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THE IDENTIFICATION, ISOLATION AND CHARACTERIZATION OF ANTIGENIC PROTEINS OF COWDRIA RUMINANTIUM

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THE IDENTIFICATION, ISOLATION AND CHARACTERIZATION OF ANTIGENIC PROTEINS OF *COWDRIA RUMINANTIUM*.

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THE IDENTIFICATION, ISOLATION AND CHARACTERIZATION OF ANTIGENIC PROTEINS OF *COWDRIA RUMINANTIUM*

by

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CONTENTS

Chapter 1

INTRODUCTION

Cowdria ruminantium, a rickettsia organism, is the causative agent of heartwater. It is a non-contagious livestock disease characterized by the development of petechiae on the conjunctiva of the eye, high fever, severe nervous symptoms, hydrothorax and hydropericardium (Van de Pypekamp & Prozesky, 1987). This disease, transmitted by some members of the tick 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 due to its presence in the Caribbean (Provost & Bezuidenhout, 1987).

The disease was first recorded in South Africa during the 1830s by the voortrekker pioneer Louis Trichardt (Neitz, 1947). In 1898 heartwater was shown to be a transmissible disease, by the subinoculation of blood (Dixon, 1898; Edington, 1898). In 1900, Lounsbury reported the disease to be transmitted by the tick *Amblyomma hebraeum*, and in 1925 the causative organism, a Rickettsia, was observed by E.V. Cowdry in sections of mammalian host tissue (Cowdry, 1925a). Until 1980 heartwater was considered to be a disease limited to Africa and a few adjacent islands. However, when the disease was diagnosed on Guadeloupe in 1980, the United States and other countries of the Western hemisphere became concerned about heartwater (Perreau *et al*, 1980).

The economic impact of heartwater on livestock production cannot be determined accurately because the disease is seldom reported, diagnosis is rarely confirmed, the incidence of the disease is masked by the use of effective dipping compounds, certain animal breeds are resistant and the existence of enzootic stability. Heartwater is a major stumbling stone against upgrading or replacing local stock or when moving stock from

heartwater free to heartwater enzootic areas (Provost & Bezuidenhout, 1987).

The disease occurs in most countries of Africa south of the Sahara. Outside the African continent heartwater occurs on several Caribbean islands (Uilenberg, 1983). Heartwater is therefore a threat to livestock on the American mainland where potential vectors are present but which do not harbour the disease and to countries where the vectors may be introduced and become established. Figure 1.1. illustrates the global distribution of heartwater (Provost & Bezuidenhout, 1987).

All proven vectors of heartwater are 3 host ticks eg. *A. hebraeum* utilises certain birds and small, medium sized and large mammals as hosts for its immature stages (nymphs and larvae) while the adult ticks prefer the larger animals. Twelve species of *Amblyomma* are presently known to be capable of transmitting *C. ruminantium*. They are: *A. variegatum, A. hebraeum, A. pomposum, A. lepidium, A. astrion, A. cohaerens, A. gemma, A. sparsum, A. marmoreum, A. tholloni, A. maculatum* and *A. cajennense*. In Africa, *A. variegatum* is the most important and widely distributed vector while the South African bont tick *A. hebraeum* is the major vector in the southern part of the continent (Walker, 1987). *A. variegatum* is the only African vector of heartwater that has established itself outside the African continent (Jongejan, 1990). The distribution of *Cowdria* therefore coincides with the distribution of it's *Amblyomma* vectors.

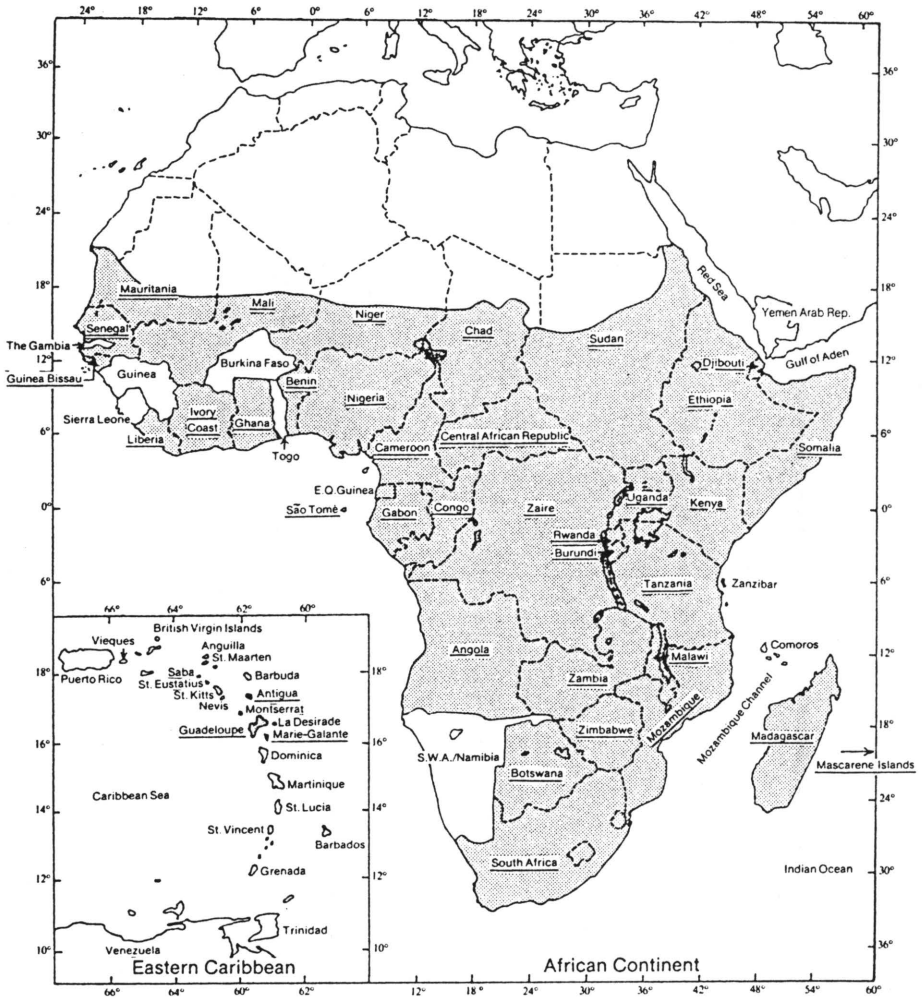


FIGURE 1.1. The global distribution of heartwater. Names of countries and islands from which the disease has been reported are underlined (Provost & Bezuidenhout, 1987).

Rickettsial organisms occupy a biological position intermediate between that of the smaller bacteria and the larger viruses and differ from the former in that they generally cannot be cultivated extracellularly in artificial media. They are therefore considered strict obligate intracellular parasites (Du Plessis, 1970a). In both the vertebrate and invertebrate hosts, *Cowdria* is consistently found within an inclusion vacuole rather than free within the host cell cytoplasm. The occurrence of this organism in colonies distinguishes it from symbiotic rickettsiae that are commonly found in ticks but unlike *Cowdria* rarely form inclusions. Organisms that live freely in the cytoplasm of cells include species of the genera *Rickettsia* and *Wolbachia*. Those found only within a membrane bound inclusion, include *Cowdria, Anaplasma, Ehrlichia* and *Coxiella* (Kocan & Bezuidenhout, 1987).

The current accepted classification of rickettsias as given in Bergy's manual is illustrated in Fig 1.2. (Krieg & Holt, 1984). The genus *Cowdria* and *Ehrlichia* are grouped together as they represent tick-borne rickettsias which occur in colonies situated in vacuoles in the host cell and multiply by binary fission. Scott (1987) was unsatisfied with this classification largely because obligate intracellular parasites, epicellular parasites and organisms that grow axenically were grouped together. He therefore proposed a reclassification of rickettsias by applying the techniques of numerical taxonomy (Fig. 1.3.; Scott, 1987).

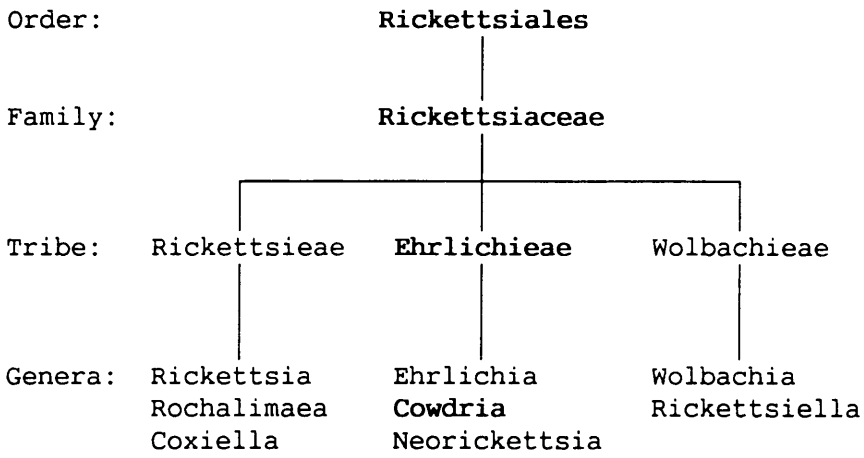


FIGURE 1.2. Current classification of rickettsias. (Krieg & Holt, 1984).

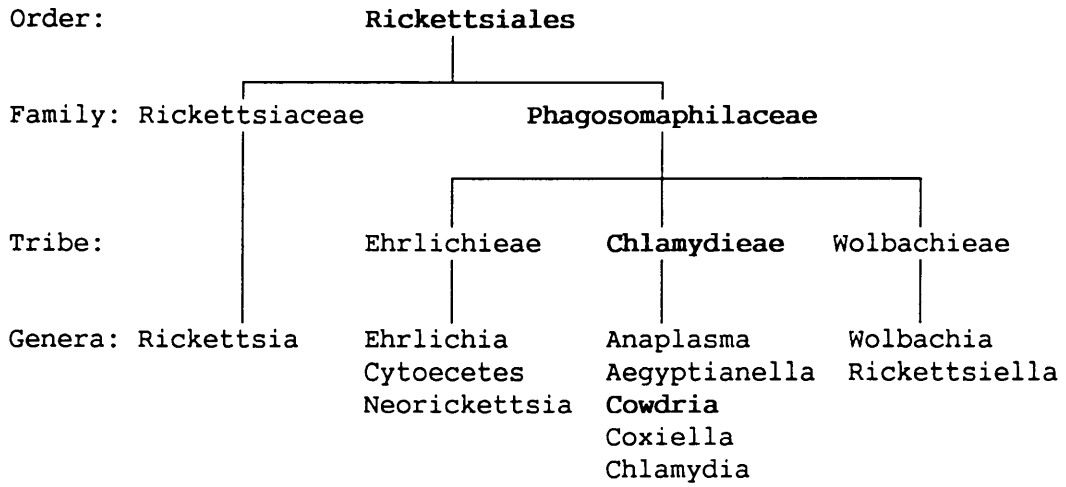


FIGURE 1.3. Proposed reclassification of the rickettsias. (Scott, 1987).

