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IDENTIFICATION OF THE ANTIGENIC PROTEINS OF *COWDRIA RUMINANTIUM*

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ABSTRACT

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Immunoblotting of *Cowdria ruminantium* proteins with sheep or bovine antiserum identified 2 antigenically conserved proteins, one being an immunodominant 31 kDa and the other a minor 27 kDa protein. These proteins are present in the electrophoretic profiles of the Welgevonden, Ball 3 and Kwanyanga stocks and are recognized by sheep antiserum to the Welgevonden, Ball 3, Kwanyanga, Mali, Comoro, Breed, Germishuys, Kümm and Mara stocks and by bovine antiserum to the Welgevonden stock of *C. ruminantium*. The stocks did not reveal identical or unique antigenic properties which could explain differences in pathogenicity and cross-immunity observed amongst the various stocks of *C. ruminantium*.

INTRODUCTION

Cowdria ruminantium, a rickettsia-like organism, is the causative agent of heartwater, a non-contagious disease of ruminants characterized by the development of petechiae on the conjunctiva of the eye, high fever, severe nervous disorders, hydrothorax and hydropericardium (Van de Pypekamp & Prozesky, 1987). This disease, transmitted by ticks of the genus *Amblyomma,* is regarded as one of the most important diseases of domestic ruminants in southern Africa. It also poses a serious threat to livestock and wildlife in the United States because of the presence of the disease and its vectors in the Caribbean (Provost & Bezuidenhout, 1987).

To date, stocks of *C. ruminantium* from geographically widely separated areas have shown either complete, partial or no cross-immunity *inter se* or against the reference Ball 3 stock, when tested in goats (Jongejan, Uilenberg & Franssen, 1988), sheep (Du Plessis, Van Gas, Olivier & Bezuidenhout, 1989) or mice (Stewart, 1989). Du Plessis, using an indirect fluorescent antibody technique, demonstrated cross-reacting antibodies between the Kümm and Ball 3 stocks (Du Plessis, 1981). These 2 stocks, however, differed with respect from their pathogenicity in sheep, cattle and mice and were not cross-protective. Antigenic differences between the stocks complicate immunization against the disease and may explain some of the disappointing immunization results that have been reported (Uilenberg, 1983). Alexander (1931) reported a variation in the virulence of the different stocks of *C. ruminantium* that may even occur for the same stock during serial passage in sheep.

Various serological tests for the detection of antibodies to *C. ruminantium* have been reported, each utilizing a different source of antigen. These sources include infected brain (Du Plessis, 1981; Ilemobade & Blotkamp, 1976), mouse peritoneal cells (Du Plessis & Malan, 1987), neutrophils (Holland, Logan, Mebus & Ristic, 1987) and infected *Amblyomma* nymph extracts (Neitz, Viljoen, Bezuidenhout, Oberem, Visser & Vermeulen, 1986). Recently, success has been achieved with the cultivation of *C. ruminantium* in cell cultures (Bezuidenhout, Patterson & Barnard, 1985). This offers a possible alternative source of suitable specific antigens with which to increase the sensitivity and specificity of existing serological tests.

Development of a subunit vaccine is of importance, because the only commercially available vaccine against heartwater is a live, blood vaccine which has various disadvantages (Oberem & Bezuidenhout, 1987).

Identification and characterization of the antigenic and immunogenic proteins of *C. ruminantium* could lead to:

- —clarification of differences observed between different stocks regarding pathogenicity and immunogenicity,
- —a sensitive, specific serological test which could be used to identify *C. ruminantium* or the different stocks thereof, and
- —a subunit vaccine protecting against antigenically different stocks.

A study was conducted to identify the antigenic proteins of *C. ruminantium,* and in this paper we report the identification of 2 common antigenic proteins of *C. ruminantium*.

MATERIALS AND METHODS

Stocks of C. ruminantium

Nine stocks of *C. ruminantium,* differing in antigenic composition, virulence, pathogenicity, serotype and origin were used in these investigations. The stocks used and their place of origin are as follows: Welgevonden, Ball 3, Breed and Mara (northern Transvaal, South Africa); Germishuys and Kümm (north-eastern-Transvaal, South Africa); Kwanyanga (eastern-Cape Province, South Africa); Mali (Mali) and Comoro (Comoro Islands), cited by Du Plessis *et al.* (1989).

