparó un anti suero, específico contra las proteínas de 31 kDa y de 27 kDa y los resultados indican que son inmunogénicas y que comparten epítopos comunes.

Palabras claves : *Cowdria ruminantium* - Proteina antigenica - Aislamiento - Amino ácido - Punto isoelectrico - Anti suero - Inmunización.