Each *Cowdria* organism is surrounded by two unit membranes separated by an electron pale area. It is suggested that the ground substance of the organism contains closely packed ribosomes (Pienaar, 1970). The size of the individual organism varies from 0,49-2,7 µm in diameter, and they are divided into four major forms: small, intermediate, large and very large (Pienaar, 1970; Prozesky *et al.*, 1986). All the forms are pleomorphic, the predominant shape observed is coccoid but ring-, comma- and horse shoe-shapes have also been identified. Within any one vacuole in the host cell cytoplasm, the organisms are generally of the same type or form (Pienaar, 1970). However it has been observed, *in vitro*, that different forms of organisms are found within a particular vacuole in cells which contain many colonies (Prozesky *et al.*, 1986). The organism has been shown to occur in various fractions of the blood (Pienaar, 1970; Ilemobade, 1976; Neitz *et al.*, 1986c); in various cell types including macrophages, monocytes, Kupffer's cells, reticulum cells of the lymph nodes, fibroblasts and connective tissue and cells of the spleen, brain, pancreas and heart in sheep, goats and cattle (Cowdry, 1925a; Du Plessis, 1970a, 1975; Ilemobade & Blotkamp, 1978). In the tick, organisms are present in the epithelial cells of the midgut and lumen of the gut (Cowdry, 1925b), salivary glands (Kocan *et al*, 1987), hypodermis, synganglion, haemolymph (Viljoen *et al*, 1988), malpighian tubules, hypodermis and rectal ampullae (Bezuidenhout, 1988).

In the vertebrate host *Cowdria* organisms initially replicate in reticulo-endothelial cells in lymph nodes and are then released into the efferent lymph stream and eventually into the blood stream where endothelial cells are parasitized. The organisms which are in contact with the cell membranes are taken up through phagocytosis, and enclosed in a vacuole. The vacuole membrane is therefore derived from cell membranes (Du Plessis, 1970a; Prozesky & du Plessis, 1987). *Cowdria* develops within the membrane bound vacuole in the cytoplasm of endothelial cells, in the mammalian host, from a single granule to a large group. This eventually causes the cell to rupture and disseminate the organisms once again into the blood, thus repeating the cycle. Multiplication appears to occur mainly by binary fission although there are indications of multiple budding and endosporulation. (Pienaar, 1970; Uilenberg, 1983).

In the tick vector *Cowdria* initially appears to develop in the midgut epithelial cells and subsequent stages of the organisms may invade and develop in salivary gland acini cells, which suggests that the organism may be transferred to the vertebrate host via the salivary glands rather than by gut regurgitation (Prozesky & Du Plessis, 1987; Bezuidenhout, 1988). It has recently been suggested that the heartwater organism spreads from the intestinal tract to other organs of the tick, including the salivary glands by means of the haemocytes (Hart *et al*, 1991).

Several researchers attempted to propagate *Cowdria in vitro* over many years, without success. It was only in 1985 that *Cowdria* was successfully cultivated in endothelial cells (Bezuidenhout *et al.*, 1985). Later success was also achieved in culturing the organism in neutrophils (Logan *et al.*, 1987). As neutrophil cultures do not divide in circulation and cannot be maintained in culture for more than a few days endothelial cell cultures are far more superior than neutrophil cultures for large scale production and long term maintenance of *Cowdria* organisms. *In vitro* cultivation of *Cowdria* was a major breakthrough as it can provide continuous production of large quantities of organisms for immunization, serology, attenuation, screening of chemotherapeutics and morphological studies of *Cowdria*.

Jongejan and co-workers recently reported on the sequential morphological development of *Cowdria* in cell culture (Fig. 1.4.; Jongejan *et al.*, 1991b). They observed that large colonies of reticulate *Cowdria* bodies resulted after division by binary fission within intracytoplasmic vacuoles. The reticulate bodies are characterized by a fine network of filaments interspersed with numerous ribosomes. After 3 to 4 days in cell culture these reticulate bodies developed into smaller intermediate bodies characterized by an electron-dense core. The intermediate bodies condensed further into electron-dense bodies shortly before disruption of the host cells releasing them into the culture medium. A new infectious cycle is thus initiated when the released elementary bodies invade other endothelial cells. Each infectious cycle lasts between 5 and 6 days. This correlates with the suggested developmental cycle observed *in vivo* where the developmental cycle of *Cowdria* in reticulo-endothelial cells is complete within 3 to 4 days (Du Plessis, 1975). There is a great deal of resemblance between the different forms of *Cowdria* found in ticks (Bezuidenhout, 1988; Hart *et al.*, 1991), mammals (Pienaar, 1970) and cell culture (Prozesky *et al.*, 1986; Jongejan *et al.*, 1991b).

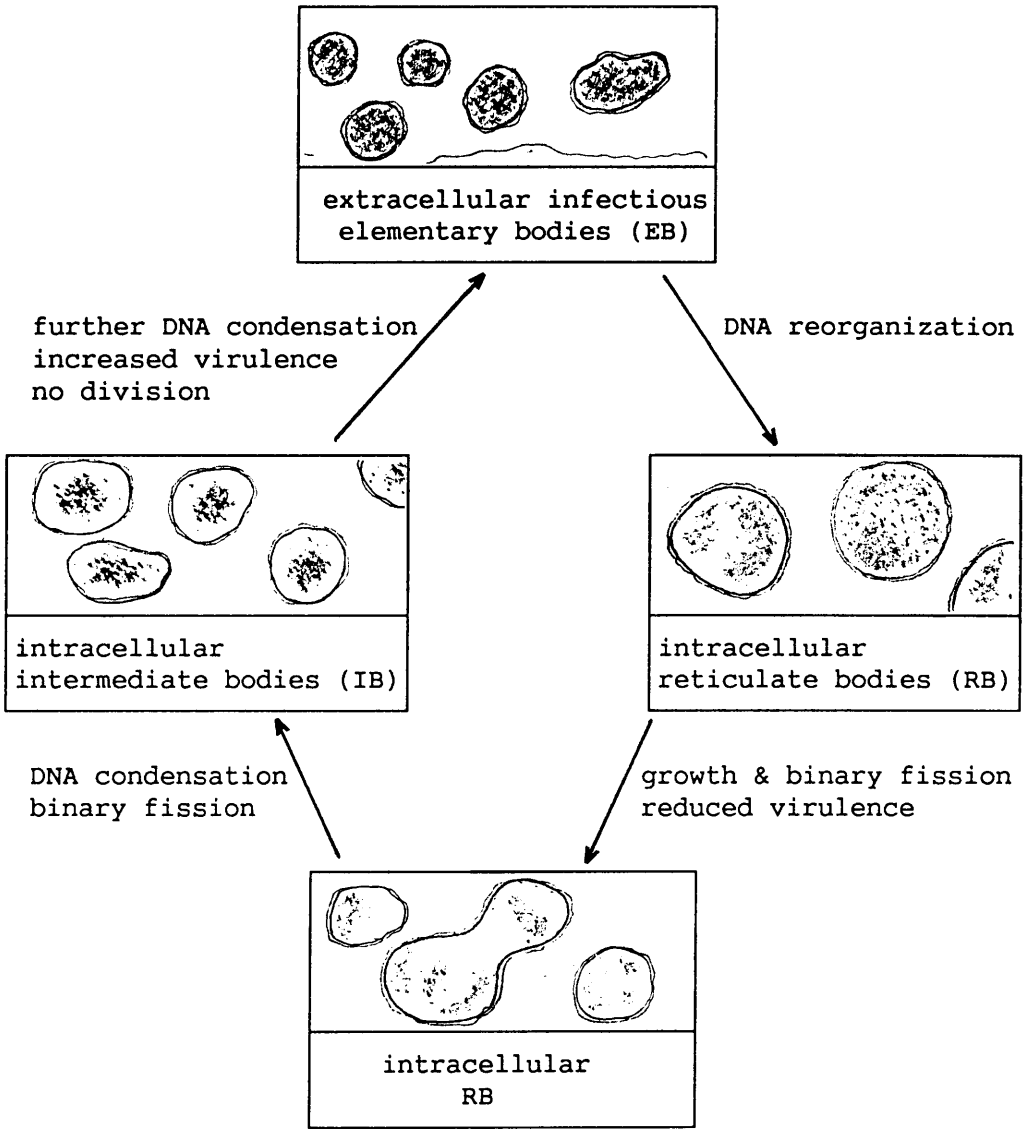


FIGURE 1.4. Proposed, diagramatic, sequential morphological development of *C. ruminantium* in cell culture according to Jongejan (1991b).

Cattle, sheep, goats and game are naturally susceptible to heartwater. Three non-ruminant hosts of *A. hebraeum* namely the leopard tortoise, the helmeted guinea fowl and the scrub hare have been identified as subclinical carriers of heartwater (Bezuidenhout, 1988). Ticks acquire the pathogen while feeding either on animals during the heartwater reaction or on carrier animals. Transmission of the disease occurs transstadially from larva to nymph, from nymph to adult, or from larva through nymph to adult (Bezuidenhout, 1987; Horak *et al*, 1987).

Transovarial transmission has only been reported once and suggested to be the exception rather than the rule (Bezuidenhout & Jacobsz, 1986). The causal agent can be maintained by one generation of ticks, infected as larvae, for a period of more than three years (Neitz, 1968). Sheep, cattle and African buffalo have shown to remain carriers of heartwater for long periods after recovery from the disease ie. for 223, 246 and 161 days respectively (Andrew & Norval, 1989). Previously it was believed that blood of infected ruminants is only infective for ticks for a limited period ie. from the start of the febrile reaction until a few weeks after recovery (Barré & Camus, 1987).