In vitro *cultivation of* C. ruminantium

The Welgevonden and Ball 3 stocks were cultured in a calf endothelial cell line (E_5 cell line) and the Kwayanga stock in a bovine aorta cell line (BA cell line), as described by Bezuidenhout *et al.* (1985).

Preparation of crude C. ruminantium *extracts from cell cultures*

C. ruminantium-infected cell cultures (80–100 % infected with intra- and extracellular organisms) were harvested with a cell scraper into the culture media. Clumps of cells were disintegrated by draw-

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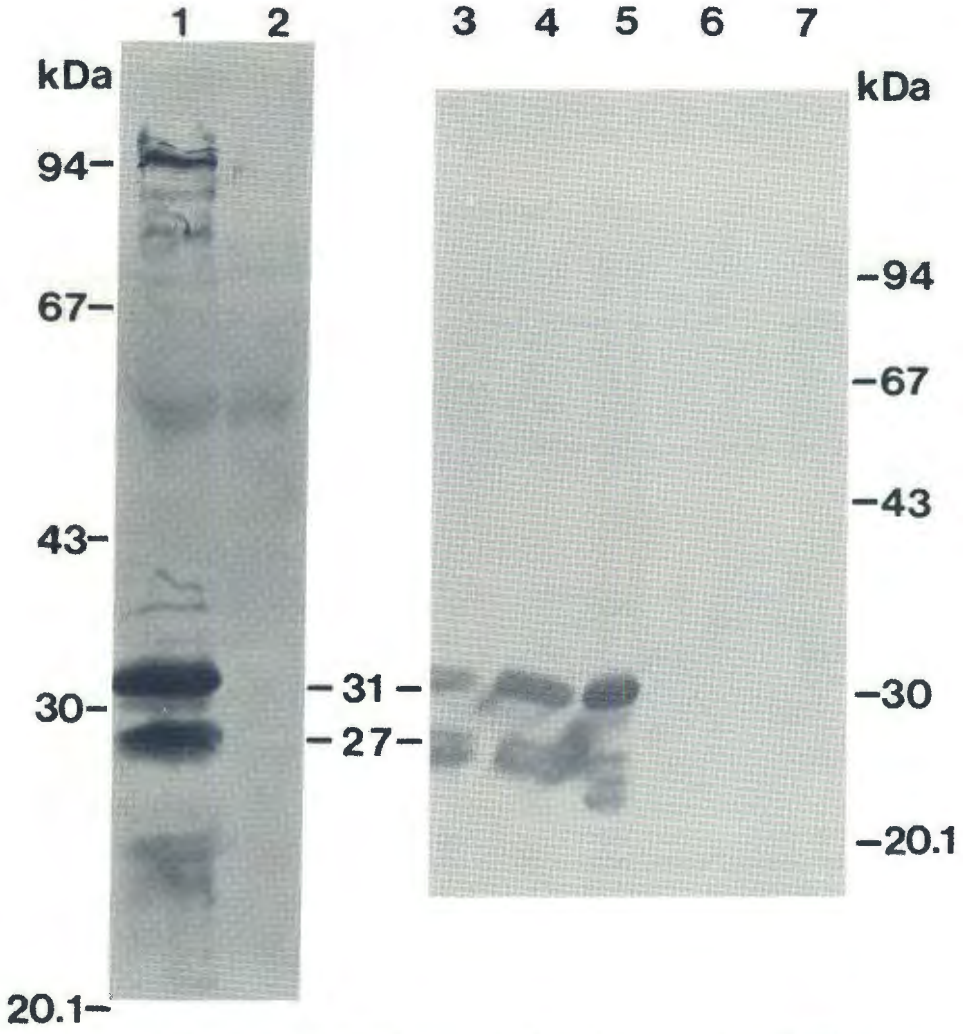

FIG. 1 Western blot analysis of *C. ruminantium*-infected crude cell culture extracts probed with 2 different hyperimmune anti-serum. Lanes 1 and 2 were probed with anti-Welgevonden hyperimmune bovine serum. Lanes 3, 4, 5, 6 and 7 were probed with anti-Welgevonden hyperimmune sheep serum. Lanes 1 and 5, Welgevonden-infected E_5 cell culture extract; Lanes 2 and 7, uninfected E_5 cell culture extract; Lane 3, Kwanyanga-infected BA cell culture extract; Lane 4, Ball 3-infected E_5 cell culture extract; Lane 6, uninfected BA cell culture extract

ing the mixture 3 times into a 20 mℓ syringe and 19 gauge needle. This material was centrifuged for 30 min at 10 000 × g. The resultant pellet was resuspended in phosphate buffered saline (PBS: 0,14 M NaCl, 1 mM KH_2PO_4, 8 mM $Na_2HPO_4.12H_2O$ and 3 mM KCl, pH 7,4). This resuspended material was stored at −75 °C and will be referred to as infected, crude, culture extracts. This procedure was also followed for the preparation of uninfected, crude, culture extracts.

Preparation of sera

Nine sheep and 1 ox was infected with 5–10 mℓ heartwater infective blood stabilate by intravenous injection. Each of the 9 sheep was infected with 1 of the following stocks of *C. ruminantium:* the Welgevonden, Ball 3, Kwanyanga, Mali, Comoro, Breed, Germishuys, Kümm and Mara stock. The ox was infected with the Welgevonden stock. The animals were treated on the 3rd day of a rising febrile reaction by intramuscular (im) injection with long-acting oxytetracycline[1] at a dosage rate of 20 mg/kg. All the animals were boosted 4 weeks after the initial inoculation. Blood collected from the immunized animals 4 weeks after final challenge, were left to clot overnight at 4 °C. The coagulated blood was then centrifuged for 10 min at room temperature at 300 × g. Sera were siphoned off and stored at −20 °C.

Protein assay

The protein content of the crude, culture extracts were determined by the Bio-Rad[2] protein micro assay using, bovine serum albumin as standard.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed by a modification of the Laemmli (1970) method. Briefly, samples for electrophoresis (40 μg protein/well) were dissolved in buffer containing 0,0625 M Tris-HCl buffer (pH 6,8), 16 % glycerol, 2 % sodium dodecyl sulphate (SDS), 2,5 % dithiothreitol and 0,001 % bromophenol blue by heating for 10 min at 100 °C. Low molecular mass standards[3] were used. The stacking gel contained 4 % acrylamide, 0,1 % bisacrylamide, 0,1 % SDS and 0,125 M Tris-HCl buffer (pH 6,8). The separating gel contained 12 % acrylamide, 0,3 % bisacrylamide, 0,1 % SDS and

[1] Liquamycin LA, Pfizer.

[2] München, FRG.

[3] Pharmacia, Uppsula, Sweden.