Within the different species of animals the incubation period of heartwater varies considerably: in naturally infected animals it varies between 9-29 days and in artificially infected animals between 5-35 days. The incubation period is influenced by the route of infection, virulence of the stock and amount of infective material administered. The morbidity and mortality rates are largely influenced by the species, breed and age of the animal, the virulence of the *Cowdria* stock, immunization and tick control programs, specific chemotherapy and the season (Van De Pypekamp & Prozesky, 1987). Indigenous breeds possess a higher degree of natural resistance. Ilemobade (1976) and Alexander (1931) reported that domestic ruminants are capable of developing resistance to heartwater. Resistance of calves and lambs is independent of the immune status of the dam (Neitz, 1939). Heartwater resistance of cattle from calf to adulthood appears to have both a specific (acquired immunity) and a non-specific component (innate immunity). The suckling calf resistance, which declines with advancing age, and conglutinin (k) associated resistance in calves, which increases with advancing age and persists lifelong, are the non specific components. The role played by acquired immunity as the specific component is determined by the time of primary infection and the frequency of re-infection (Du Plessis, 1985b). Once an animal has recovered from heartwater, immunity lasting for many months may be expected (Neitz, 1939). The duration of immunity to heartwater varies from six months to four years, depending on the species of animal (Stewart, 1987). The mechanism by which the immune response develops is largely unknown but cellular rather than humoral immunity has been

suggested (Alexander, 1931; Du Plessis, 1970b; Stewart, 1987). In order to analyse the mechanism of immunity to heartwater, it is essential that purified *Cowdria* antigens become available (Ambrosio *et al.*, 1987). Recently studies indicated cell-mediated immunity in heartwater (Du Plessis *et al*, 1991). They observed that athymic nude mice were unable to make a drug aided recovery from infection and were unable to mount an immune response. Adoptive transfer of immunity was achieved by transfer of spleen cells of immune mice. Macrophages, Lyt-2^+ and the opsonic effect of antibodies were also suggested to play a role in immunity.

In 1971 Du Plessis and Kümm identified the first stock of *Cowdria* to be pathogenic to laboratory mice (Du Plessis & Kümm, 1971), designated the Kümm stock. A complete range of pathogenicity for laboratory mice has since been observed in isolated stocks of *Cowdria*. Heartwater in laboratory mice is complicated by a variable degree of pathogenicity between different stocks of *Cowdria*, variation in susceptibility of strains of laboratory mice and variation in pathogenicity of some stocks depending on the route of infection. Heartwater in laboratory mice is characterized by the absence of pyrexia and a marked hypothermia (Mackenzie & McHardy, 1987). The highest concentration of *Cowdria* organisms have been indicated in the lungs and heart of laboratory mice (Prozesky & Du Plessis, 1985). The murinotropic stocks of *Cowdria* provide a suitable model for laboratory investigations of the organism which can be conducted at a reasonable cost.

Diagnosis of heartwater is often difficult in the live animal because clinical signs of the disease are not pathognomonic and the course of the disease is usually too fast for early treatment. A tentative diagnosis of heartwater can be made by considering the epidemiology, symptomatology and lesions at autopsy. But a definite diagnosis depends upon the microscopic demonstration of the causative organism in the vascular endothelial cells of the brain. Subinoculation of blood, infection by ticks collected from sick animals or brain biopsies can be used to make a diagnosis in the live animal. In the field the most reliable method of diagnosis remains the examination of brain smears from the first animal that has died followed by daily rectal temperatures of all animals (Camus

& Barré, 1987). Treatment of heartwater during the early febrile stages, which is usually not detected, presents few problems and recovery can be expected when either sulphonamides or tetracyclines are used. Treatment of heartwater once neurological signs have developed, becomes more difficult as not enough is known about the pathophysiology to make supportive treatment really effective (Van Amstel & Oberem, 1987).

Development of a serological assay can be useful for the diagnosis of heartwater, identification of the aetiological agent in blood, screening tick material for infectivity and monitoring of the time course of the immune response. The capillary flocculation test was investigated in 1976 as a diagnostic test for heartwater (Ilemobade & Blotkamp, 1976). When using this assay to screen for antibodies to *Cowdria*, antibodies were first detected 1-4 weeks after clinical recovery or after treatment. The antibodies were observed to persist 1-4 weeks after treatment but the animals were immune 4 months after infection. Therefore the capillary flocculation test cannot be used to determine the immune status of animals and was found to be restricted to the detection of recent infections. This therefore limited its usefulness in diagnosis and epidemiological studies.

Neitz and his co-workers developed an enzyme-linked immunosorbent assay (ELISA) utilizing wheat germ lectin purified organisms from infected nymphae as antigen. They were able to detect antibodies to *Cowdria* from day 8 post infection and up to day 28. The ELISA was specific for *Cowdria* antigens and was suggested to be useful for diagnosis, immunological studies, screening samples for antigen and epidemiology studies (Neitz *et al*, 1986a, 1986b, 1986c; Viljoen *et al*, 1988).

In 1987 two different research groups reported an indirect fluorescent antibody (IFA) assay for the detection of heartwater antibodies in sera of animals (Du Plessis & Malan, 1987; Holland *et al*, 1987). The IFA assay developed by Holland *et al* used primary blood neutrophil cultures as antigen and they demonstrated an antigenic relationship between *Cowdria* and *Ehrlichia*. Du Plessis and coworkers on the other hand used peritoneal macrophages of mice as antigen and they demonstrated a lack of cross

reactivity with immunologically related organisms such as *Chlamydia, Rickettsia* and *Coxiella* (Du Plessis & Malan, 1987), and cross reactivity with *Ehrlichia* (Du Plessis *et al.*, 1987). Therefore the application of the IFA assay for the diagnosis and epidemiological studies of heartwater is complicated by cross reactions with *Ehrlichia*. However the IFA assay can be a valuable tool for monitoring artificial infection of experimental animals.

Recently, a competitive ELISA (CELISA) which detected specific *Cowdria* antibodies in goat, sheep and cattle sera was developed (Jongejan *et al.*, 1991a). They used a monoclonal antibody, which reacts with a 32 kDa *Cowdria* protein in western blots, and sonicates of *Cowdria* infected endothelial cell cultures as antigen. They found a 89 % agreement between the CELISA and the IFA test for which infected endothelial culture cells were used as antigen. In addition, they found no cross reactions with *Ehrlichia* or *Anaplasma*. This lack of cross-reactivity and detection of antibodies to eight geographically widely distributed stocks of *Cowdria* makes the CELISA a promising test for use in heartwater endemic areas (Jongejan *et al.*, 1991a).

The detection of antibodies to *Cowdria* in serum is important for studies on immunological aspects of heartwater, in epidemiological investigations and in the diagnosis of the disease. Since the sensitivity and specificity of serological tests depend on the source and the method employed for the preparation of the antigen as well as the detection method for antibody-antigen interaction, alternative tests should still be investigated (Neitz *et al.*, 1986b).

Control of heartwater remains a serious problem. Intensive dipping with acaricides is expensive and only effective under certain conditions. Protective immunity against heartwater can be induced in ruminants by infection with virulent blood and subsequent treatment of the reaction with antibiotics to prevent a serious course of the disease. Development of a subunit vaccine is of great importance as the only commercial vaccine available against heartwater is a live blood vaccine. This vaccine has various disadvantages including high cost of production,

extreme liability of the organism necessitating storage and transport at -76°C, intravenous route of administration, losses of vaccinated animals due to the virulence of the vaccine, the unwanted transmission of other diseases and the unsuitability of its use in countries where potential vectors are present but where the disease organism is absent (Oberem & Bezuidenhout, 1987). Heartwater will remain a disease of major importance until such time as an effective and safe method of vaccination becomes available (Van de Pypekamp & Prozesky, 1987).

The aim of this project therefore was to identify, purify and characterize antigenic proteins of *Cowdria* that may be suitable for the development of a specific diagnostic assay and which may be used as protective immunogens for the purpose of vaccination.

Chapter 2

PARTIAL PURIFICATION OF *COWDRIA* ORGANISMS

2.1 INTRODUCTION

Purification of *Cowdria* is important for several practical and analytical reasons (Viljoen *et al.*, 1985). It is essential for identification and characterization of antigenic and immunogenic determinants. This may lead to elucidation of the nature of immunity to heartwater, the development of a sensitive, specific diagnostic assay and production of a specific vaccine through recombinant DNA technology. Furthermore, availability of purified organisms may facilitate accurate taxonomic classification, morphological and genetic studies and investigations of the presumed toxin produced by these pathogens. Additionally the developmental sequence in the vector and vertebrate host including the relationship between developmental stages and pathogenicity could also be determined.

The purification of sufficient amounts of viable *Cowdria* organisms has been hampered for many years by their labile nature and initial difficulties experienced in the cultivation of the organism *in vitro* (Uilenberg, 1983). Success has since then been achieved in cultivation of *Cowdria* in cell cultures (Bezuidenhout, *et al.*, 1985) and neutrophils (Logan, *et al.*, 1987). Infected endothelial cell cultures are more suitable for large scale production of *Cowdria* organisms than neutrophil cultures as the latter do not divide in circulation and cannot be maintained in culture for more than a few days (Logan, *et al.*, 1987). Purification of organisms from a cell culture source is advantageous since the infectious cycle may be monitored by light microscopy. This enables harvesting of the cultures at a stage when they are maximally

infected with organisms. Conventional sources of *Cowdria* such as infected tick tissue (Bezuidenhout, 1981; Neitz *et al.*, 1986a & b), brain (Viljoen *et al.*, 1985; Neitz *et al.*, 1986a), blood fractions (Neitz *et al.*, 1986c) and mouse tissue (Du Plessis & Kümm, 1971) do not have this advantage.

Various methods of purifying pathogenic rickettsiae have been reported. These include ether extraction (Craigie, 1945), celite treatment (Shepard & Topping, 1947), differential centrifugation (Bovarnick & Snyder, 1949), anion (Hoyer *et al.*, 1958) or cation chromatography (Hara, 1958) and sucrose (Ribi & Hoyer, 1960), Percoll (Neitz *et al.*, 1987) or renografin density gradient centrifugation (Weiss *et al.*, 1975).

Two methods have been reported to purify *Cowdria* organisms but both methods have disadvantages:

1. Percoll density gradient certrifugation was performed on crude sheep brain, mouse liver and spleen, and nymph extracts, which resulted in successful recovery of partially purified, viable organisms possessing different densities ranging from 1,134 - 1,034 g/ml. This exemplifies the pleomorphism and different stages of development of the organism. The method does not result in pure organisms and therefore necessitates combination with an alternative purification method (Neitz *et al.*, 1987).

2. Wheat germ lectin chromatography was performed on crude brain, nymph and endothelial culture extracts and resulted in viable, partially purified organisms. This method was limited by low binding capacity as only with a suspension of ≤ 42 mg protein could all the *Cowdria* organisms (ie. 12 mg) be retained on the column. Additionally lectins are not as specific as antibodies in recognizing different cell populations (Vermeulen *et al.*, 1987).