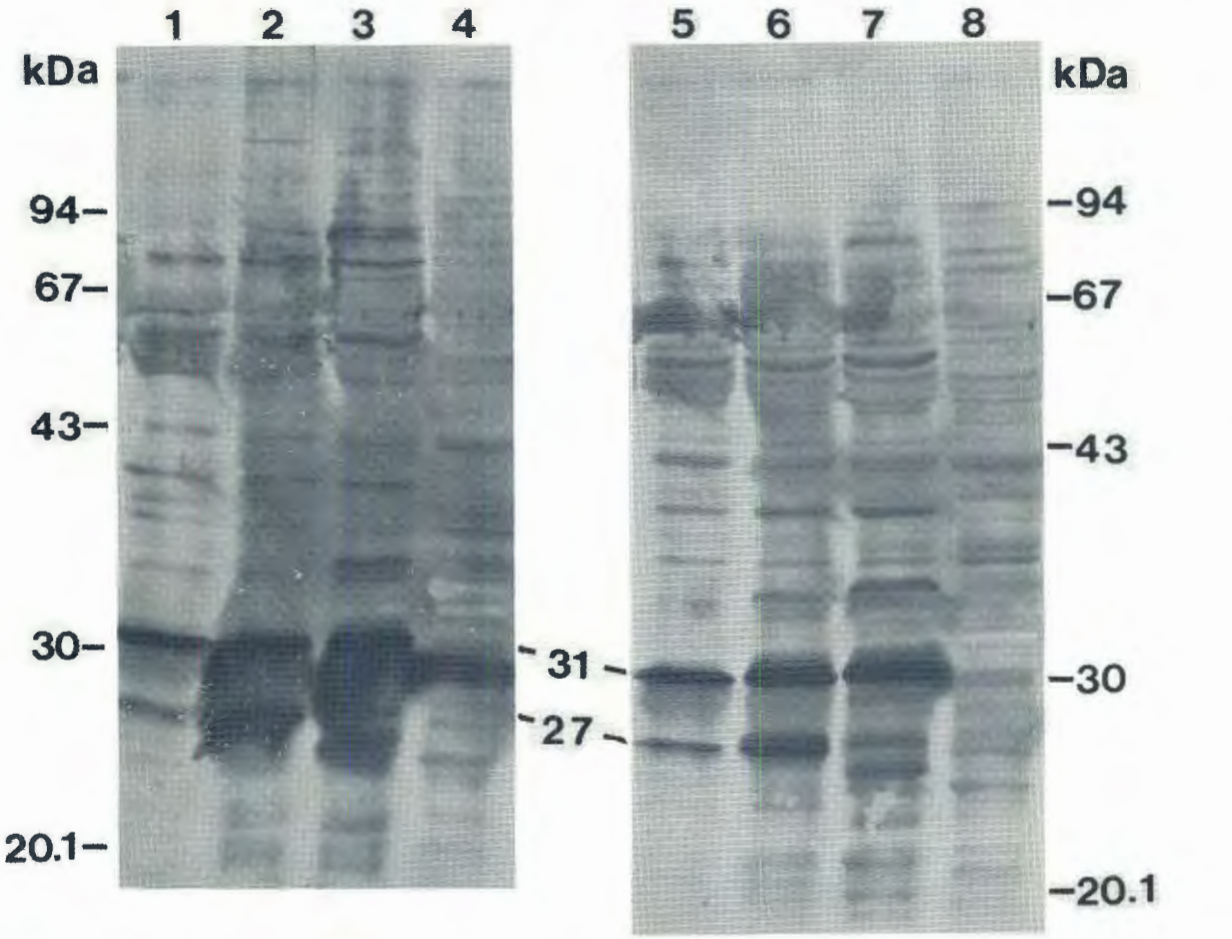

FIG. 2 Western blot analysis of *C. ruminantium*-infected crude cell culture extracts probed with 2 different hyperimmune anti-serum. Lanes 1, 2, 3, 4 and 5 were probed with anti-Kwanyanga hyperimmune sheep serum. Lanes 6, 7, 8, 9 and 10 were probed with anti-Ball 3 hyperimmune sheep serum. Lanes 1 and 6, Kwanyanga-infected BA cell culture extract; Lanes 2 and 7, Ball 3-infected E_5 cell culture extract; Lanes 3 and 8, Welgevonden-infected E_5 cell culture extract; Lanes 4 and 9, uninfected BA cell culture extract and Lanes 5 and 10, uninfected E_5 cell culture extract.

0,375 M Tris-HCl buffer (pH 8,8). Each gel was polymerized by the addition of ammonium persulphate and tetramethylenediamine at final concentrations of 0,05 % and 0,1 %, respectively, in the stacking gel and 0,05 % each in the separating gel. Gels of 1,5 mm × 12 cm × 16 cm were prepared. Electrophoresis was performed with an electrode buffer containing 0,016 M Tris-HCl, 0,125 M glycine and 0,06 % SDS, pH 8,3 for about 2 h at a constant electric current of 45 mA, whilst the mobile front was in the stacking gel. Once the mobile front entered into the separating gel, electrophoresis was performed at a constant electric current of 60 mA for about 3 h.

Bands were visualized by staining the gel for 18 h with 0,25 % Coomassie brilliant blue, 5 % methanol, 1 % acetic acid and 5 % distilled water. The gels were then destained with 5 % methanol, 1 % acetic acid and 5 % distilled water.

Western blotting

Electrophoretic transfer of SDS-PAGE-separated polypeptides was achieved by a modification of the procedures of Moos, Nguyen & Liu (1988). Proteins were transferred to a polyvinilidene difluoride[4] (PVDF) membrane by electrophoresis in 10 mM cyclohexylamino propane sulphonic acid (CAPS) buffer (pH 9,0) at 0,12 A to 0,25 A for 165 min. A portion of the blotted sheet containing the low molecular mass standards were removed and the remaining portion was air dried and stored at room temperature until further use. The molecular mass standards were visualized by staining the membrane with 0,25 % Coomassie brilliant blue, 5 % methanol, 1 % acetic acid and 5 % distilled water for 10 min. The membrane was destained in 5 % methanol, 1 % acetic acid and 5 % distilled water for 10 min and rinsed in distilled water for a further 10 min, after which it was left to dry.

Immunoblotting

C. ruminantium antigens on the PVDF membranes were identified by immunoblotting techniques with hyperimmune sera against 9 stocks of *C. ruminantium*. Dried, western blotted, PVDF membranes were rewetted in methanol and rinsed briefly in distilled water. The membranes were incubated in sera diluted 1:200, with a solution containing 1 % milk powder[5], 0,05 % Tween 20, 20 mM Tris-HCl and 0,9 % NaCl, pH 7,4 (incubation buffer), with gentle agitation for 90 min at room temperature. The membranes were washed 3 times, 5 min/wash, with 0,1 % milk powder in 20 mM Tris-HCl, pH 7,4 (wash buffer). This was followed by gentle agitation of the membrane for 90 min at room temperature in peroxidase-conjugated anti-bovine or anti-sheep

[4] Millipore, South Africa (Pty) Ltd.

[5] Elite, South Africa.

[6] Cappel laboratories, West Chester, USA.

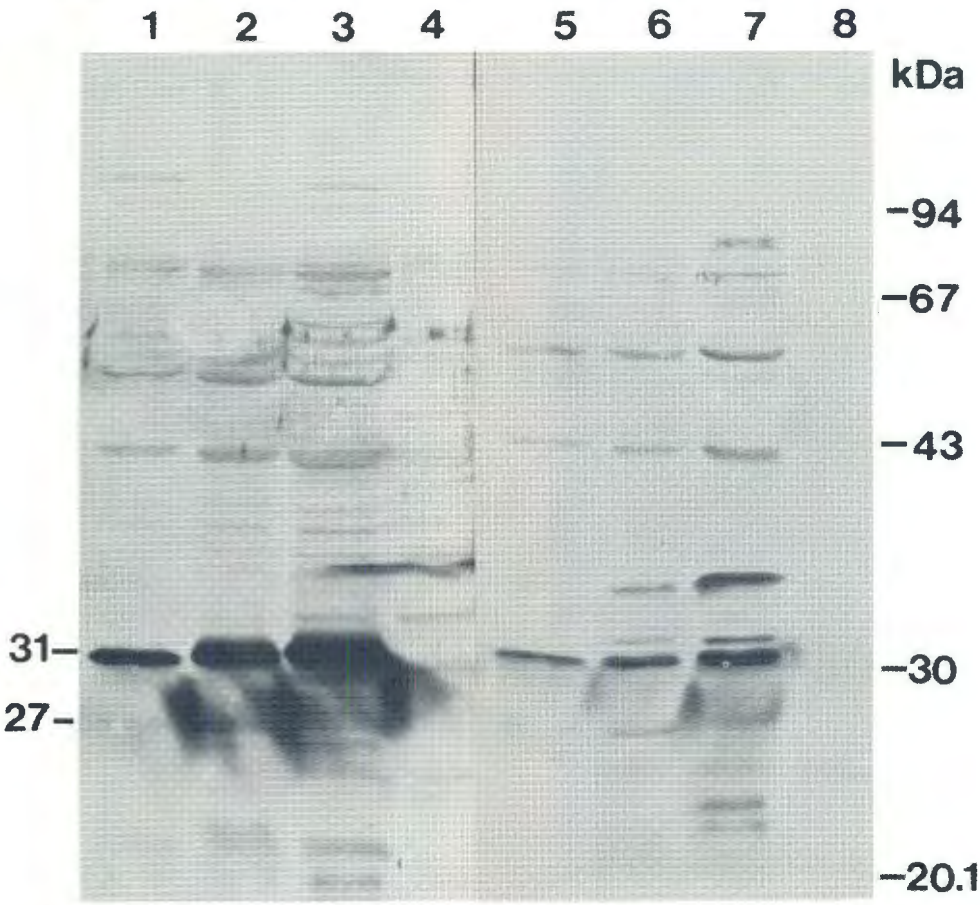

FIG. 3 Western blot analysis of *C. ruminantium*-infected crude cell culture extracts probed with 2 different hyperimmune anti-serum. Lanes 1, 2, 3 and 4 were probed with anti-Mara hyperimmune sheep serum. Lanes 5, 6, 7 and 8 were probed with anti-Germishuys hyperimmune sheep serum. Lanes 1 and 5, Kwanyanga-infected BA cell culture extract; Lanes 2 and 6, Ball 3-infected E_5 cell culture extract; Lanes 3 and 7, Welgevonden-infected E_5 cell culture extracts and Lanes 4 and 8, uninfected E_5 cell culture extract.