Cowdria is reported to sediment at centrifugal forces of 30 000 xg when obtained from mammalian host or tick tissue (Neitz *et al.*, 1987). This enabled researchers to employ differential centrifugation as an initial step in partially purifying and concentrating the organism before Percoll density gradient centrifugation and wheat germ lectin chromatography (Neitz *et al.*, 1987; Vermeulen *et al.*, 1987). However preliminary experiments have indicated that *Cowdria* may sediment at lower centrifugal

forces when obtained from cell cultures. The behaviour of *Cowdria* during differential centrifugation, when derived from cell culture, should be determined. Additionally, intracellular organisms should be released from within the cell by mild sonication conditions and both released intracellular and extracellular organisms concentrated by centrifugation before further purification.

Immunoadsorbent purification techniques are becoming increasingly popular as a result of simplicity, high degree of specificity, selective affinities, rapid, single-step isolation and minimal contamination by non-specific proteins. Immunosorbent separation is based on the principle of molecular recognition, defined as the formation of a complex between 2 specific molecules and exemplified by the immunological complex between antigen and antibody (Calton, 1984). Antibodies are used either to specifically select and purify a particular component from a heterogeneous mixture (positive selection) or to deplete a heterogeneous mixture of contaminating components (negative selection).

Various methods have been investigated to desorb bound components in the case of positive selection. These methods either involve enzymatic digestion of the matrix (Scholossman & Hudson, 1973; Webb *et al.*, 1975) or reduction of disulfide bonds which may be used to couple antibodies to matrixes (Kiefer, 1975; Stephan *et al.*, 1966). This results in liberation of the whole antigen-antibody complex which then needs to be dissociated to release the antigen from the complex. These methods do not permit regeneration of the immunoadsorbent. Direct dissociation of the antigen from the immunoadsorbent would be advantageous in this respect. However mild dissociation of the antigen from the immunoadsorbent is difficult as strong (10^{10} M^{-1}) association constants may exist requiring harsh desorption conditions (Stewart, 1977). Furthermore, the degree of association is dependent on the number of bonds and proportion of the hydrophilic and hydrophobic groups involved (Cohn & Ferry, 1943). The stability of these bonds are affected in opposite ways for eg. by increasing the ionic strength the electrostatic interactions are decreased but the hydrophobic interactions are increased (Hofstee, 1976). Therefore desorption conditions must first be optimized. Using the

positive selection method to recover bound *Cowdria* organisms in a viable form would be detrimental considering the harsh desorption conditions that may have to be employed. Such conditions are eliminated when negative selection is employed as the required antigen may be eluted under mild conditions as no dissociation of antigen-antibody complexes is required (Neitz & Vermeulen, 1987).

Negative selection immunoadsorbent chromatography (NSIAC) requires anti-serum with high affinity and broad specificity directed against the contaminating material. Should an anti-serum be required to recognise the major and minor contaminating components, then repeated immunization with large doses of antigen mixture will be called for. Repeated injections of antigen can increase antibody levels in serum but a point is reached when no further increase is observed (Eisen & Siskind, 1964). Boosting 3-4 times at 4 weekly intervals will lead to maturation of the immune response and yield high-affinity preparations which will be advantageous (Harlow & Lane, 1988). Increasing the affinity with time could also lead to the frequent observation that specificity of antisera decreases with progressive immunization. This occurs as antibodies of high affinity should be capable of displaying a wider range of cross-reactions than antibodies with low affinity (Eisen & Siskind, 1964). For rare antigens, 1 or 2 injections with a low dose to prime the animals followed by a larger secondary boost have often shown to be effective for antibody production. The use of Freunds adjuvant will be advantageous when small amounts of the antigen are available. The depot action of the adjuvant requires smaller doses of antigen as it protects the antigen and allows slow release ensuring a persistent antibody response (Harlow & Lane, 1988).

NSIAC therefore offers ideal conditions for the purification of viable *Cowdria* organisms from cell cultures, and was therefore investigated.

2.2 MATERIALS AND METHODS

2.2.1 In vitro cultivation of *Cowdria*

In vitro cultures of *Cowdria* served as a source of *Cowdria* organisms for further investigations. The Welgevonden stock was cultivated in an E5 cell line as described by Bezuidenhout *et al.*, 1985. This stock was used in all our investigations unless otherwise stated. It was chosen because the pathological patterns caused by this stock resembles those caused by the Ball3 stock presently used in the live vaccine (Prozesky & Du Plessis, 1985). Additionally, unlike the Ball3 stock, the Welgevonden stock is pathogenic to mice, permitting viability tests to be determined in these animals.

Passaging of cell cultures: Cell cultures were propagated in modified Eagles medium (prepared and supplied by the Medium Preparation Section, OVI, Onderstepoort), containing 10 % bovine serum, sodium benzylpenicillin (0,12 mg/ml), streptomycinsulphate (0,198 mg/ml) and Amphotericin B (0,004 mg/ml), at 37°C in plastic culture flasks and will be referred to as Complete Medium. The cell cultures were passaged 1:2 every 7 days using standard techniques. The culture medium was poured off the cells and the flask rinsed with 20% ATV (ATV = 16 g KCl, 320 g NaCl, 44 g glucose.$1H_2O$, 22,2 g $NaHCO_3$, 20 g trypsin, 8 ml phenol red and 4 l water). Fresh ATV was added at 3 ml/75 cm^2 flask and incubated at 37°C for 5 min. When the cells were dislodged the ATV was inactivated with 5 ml Complete Medium and the mixture drawn up 4 times through a 19G needle and dispensed 1:2. Fresh medium was then added to each flask to a total volume of 50 ml/75 cm^2 flask and incubated at 37°C.

***Primary inoculation of cell cultures with* Cowdria:** Heartwater susceptible sheep were inoculated with 5 ml tested heartwater inoculum of specified *Cowdria* stock (Welgevonden). The inoculum was prepared as described by Oberem and Bezuidenhout (1987). Daily rectal temperatures were taken and blood was collected, in heparin, on the 3rd day of a rising febrile reaction. The infected blood was thoroughly mixed by rapidly drawing the blood up through a 21G needle. The mixed blood was then added to a

monolayer of irradiated (9Kr) E5 cells at 2 ml blood/75 cm^2 flask, and the flasks placed on a horizontal rocker and incubated at 37°C for 2 h. The blood was then thoroughly rinsed off from the cells with Complete Medium. Fresh Complete Medium was added to each flask to a total volume of 20 ml/75 cm^2 flask and returned to the rocker. Cultures infected with fresh blood took 2-4 cycles before the cultures were highly infected. If the cultures took longer than 7 days to become highly infected half of the medium was replaced at weekly intervals with fresh Complete Medium. This was done at a developmental stage when no or a minimum extracellular organisms were present. The infected cultures were monitored on a regular basis by light microscopic examinations of Rappi-Diff (Clinical Science Laboratories) stained culture smears.

Passaging of infected cultures (secondary infected cell culture inoculum): The infected cell cultures were ready for passage when at least 80% of the cells were infected with *Cowdria* colonies and maximum extracellular organisms were released into the culture media. Cells were scraped off culture flasks with a cell scraper into the culture medium and dispersed by drawing the suspension up 3 x through a 19G needle. The suspension was then equally dispensed 1:2 into flasks containing a new monolayer of cells. Fresh Complete Medium was added to each flask equalling the volume of infected material added and the flasks were then placed onto the rocker at 37°C for 2-16 h. Hereafter the culture medium was replaced by fresh Complete Medium.

Storing of infected cultures: On day 2 post infection, when the cells contained *Cowdria* colonies, the cells were trypsonized with 20 % ATV and centrifuged at 200 xg for 5 min. The supernatant was discarded and the pellet resuspended in cold (4°C) Eagles medium containing 20 % serum, thereafter an equal volume of cold (4°C) Eagles medium containing 10 % DMSO was added slowly while mixing. A total volume of 1 ml/75 cm^2 flask is recommended. This suspension was put into cryotubes and wrapped in 3 layers of heavy duty tin foil and stored at -70°C. Uninfected cell cultures were stored in the same manner as infected cells at a stage when the cells had formed a monolayer.

Secondary inoculation of cells with frozen cell culture inoculum: The frozen cell cultures were fast thawed in a 37°C water-bath and centrifuged at 200 xg for 5 min to rid the cells of the DMSO. The pellet was resuspended in Complete Medium and dispensed onto the same surface area from which the cells were removed and Complete Medium was added to a final volume of 10 ml/75 cm^2 flask.

2.2.2 Production of polyclonal anti-*Cowdria* sera in bovine, sheep, mice and rabbits

Preparation of polyclonal anti-*Cowdria* sera in bovine, sheep, mice and rabbits were investigated for use in a serological assay to screen samples during the process of purification and for use in the immunoadsorbent chromatography.

Preparation of polyclonal sera in bovine and sheep: Adult sheep and cattle were infected with 5-10 ml Welgevonden stock infective blood (Oberem & Bezuidenhout, 1987) by intravenous (i.v.) injection. Animals showing rise in rectal temperature during the 24-day observation period were treated, on the third day of a rising febrile reaction, by intramuscular (i.m.) injection with long acting oxytetracycline (liquamycine LA from Pfizer), at a dosage rate of 20 mg/kg. All the animals were boosted 4 weeks after the initial inoculation. Blood was collected 4 weeks after the final challenge and sera prepared as described below (Neitz *et al.*, 1986b).

Preparation of polyclonal sera in mice: White mice, six weeks of age, were infected each with 0,2 ml Welgevonden stock infected mice organ suspensions (heart, spleen and liver). The organs were prepared as described by Du Plessis (1985a). The heartwater reaction was blocked with 40 µg tetracycline/mouse on the eighth day post infection. The mice were boosted 4 weeks after the initial inoculation. Blood was collected 4 weeks after the final challenge and sera prepared as described below.

Preparation of polyclonal sera in rabbits: Welgevonden stock infected and uninfected E5 cell cultures were harvested with a cell scraper into their respective culture media. The clumps of cells were dispersed by drawing the mixture up 3 times through a 18G needle. DMSO was added to a final concentration of 7% as a cryopreservant and stored at -70°C. The material was thawed and sonicated with a Branson cell disruptor B-30 and microtip at 40% duty cycles using 5 on the output control for 5 seconds. The sonicated material was centrifuged at 200 xg for 10 min at 4°C. The resultant supernatant was centrifuged at 10 000 xg for 30 min at 4°C. The 10 000 xg pellet was resuspended in 10 ml PBS, pH 7,4 (PBS: 0,14 M NaCl, 1 mM KH_2PO_4, 8 mM $Na_2HPO_4.12\ H_2O$ and 3 mM KCl, pH 7,4). The protein content was determined by a Bio-Rad protein assay (subsection 2.2.6.). The immunization protocol as set out in Table 2.1. was followed using 2 rabbits for each immunization protocol. Both infected and uninfected material were used as antigen. One rabbit was not immunized and served as control. Blood was drawn, with a needle and syringe, from each rabbit's ear prior to each immunization and on day 56 the rabbits were bled from the ear under vacuum. Sera was prepared as indicated below.