IgG[6], which was diluted 1:500 with incubation buffer. After washing 3 times with wash buffer, 5 min/wash, proteins were detected by immersing the membrane into a solution containing 0,06 g of chloronaphtol/20 mℓ of cold methanol in 0,06 mℓ H_2O_2/100 mℓ 20 mM Tris-HCl, pH 7,4 for 20 min. After terminating substrate development by rinsing the membrane in distilled water the membrane was air-dried and stored at −20 °C.

RESULTS

The immuno blots with sheep anti-sera to the 9 stocks showed both similarities and differences between the 3 stock antigens and the 9 anti-sera (Fig. 1, 2, 3, 4 and 5).

The only similarities observed between the stocks are the 27 and 31 kDa proteins. These proteins are present in the antigenic protein profiles of the Welgevonden, Ball 3 and Kwanyanga stocks and are recognized by sheep anti-sera to the Welgevonden, Ball 3, Kwanyanga, Mali, Comoro, Breed, Germishuys, Kümm and Mara stocks, and bovine anti-sera to the Welgevonden stock. The 31 kDa protein is always recognized by all 9 sheep antisera, but the intensity of the 27 kDa band varied according to the stock of *C. ruminantium* used for the detection.

The average number of antigenic proteins detected for the Welgevonden, Ball 3 and Kwanyanga stocks by the 9 sheep anti-sera are 20, 12 and 9, respectively. No 2 stocks show identical antigenic properties.

DISCUSSION

The identification of *C. ruminantium* antigenic proteins by immunoblotting procedures revealed 2 common antigens of 27 kDa and 31 kDa. These proteins are present in the protein profiles of the Welgevonden, Ball 3 and Kwanyanga stocks. They are recognized by sheep antiserum to the Welgevonden, Ball 3, Kwanyanga, Mali, Comoro, Breed, Germishuys, Kümm and Mara stocks and by bovine antiserum to the Welgevonden stock. Recently, a 32 kDa immunodominant, antigenically conserved, *C. ruminantium* protein was identified (Jongejan & Thielemans, 1989). This protein was demonstrated in goat choroid plexus, infected with either the Senegal or Welgevonden stocks and recognized by goat anti-sera against Senegal, Sudan, Kenya, Nigeria, Guadeloupe, Ball 3, Kümm, Kwanyanga and Welgevonden stocks. Molecular mass determination by SDS-PAGE may be determined with an accuracy of at least 10 % (Weber & Osborn, 1969). Therefore, the probability exists that this 32 kDa protein is the same as the 31 kDa immunodominant protein identified in these investigations.

It was shown that sheep anti-sera to the 9 stocks identified an average of 20 antigenic proteins for the Welgevonden stock and an average of 12 for the Ball 3 stock. The Welgevonden stock, therefore possesses a larger number of common antigens to the other 7 stocks than does Ball 3. This is consistent with the observation that the Welgevonden stock elicits total immunity against 4 and partial immunity against 5 stocks, while Ball 3 elicits total immunity