Preparation of serum from blood: Blood collected from the immunized animals were left to clot for 2 h at room temperature and thereafter overnight at 4°C. The coagulated blood was then centrifuged at 300 xg in a Piccola bench top centrifuge for 10 min at room temperature. The sera was siphoned off and stored at -20°C (Neitz *et al.*, 1986b).

TABLE 2.1. Immunization protocols for production of rabbit polyclonal serum.

Protocol no.	day	Antigen	Route injected	ml injected per site	Total mg protein injected
1	0	0,5 mg prot/ml PBS mixed 1:1 with FCA	s.c. & i.m.	0,4	0,2
	14	0,5 mg prot/ml PBS mixed 1:1 with FIA	s.c. & i.m.	0,8	0,4
	28	0,5 mg prot/ml PBS	s.c. & i.m.	0,6	0,6
	42	1 mg prot/ml PBS	i.v.	1,0	1,0
	56	bled			
2	0	0,5 mg prot/ml PBS mixed 1:1 with FCA	s.c. & i.m.	1,0	0,5
	56	bled			
3	0	0,5 mg prot/ml PBS	i.v.	1,0	0,5
	56	bled			

2.2.3 Release of intracellular *Cowdria* organisms from E5 cells by sonication

The use of sonication as a method to release whole *Cowdria* organisms from E5 cells for further purification was investigated. Crude Welgevonden stock infected culture extracts were prepared. The Welgevonden stock infected cell cultures (80-100% infected with intra- and maximum extracellular organisms) were harvested with a cell scraper into their culture media. The clumps of cells were dispersed by drawing the mixture up 3 times through a 19G needle. Fresh preparations of crude extracts were divided into 5 equal volumes and sonicated on ice with a Branson

cell disruptor B-30, with a microtip, as indicated in Table 2.2. Directly after sonication the samples were centrifuged at 30 000 xg for 30 min at 4°C. The resultant pellets obtained were prepared for electron microscopic studies as described in subsection 2.2.9.

TABLE 2.2. Sonication conditions at 5 % Duty cycles.

Sample no.	Output control setting	Time sec.
1	3	5
2	3	15
3	6	5
4	6	15
5	0	0

2.2.4 Partial purification of *Cowdria* organisms from E5 cells by differential centrifugation

The following differential centrifugation procedure was investigated as an initial step in partial purification of *Cowdria* organisms. The Welgevonden stock infected cell cultures (80-100% infected with intra- and maximum extracellular organisms) or uninfected cell cultures as control were harvested with a cell scraper into their culture media. The mixture was diluted 1:1 with PBS, pH 7,4. The clumps of cells were dispersed by drawing the mixture up 3 times through a 19G needle. This material was either used immediately or stored frozen at -70°C. The infected or uninfected crude culture extracts were centrifuged at 200 xg for 30 min. The supernatants were then centrifuged at 1000 xg for 30 min. These supernatants were then further centrifuged at 10 000 xg for 30 min. and the supernatants centrifuged at 30 000 xg for 30 min. All centrifugation steps were performed at 4°C. The pellets that were obtained after each centrifugation step were prepared for electron microscopy studies as described in subsection 2.2.9.

2.2.5 Negative selection immunoadsorbent chromatography

NSIAC was investigated for purification of *Cowdria* organisms from cell cultures.

Purification of IgG by DEAE-Cibacron blue 3-G-Agarose: Purification of IgG by DEAE-Cibacron blue 3-G-Agarose was performed according to the Bio-Rad catalogue. The anti-E5 cell polyclonal rabbit serum sample (prepared as described in subsection 2.2.2.) was dialyzed against a buffer containing 0,02 M Tris-HCl, 0,028 M NaCl, 0,02% (w/v) NaN_3, pH 8,0 (starting buffer). A column (15 x 360 mm) of DEAE-Cibacron blue 3-G-Agarose was prepared using 4 ml gel/1 ml serum. The serum sample was applied to the column and eluted with 3 bed volumes of starting buffer. Fractions with approximately the same volume or half that of the sample applied to the column, were collected. The absorbance of the fractions were recorded at 280 nm. Fractions of the unbound peak were combined. The absolute amount of IgG obtained was determined by converting the absorption values obtained into concentration values according to literature, Absorbance at 280 nm = 1,0 (1 cm pathlength) = 0,75 mg IgG/ml (Robyt & White, 1987). Microzone electrophoresis was performed on selected fractions obtained from DEAE-Cibacron blue 3-G-Agarose chromatography purification of anti-E5 polyclonal rabbit serum and whole rabbit serum to test the purity of the IgG fraction. The procedure was performed according to the Beckman instruction manual.

Coupling of IgG to CNBr-activated Sepharose 6MB: Coupling of purified anti-E5 cell IgG to CNBr-activated Sepharose 6MB was performed according to the method described by Pharmacia (1983). The pooled fraction of purified anti-E5 cell IgG (protein sample) was dialyzed against three changes of 1l each of 0,2 M $NaHCO_3$, 0,5 M NaCl, pH 8,5 (coupling buffer). The protein content of the sample was determined at 280 nm. A concentration of 5-10 mg protein/ml gel is recommended and the volume of the protein sample should be that giving a gel:sample ratio of 1:2. If concentration of the protein sample was necessary it was done with an immersible CX-10 ultrafilter NMWL 10000 Daltons (Millipore). Two grams of freeze dried powder (CNBr-activated Sepharose 6MB, Sigma) was swollen for

15 min in 1 mM HCl at room temperature. The swollen gel was washed on a sintered glass filter, G3, with 400 ml of 1 mM HCl, added in several aliquots. Hereafter the gel was washed with 10 ml of coupling buffer. The washed gel was immediately transferred to a solution of the protein sample. The protein solution and gel suspension was mixed at room temperature for 2 h with an automatic back and forth shaker, and then transferred to a buffer containing 1 M ethanolamine in coupling buffer and left to stand for 2 h at room temperature. Absorbance of the supernatant was measured at 280 nm to determine the amount of IgG that coupled to the gel. The gel was washed 10 times with 50 ml aliquots of alternatively 0,1 M acetate buffer, 0,5 M NaCl, pH 4 and coupling buffer, and stored in the latter buffer containing 0,02% (v/v) NaN_3 at 4°C.

Negative selection immunoadsorbent chromatography (NSIAC): NSIAC was performed according to the method described by Pharmacia (1983). The anti-E5 cell IgG coupled gel was packed into a column (170 x 15 mm) and equilibrated with PBS buffer, pH 7,4 at 4°C. Fresh media from the Welgevonden isolate infected E5 cell cultures, containing maximum extracellular organisms were centrifuged at 10 000 xg for 30 min at 4°C. One pellet was prepared for electron microscopic examinations (subsection 2.2.9.) and the remaining pellets were resuspended in PBS, pH 7,4. Protein concentration (subsection 2.2.6.) and infectivity (subsection 2.2.10.) of the resuspended sediments were determined. A volume not exceeding 1 ml containing 4-5 mg protein, was applied onto the column and incubated for 30 min. The unbound material was eluted from the column with PBS, pH 7,4 at a flow rate of 11 ml/60 min and 1 ml fractions were collected. The material that bound to the column was eluted with 3 M KSCN, 50 mM Tris-HCl, pH 9,0. The absorbance of the fractions were recorded at 280 nm. The column was regenerated with 10 bed volumes of 0,1 M Tris-HCl, 0,5 M NaCl, pH 8,4 followed by 0,1 M sodium acetate, 0,5 M NaCl, pH 4,5 and equilibrated with 10 bed volumes of PBS, pH 7,4 containing 0,02% (w/v) NaN_3. The column was stored in the latter buffer. Before reuse the column was regenerated with 20 bed volumes of PBS, pH 7,4.

Fractions were pooled into 2 categories: unbound material (NSIAC peak 1) and bound material (NSIAC peak 2). The pooled material was centrifuged at 10 000 xg for 30 min. Samples of the pellets of NSIAC peak 1 and NSIAC peak 2 were prepared for electronmicroscopic studies (subsection 2.2.9.) The remaining sample pellets were resuspended in 1 ml PBS, pH 7,4 and, together with the crude extracts analyzed for protein content (subsection 2.2.6.), antigenicity (ELISA subsections 2.2.7. and immunoblots, subsections 2.2.12. and 2.2.13.) and infectivity tests in mice (subsection 2.2.10.).

2.2.6 Bio-Rad protein assay

The protein content of the various samples were determined by the Bio-Rad protein assay (Bio-Rad, 1984). Several dilutions of bovine serum albumin protein standard containing 1-25 µg/ml were prepared. Quantities of 0,8 ml of standards and appropriately diluted samples were placed in clean dry test tubes. Sample buffer was used as a blank. To this 0,2 ml Bio-Rad Dye Reagent Concentrate was added and mixed several times avoiding excess foaming. The absorbance at 595 nm versus reagent blank was measured.

2.2.7 Enzyme-linked immunosorbent assay

Polyclonal antisera were screened for antibody titers and various crude extracts including the fractions obtained from immunoadsorbent chromatography were screened for antigenic properties by an enzyme-linked immunosorbent assay. The ELISA I protocol was developed at Onderstepoort and although it still required optimization it was initially used for screening samples. In ELISA I the antigen was incubated overnight in the wells and thereafter fixed with glutaraldehyde. During the course of our investigations reaserchers at the University of Pretoria developed a different ELISA protocol (ELISA II; Verschoor *et al.*, 1989). In ELISA II the antigen is dried in the wells and then fixed with methanol. A Casein/PBS buffer is used throughout ELISA II instead of various different buffers as in ELISA I. The 2 protocols were tested and initially found to compare favourably. Since reagents for ELISA II were more readily available it was later used for screening the samples.

Welgevonden stock infected and uninfected cell culture extracts were used as antigen for antibody titer determinations of serum. Bovine anti-Welgevonden stock serum was used to screen samples for antigenic properties.

ELISA I: Linbro microtiter plates (Flow laboratories) were coated with 0,1 ml antigen at concentrations of 5; 0,5 and 0,05 µg protein/ml of a 0,05 M bicarbonate buffer, pH 9,6. The plates were incubated for 18 h at 4°C thereafter they were washed once with distilled water and once with PBS, pH 7,4. Hereafter the antigen was fixed by coating the plates with 0,1 ml 2,5% glutaraldehyde in 0,1 M Millonig's phosphate buffer, pH 7,4 (Millonig's phosphate buffer = 2% (w/v) $NaH_2PO_4.H_2O$, 0,4% (w/v) NaOH and 0,54% (w/v) glucose) and incubated for 3 min at room temperature. Hereafter the plates were washed 4 times with 100 mM glycine in PBS, pH 7,4. The blocking buffer, 1% Elite milk powder in PBS, pH 7,4 was applied in 0,3 ml quantities to block all the vacant sites. After an incubation period of 2 h at room temperature they were washed once with PBS, 0,05% Tween 20, pH 7,4 (washing solution). Serial dilutions of serum (diluted 1:20, 1:100, 1:500 and 1:2500 with PBS, 0,05% Tween 20, 5% Elite milk powder, pH 7,4) was added in 0,1 ml quantities and incubated at room temperature for 90 min. The plates were washed 4 times with the washing solution. The conjugate (peroxidase conjugated anti-sheep, bovine, mice or rabbit IgG immunoglobulins, Cappel), diluted 1:1000 with PBS, 0,05% Tween 20, 5% Elite milk powder, pH 7,4 was added in 0,1 ml quantities and incubated for 60 min at room temperature. The plates were then washed 4 times with washing buffer. A 0,08 mg quantity of H_2O_2 was added to 10 ml 0,1 M citrate buffer pH 4,5 containing 1 mg/ml OPD. This solution was applied in 0,1 ml quantities to the plates and the colour development monitored with a Titertek Multiscan MC at 690 nm and 405 nm. Signal to background ratio's were calculated from absorbances obtained for infected and corresponding non-infected fractions. The antibody titers were evaluated as the highest serum dilution still giving a positive signal (≥ twice background).

ELISA II: Sterlin microtitre plates (nunc) were coated with 0,1 ml antigen at a concentration of 5, 0,5 and 0,05 µg/ml of a PBS buffer pH

7,4. The plates were placed under a lamp and fan to dry for 2 h thereafter the antigen was fixed with 0,2 ml 70% (v/v) absolute methanol for 10 min. A 0,5% (w/v) casein in PBS buffer, pH 7,4 (buffer A) was applied to the plates in 0,2 ml quantities to block all the vacant sites. After an incubation period of 60 min at room temperature plates were washed once with buffer A. Serial dilutions of serum, 1:20, 1:100, 1:500 and 1:2500 with buffer A, were added in 0,05 ml quantities and incubated for 45 min. Hereafter the plates were washed 3 times with buffer A. Peroxidase conjugated immunoglobulins (Cappel), diluted 1:1000 with buffer A was added in 0,05 ml quantities. After an incubation period of 30 min, the plates were washed 3 times with buffer A. An OPD substrate prepared as described above was added in 0,05 ml quantities. The colour reaction was monitored and interpreted as described above.

2.2.8 Indirect fluorescent antibody assay

The anti-*Cowdria* sera of mice, sheep, cattle and rabbits were subjected to the indirect fluorescent antibody (IFA) test as described by Du Plessis & Malan, 1987. The IFA assay was performed in addition to the ELISA as sheep serum resulted in high background when used in the ELISA. The IFA assay therefore allowed serum titers from mice, cattle, rabbits and sheep to be compared. The test was kindly carried out at the OVI, Onderstepoort by Mrs van Gas and Dr du Plessis. The test procedure will briefly be described here.

The peritoneal macrophages of mice infected with the Kümm stock of *Cowdria* were used as antigen. The Kümm stock, used as antigen in the IFA assay, can detect antibodies to different stocks of *Cowdria* (Du Plessis *et al.*, 1989). A droplet of peritoneal macrophage cell suspension was placed in wells of slides with a platinum wire loop. The slides were stored in liquid nitrogen. Immediately before use, they were exposed to air for a few seconds and plunged into cold methanol for 1-3 sec. A drop of serum at the appropriate serial dilution was placed in each well and incubated for 30 min in a moist chamber at 37°C. The slides were washed in a dish of 10 fold diluted buffer (buffer: 80 g NaCl; 27 g $HNa_2PO_4.12H_2O$; 4 g $H_2NaPO_4.2H_2O$; in 1 l distilled water, pH 7,2), whilst

stirring, for 15 min. the slides were then flooded with the appropriate antispecies conjugate and again incubated for 30 min at 37°C. After a second washing for 15 min, the slides were mounted in 50% glycerine and examined under a binocular microscope equipped with HBO50 mercury burner and an BP 390-490 exciter filter.

2.2.9 Electron microscopy

Cowdria infected E5 cell culture crude extracts representing controls and extracts subjected to sonication, differential centrifugation, freeze/thawing and fractions obtained from NSIAC were prepared for electron microscopic (EM) studies. Preparation of these samples was kindly performed by the staff of the EM section at the OVI, Onderstepoort.

Aliquots of the samples to be examined were centrifuged at 10 000 xg for 30 min at 4°C. The sediment of each fraction was fixed with 2,5% glutaraldehyde in 0,1 M Millonig's phosphate buffer pH 7,4 for 24 h at 4°C. All subsequent procedures were performed at room temperature unless otherwise stated. Two rinses of 10 min each were performed with Millonig's phosphate buffer. Post fixing was carried out in 1% osmium tetroxide in Millonig's phosphate buffer for 45 min. Two rinses of 10 min each were performed with 0,1 M Millonig's phosphate buffer pH 7,4. Dehydration was achieved with stepwise addition of 50%, 70%, 90%, 95% and 3 x 100% ethanol for 15 min each. A solution of 50:50 (propylene oxide:ethanol) was then added and incubated for 10 min followed by 2 x incubation in 100% propylene oxide for a further 10 min each. The fractions were then incubated in stepwise addition of resin:propylene oxide (30:70, 50:50, 70:30 and 2 x 100% resin) for 15 min each. The fractions were left in capsules to polymerise for 24 h at 65°C. Ultra-thin sections of 0,05 µm were cut with a Reichert-Jung Ultracut microtome. These sections were stained for 30 min in aqueous solutions of 5% uranyl acetate and for 3 min in lead citrate. Hereafter the sections were rinsed with 0,02% NaOH followed by double distilled water and left to dry. The grids were examined with a Jeol JEM 1200 EX electron microscope.

2.2.10 Determination of infectivity

The viability of *Cowdria* organism before and after purification by NSIAC was determined by infectivity tests in mice. Mice were injected with tenfold serial dilutions in PBS, pH 7,4 of crude extracts and the unbound fractions obtained from NSIAC at 0,2 ml/mice, i.v. using 5 mice for each dilution. The control mice were injected with PBS, pH 7,4. The fifty percent mortality endpoints were calculated as described by Reed & Muench, 1938.

2.2.11 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-PAGE was performed on crude infected and uninfected cell culture extracts before and after NSIAC to compare Coomassie stained protein patterns. SDS-PAGE was performed as described by Laemmli (1970). The stacking gel contained 4% acrylamide, 0,1% bisacrylamide, 0,1% SDS in 0,1 M Tris-HCl buffer (pH 6,8). The separating gel contained 12% acrylamide, 0,3% bisacrylamide, 0,1% SDS in 0,4 M Tris-HCl buffer (pH 8,8). Each gel was polymerized by the addition of ammonium persulphate and TEMED 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 x 120 x 160 mm were prepared. Samples for electrophoresis, at concentrations of 40 µg/well unless otherwise stated and LMW standards (Pharmacia), were dissolved in 0,06 M Tris-HCl buffer (pH 6,8), 16% glycerol, 2% SDS, 2,5% DTT and 0,001% bromophenol blue by heating at 100°C for 10 min. Electrophoresis was performed in an electrode buffer containing 0,02 M Tris-HCl, 0,1 M glycine and 0,06% SDS, pH 8,3 for about 2 h at a constant current of 45 mA whilst the mobile front was in the stacking gel. Once the mobile front entered into the separating gel the electrophoresis was performed at a constant current of 60 mA for about 3 h.

Coomassie stain: 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.

2.2.12 Western blotting

Electrophoretic transfer of SDS-PAGE separated polypeptides was achieved as described by Moos *et al.*, 1988. Proteins were transferred to a PVDF membrane (Millipore) by electrophoresis in 10 mM CAPS buffer, pH 9,0 at 0,12 A to 0,25 A for 1 h 45 min. The blotted sheet was air-dried and stored at -20°C until further use.

2.2.13 Protein- and immuno-staining of Polyvinylidine difluoride membranes

Protein staining of PVDF membranes: The LMW standards and/or separated samples 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 and stored at -20°C.

Immunostaining of PVDF membranes: *Cowdria* antigens, in infected crude cell cultures before and after NSIAC, were identified by immunoblotting techniques (Millipore) with polyclonal sera against *Cowdria*. Dried, western blotted, PVDF membranes were rewetted in methanol and rinsed briefly in distilled water. The membranes were incubated in sera diluted 1:200 (unless otherwise stated), with a solution containing 1 % Elite milk powder, 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 % Elite 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 rabbit anti-bovine, goat or sheep IgG (Cappel), 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 of 30% H_2O_2/100 ml 20 mM Tris-HCl, pH 7,4 for 20 min. After termination of substrate development by rinsing the membrane in distilled water, the membrane was air-dried and stored at -20 °C.

2.3 RESULTS

2.3.1 *In vitro* cultivation of *Cowdria*

During *in vitro* cultivation of *Cowdria* the following general observations were made: After primary infection of the cell cultures with infected blood it took between 14-21 days for the cells to become highly infected and ready for passaging. However, after secondary infection of cell cultures, with infected cell culture inoculum, it took only 7 days for the cultures to become highly infected and ready for passaging. The presence of extracellular organisms coincided with E5 cell rupture and eventual release of the cell debri into the culture medium.

With Rappi-Diff staining of cell culture smears the cytoplasm of the cells stained light mauve and the nucleus stained pink. The smaller colonies of *Cowdria* stained dark blue and as they grew larger they stained a lighter blue. The elementary bodies that broke free from these colonies stained a light to pinkish blue (Fig. 2.1.).

The infectious cycle of *Cowdria*, as observed with light microscopy, in cell cultures after secondary infection was as follows:

Day 1 post infection: elementary bodies were taken up by the cell and it was difficult to distinguish between cell granules and organisms.

Day 2 post infection: small colonies staining dark blue were found in the cytoplasm of the cell.

Day 3-5 post infection: colonies were increasing in size.

Day 6-7 post infection: colonies were still increasing in size. The colonies began to rupture releasing elementary bodies into the cell culture medium, and subsequently new cells became infected and the cycle repeated.