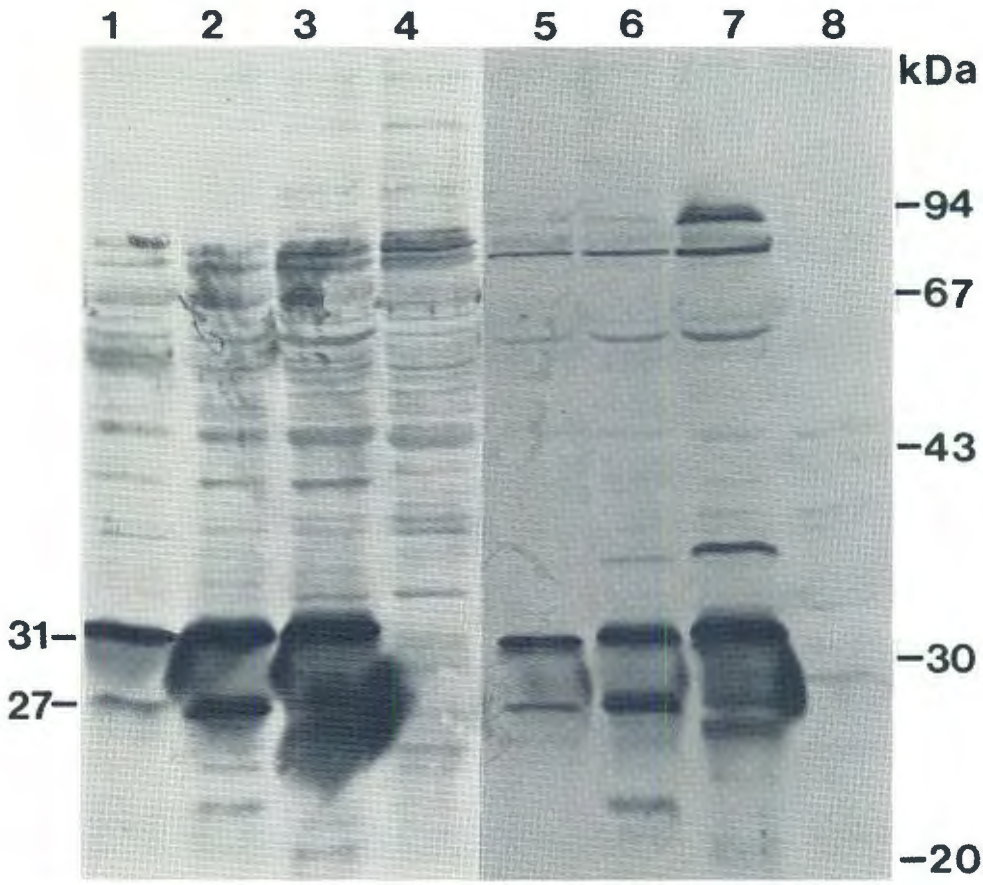

FIG. 4 Western blot analysis of *C. ruminantium*-infected crude cell culture extracts probed with 2 different hyperimmune anti-serum. Lanes 1, 2, 3 and 4 were probed with anti-Kümm hyperimmune sheep serum. Lanes 5, 6, 7, and 8 were probed with anti-Breed hyperimmune sheep serum. Lanes 1 and 5, Kwanyanga-infected BA cell culture extract; Lanes 2 and 6, Ball 3-infected E_5 cell culture extract; Lanes 3 and 7, Welgevonden-infected E_5 cell culture extracts and Lanes 4 and 8, uninfected E_5 cell culture extract.

only against 2 and partial immunity against 4 of the 8 stocks of *C. ruminantium* used in our investigations (Du Plessis *et al.*, 1989). As complete, partial or no cross-protection is observed between the various stocks of *C. ruminantium*, it would be unlikely that the 27 and 31 kDa proteins play a role in protection. Furthermore, no correlation could be found between the serologically detectable antigens and cross-protection of certain stocks. The Ball 3 and Comoro and the Ball 3 and Germishuys stocks are totally cross-protective in sheep. Yet these 3 stocks do not possess any unique antigens to explain the cross-protection.

It is suggested that virulence of certain *Anaplasma* isolates may be associated with specific antigens and that identification of these antigens may form the basis for strain differentiation (Adams, Smith & Kühlenschmidt, 1986). Antigenic heterogenicity and the presence of common antigens have been demonstrated among isolates of *Rickettsiae* (Tamura, Ohashi, Urakami, Takahashi & Oyanagi, 1985), Chlamydia (Caldwell & Judd, 1982) and *Anaplasma* (Adams *et al.*, 1986). Antigenic heterogenicity allows grouping of different isolates. A panel of isolate-restricted, monoclonal antibodies could be used to classify rickettsial organisms. This could allow the correlation of geographical distribution to antigenic differences and the identification of organisms in cross-immunity experiments as being a primary persistent or a secondary heterogeneous challenge (McGuire, Palmer, Goff, Johnson & Davis, 1984). Monoclonal antibodies recognizing common determinants can be used to identify organisms in vertebrate and invertebrate host tissues, including cell culture. In addition, common determinants can form the basis for specific serological tests and for the synthesis of probes to screen genomic libraries.