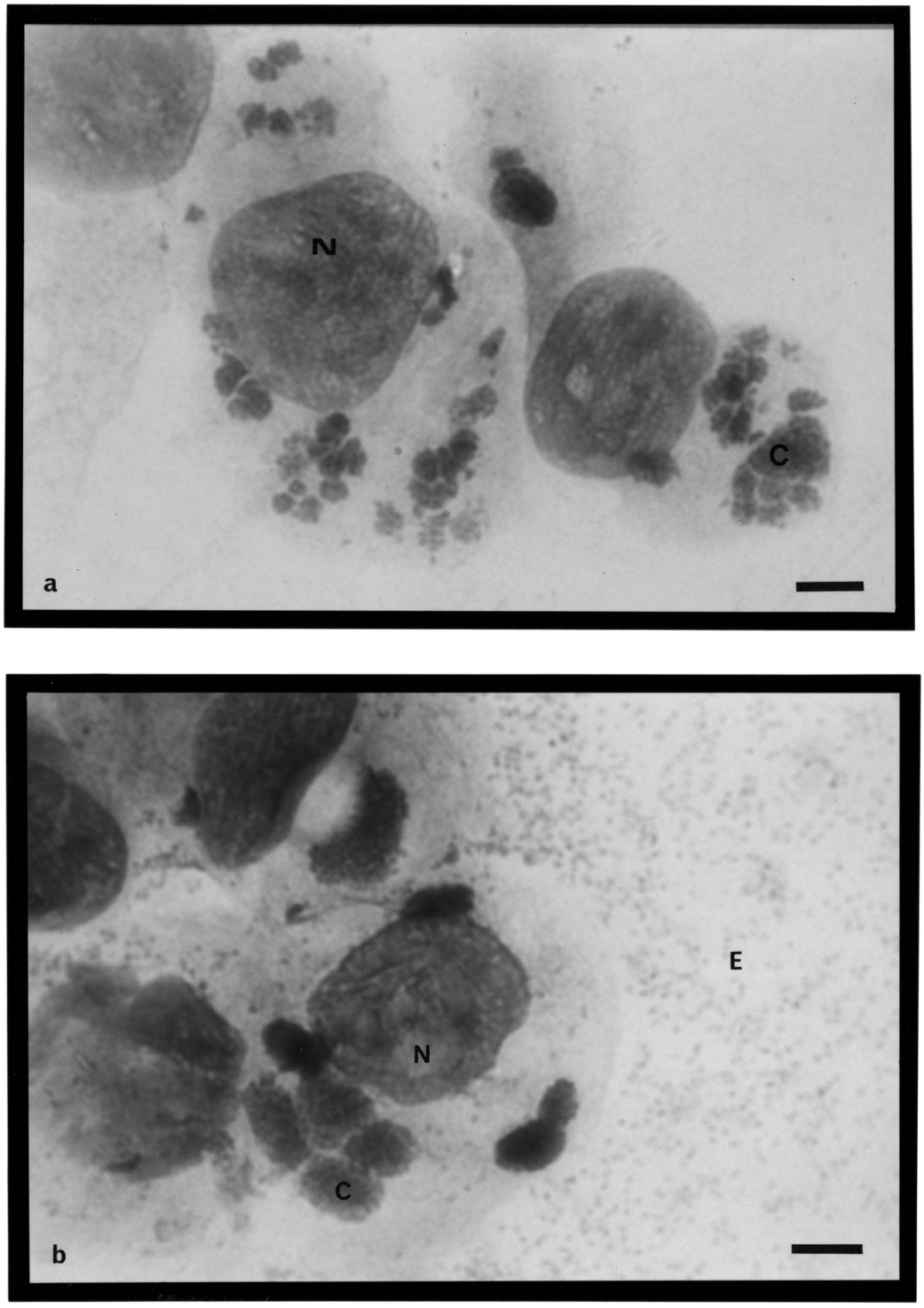


FIGURE 2.1. Welgevonden stock infected E5 cell culture smears stained with Rappi-Diff. a) day 3 post secondary infection: colonies of organisms in the cytoplasm of the cells. b) day 7 post secondary infection: colonies in the cytoplasm of the cells and extracellular individual organisms. N = nucleus. C = *Cowdria* colony. E = extracellular *Cowdria* organism. Bar = 5 µm.

2.3.2 Production of polyclonal anti-Cowdria sera in bovine, sheep, mice and rabbits

Polyclonal bovine, sheep and mice anti-Welgevonden serum were prepared and these various serum sources were tested in ELISA I. Antibody titers of 1:2500 were obtained for mouse and bovine serum (Table 2.3.). No positive results could be obtained when sheep serum was tested by ELISA I as a result of high unspecific background. Sheep serum gave satisfactory results when tested in the IFA assay (subsection 2.2.8.) resulting in antibody titers of 1:320 (Table 2.3).

TABLE 2.3. ELISA I and IFA assay reciprocal titers for various serum sources. (* - protocol no see Table 2.1.)

	ELISA I			IFA
Serum source	µg protein/ml	Conjugate dilution	Titer	Titer
mouse	0,5	1:5000	2500	320
bovine	5,0	1:1000	2500	320
sheep	0,05-5,0	1:1000-1:5000	neg.	320
rabbit 1*	0,5	1:5000	2500	100
rabbit 2*	0,5	1:5000	500	10
rabbit 3*	0,5	1:5000	neg.	10

An alternative serum source and preparation was examined viz. polyclonal rabbit antisera. Three immunization protocols were used to prepare polyclonal serum against Welgevonden stock infected and non-infected E5 cell cultures (Table 2.1.). Evaluation of polyclonal rabbit serum by ELISA I and IFA assay, indicated that immunization protocol 1 resulted in sera with the highest antibody titer of 1:2500 and 1:100 respectively (Table 2.3). The ELISA I titers obtained for immunization protocol 1 are similar to those obtained for mice and cattle anti-serum.

In the early stages of the immunization program of the rabbits no antibody titers were obtained (Table 2.4). Immunization protocol 1 showed an antibody titer of 1:500 2 weeks after the second inoculation with adjuvant. After the third inoculation with antigen the antibody titer increased to 1:2500 and remained constant even after a fourth

inoculation. Immunization protocol 2 only showed an antibody response (1:500) 42 days after the initial single inoculation of antigen with FCA. Immunization protocol 3 consisting of a single inoculation without adjuvant failed to produce an antibody response.

TABLE 2.4. ELISA I antibody titers obtained for rabbit sera during an immunization program. Titers were determined by ELISA I using Welgevonden infected E5 cell culture extracts as antigen at 0,5 µg protein/ml buffer. * - titer determined by ELISA II using uninfected E5 cell culture extracts as antigen at 0,5 µg protein/ml buffer. # - Control = unimmunized, naive rabbit serum; nd. - not done because of lack of serum.

Rabbit serum samples compared	Reciprocal ELISA I titers				
	Day				
	0	14	28	42	56
Protocol 1: anti-Wlg/E5 : anti-E5	neg	neg	500	2500	2500
anti-Wlg/E5:control#	nd	nd	nd	nd	2500
anti-E5:control#	nd	nd	nd	nd	2500
	nd	nd	nd	nd	*2500
Protocol 2: anti-Wlg/E5 : anti-E5	neg	neg	neg	2500	500
Protocol 3: anti-Wlg/E5 : anti-E5	neg	neg	neg	neg	neg

At this stage of our investigations we became aware of an alternative ELISA protocol that compared favourably with ELISA I and whose reagents were easily available. ELISA II was therefore used in further investigations. Polyclonal rabbit antisera to uninfected E5 cell cultures (immunization protocol 1) showed antibody titers of 1:2500 when tested in the ELISA II using uninfected E5 cell cultures as antigen. These results suggest possible use of this polyclonal E5 cell rabbit antisera in NSIAC for purification of whole *Cowdria* organisms from E5 cell cultures.

As the polyclonal bovine anti-Welgevonden serum gave satisfactory titers and was available in larger quantities than either the mice or rabbit

serum it was subsequently used in ELISA and immunoblot examinations of purification samples.

2.3.3 Release of intracellular *Cowdria* organisms from E5 cells by sonication

Sonication of Welgevonden stock infected E5 cell cultures was examined as a means whereby whole *Cowdria* organisms could be released from within the cell for further purification. Electronmicroscopic examinations of material subjected to various sonication conditions (Table 2.2.) revealed damaged and poorly defined organisms after even the mildest conditions of sonication (Fig 2.2.). Few clearly defined undamaged membranes could be identified and mostly clumps of what appears to be *Cowdria* matrix were present. Thus *Cowdria* organism were disrupted by mild sonication conditions using an output control of 3 and 5% duty cycle for 5 sec.

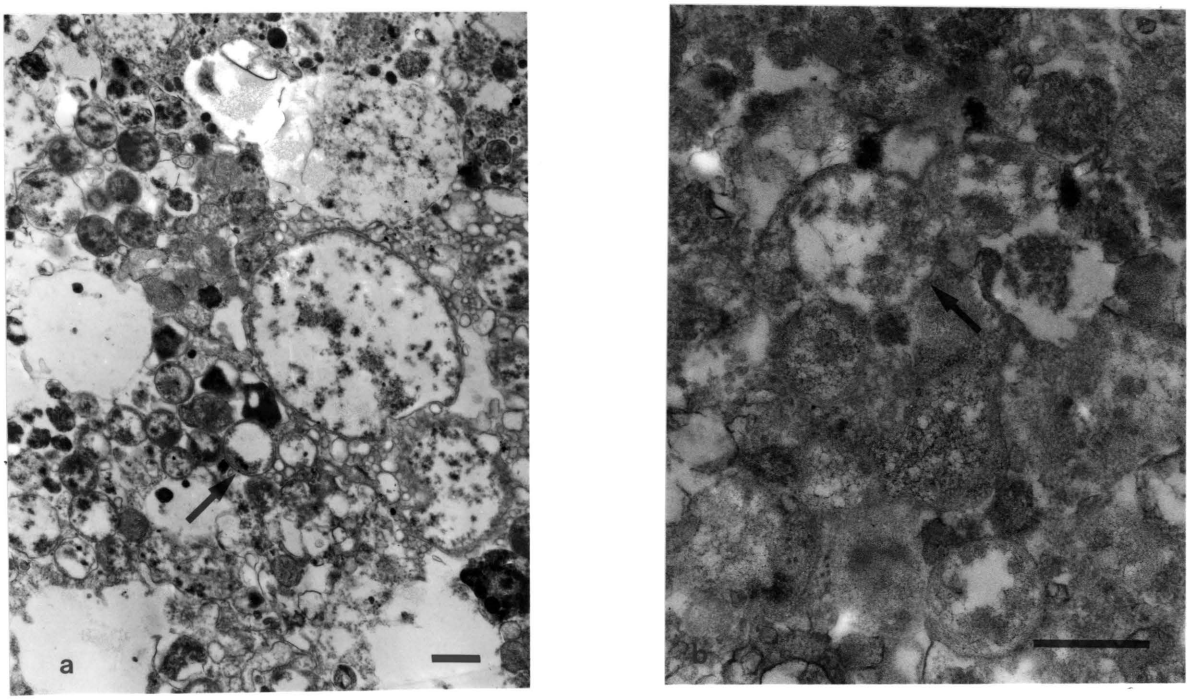


FIGURE 2.2. Electron micrographs of *Cowdria* infected crude E5 cell culture extract subjected to sonication. a) Control. not sonicated. b) Sonicated for 5 sec at output control setting 3 and 5% duty cycle. ----› *Cowdria* organism. Bar = 0,5 µm.