The 27 and 31 kDa common antigenic proteins should be investigated for possible use in an enzyme-linked immunosorbent assay (ELISA) whereby the presence of anti-*C. ruminantium* antibodies can be detected in sera. This assay can be employed during epidemiological surveys and possibly also be used to screen hybridoma clones for specific monoclonal antibody production. The partial amino acid sequence of the 27 and 31 kDa proteins should be determined and an appropriate probe developed for screening *C. ruminantium* genomic libraries. Furthermore, production of monoclonal antibodies against the 27 and 31 kDa proteins of *C. ruminantium* could be useful in screening *C. ruminantium* genomic expression libraries and for the development of a specific ELISA test, whereby the presence of *C. ruminantium* antigens could be identified in tissues.

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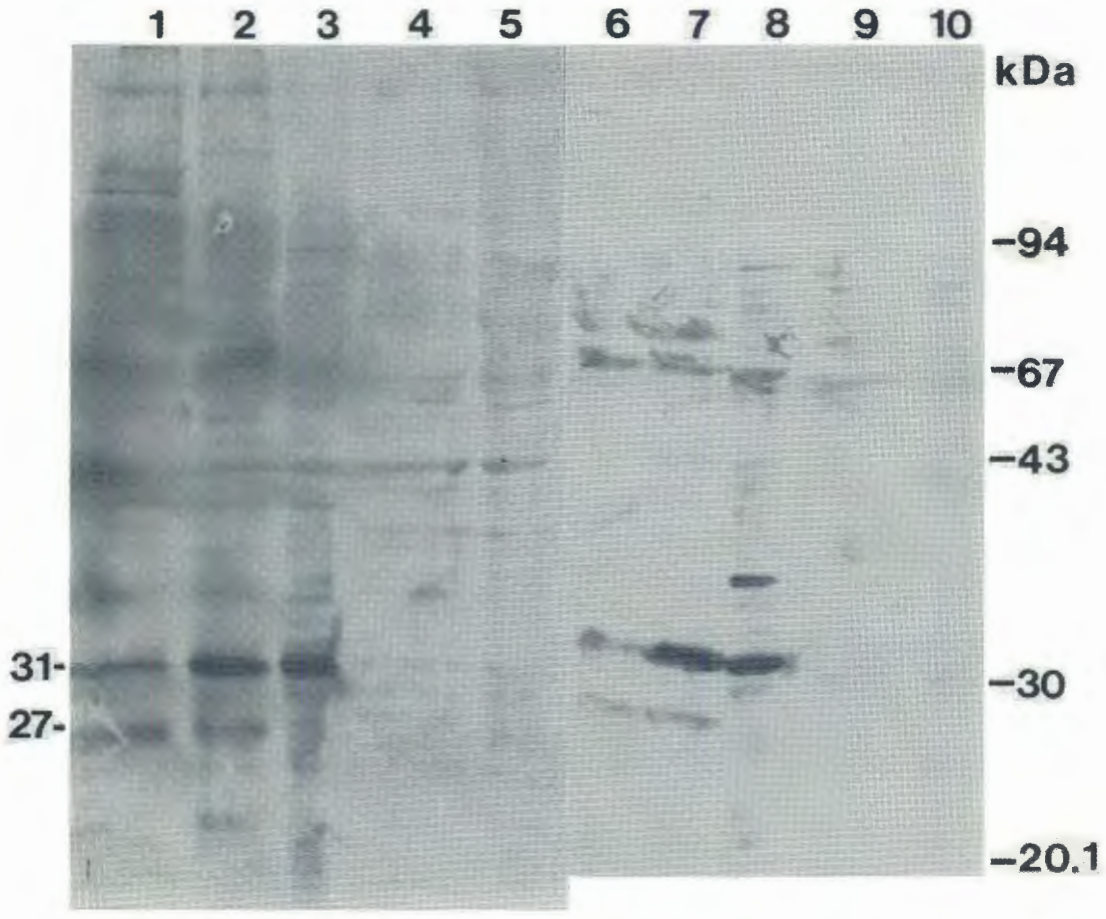

FIG. 5 Western blot analysis of *C. ruminantium*-infected crude cell culture extracts probed with 2 different hyperimmune anti-serum. Lanes 1, 2, 3 and 4 were probed with anti-Mali hyperimmune sheep serum. Lanes 5, 6, 7 and 8 were probed with anti-Comoro hyperimmune sheep serum. Lanes 1 and 5, Kwanyanga-infected BA cell culture extract; Lanes 2 and 6, Ball 3-infected E_5 cell culture extract; Lanes 3 and 7, Welgevonden-infected E_5 cell culture extracts and Lanes 4 and 8, uninfected E_5 cell culture extract.

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