2.3.4 Partial purification of *Cowdria* from E5 cells by differential centrifugation

Differential centrifugation was examined as an initial step in purification of *Cowdria* from E5 cell cultures for NSIAC. Electron microscopic examination of the pellets obtained as described in subsection 2.2.4, revealed heartwater organisms predominantly present in the pellets obtained at 200, 1000 and 10 000 xg and to a lesser extent at 30 000 xg (Fig 2.3.). Electron dense and electron pale heartwater organisms, varying in size, and considerable E5 cell debri appeared throughout the differential centrifugation range. The average size of the organisms in the 4 pellets varied between 0,1 - 2,0 µm in diameter and was independent of the centrifugal force. Freeze-thawing did not result in loss of integrity of the *Cowdria* membrane. The only difference observed between fresh and freeze/thawed material was the presence of whole endothelial cells and whole colonies of organisms at 200 xg when fresh material was used (Fig 2.3.).

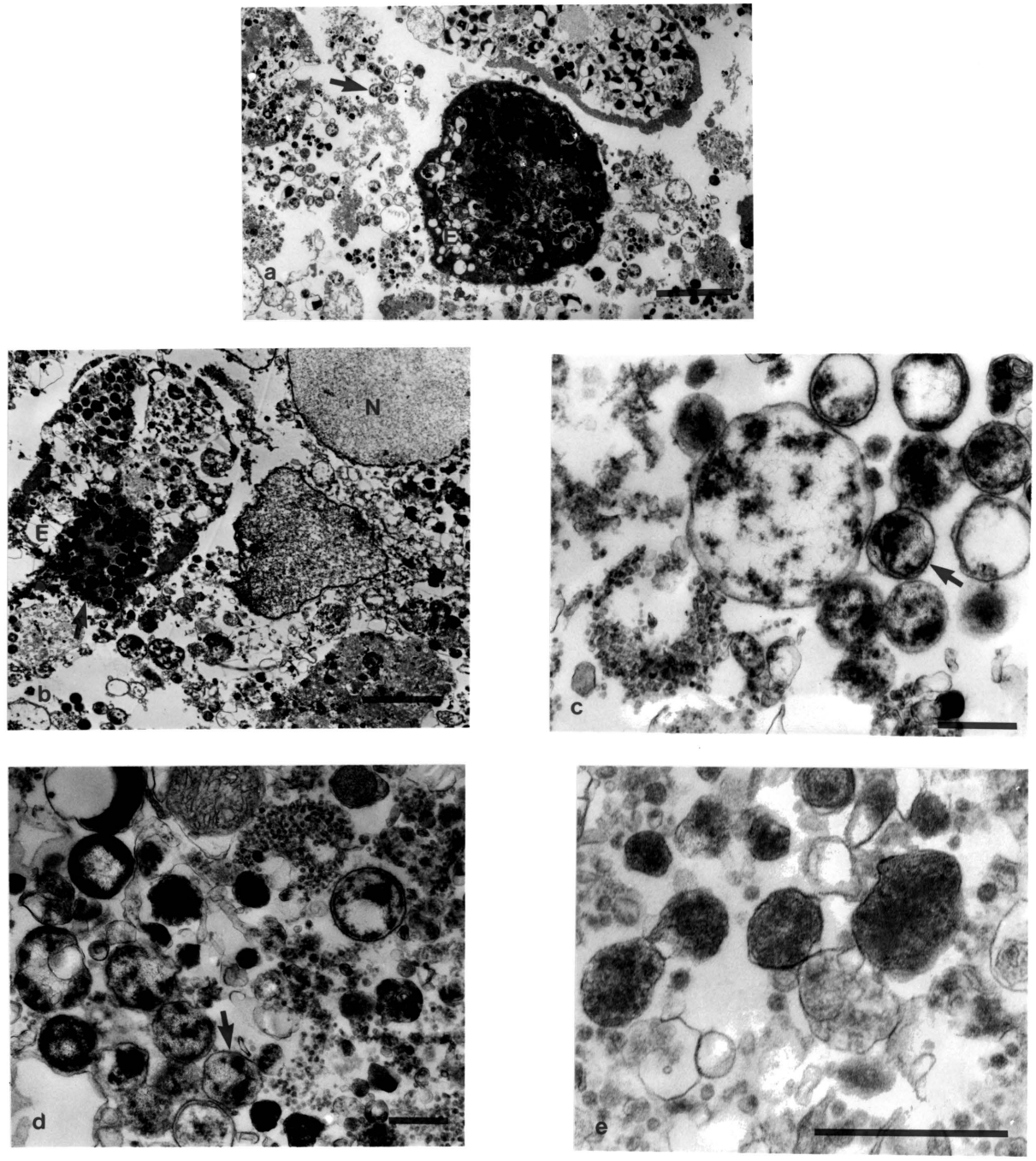


FIGURE 2.3. Electron micrographs of *Cowdria* infected E5 cell culture crude extracts subjected to differential centrifugation. a) 200 xg centrifugation pellet of fresh material. b) 200 xg centrifugation pellet of freeze/thawed material. c) 1000 xg centrifugation pellet of the 200 xg supernatant. d) 10 000 xg centrifugation pellet of the 1000 xg supernatant. e) 30 000 xg centrifugation pellet of the 10 000 xg supernatant. ----> = *Cowdria* organism; E = whole E5 cell; N = nucleus. a) & b) Bar = 3 µm. c), d) & e) Bar = 0,5 µm.

2.3.5 Negative selection immunoadsorbent chromatography

The anti-E5 polyclonal sera produced in rabbits according to immunization protocol I was used for the preparation of an immunoadsorbent for use in NSIAC. It was determined that a total of 23 mg anti-E5 IgG was purified from 12 ml polyclonal sera by DEAE Cibacron blue 3-G Agarose chromatography (Fig 2.4.). The purified immunoglobulins were obtained by combining fractions no. 10 - 34. Microzone electrophoresis was performed on samples of fractions no. 12, 15, 18, 21, 27 and 33. All the samples excepting no. 12 contained gamma globulins while samples no. 18 and 21 also contained beta globulins.

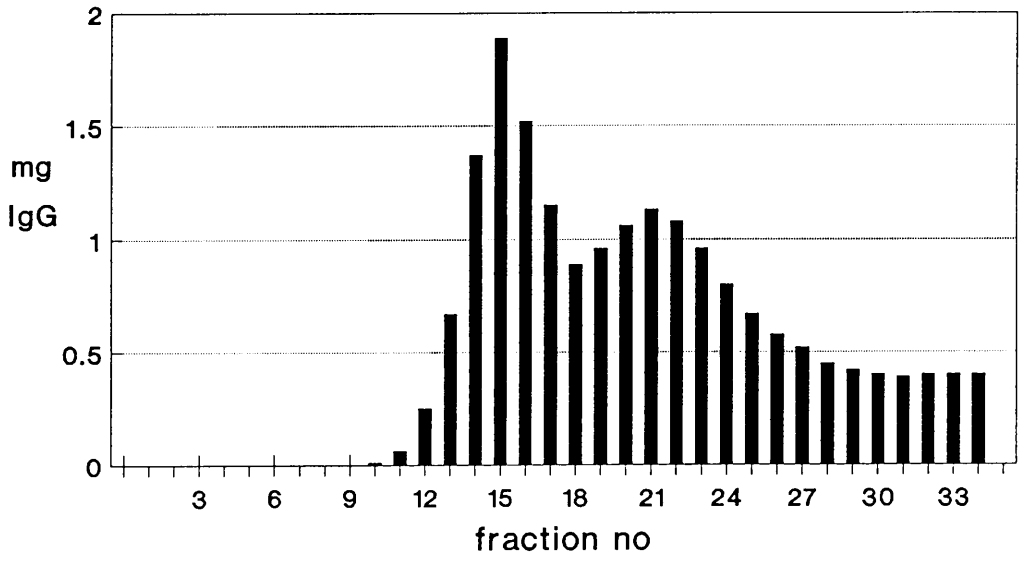


FIGURE 2.4. Representation of the elution pattern obtained after DEAE Cibacron blue 3-G-Agarose chromatography of anti-E5 cell rabbit serum. Two ml fractions were collected. Fractions no. 12, 15, 18, 21, 27 and 33 were investigated by microzone electrophoresis. The IgG concentration was determined from the Abs_{280nm} values.

The purified anti-E5 IgG was coupled to CNBr-activated Sepharose 6MB with a calculated 91 % efficiency. NSIAC was performed using crude Welgevonden isolate infected E5 cell cultures (Fig. 2.5.). The isolation procedure was repeated and results obtained tabulated in Table 2.5. Protein assays

indicated that the NSIAC purification procedure removed 10-19% protein from crude extracts (ie. between 0,4 and 0,9 mg protein; Table 2.5.). An increase in antibody titer was observed by ELISA II for NSIAC fractions: crude extracts (1:100), NSIAC peak 1 (1:500) and NSIAC peak 2 (1:2500). Negative ELISA II results were obtained when the NSIAC experiment was repeated. However antibody titers by ELISA I gave a titer of 1:500 for both the crude and NSIAC peak 1 samples and negative results for NSIAC peak 2. Electron microscopic examination of crude extracts and NSIAC peak 1 revealed that most of the cellular debri was removed by the immunoadsorbent (Fig 2.6.). However small organelles and cellular debri were still observed in the unbound fraction, together with *Cowdria* organisms. Another observation made during these studies was that relatively fewer *Cowdria* organisms were present after concentration of the NSIAC fractions by centrifugation at 10 000 xg. The protein loss was determined to be between 60% - 80% (Table 2.5.).

Coomassie stained SDS-PAGE protein profiles revealed only 2 differences between crude extracts and immunoadsorbent peak 1 (Fig 2.7.). Albumin (67 kDa) was almost completely removed while a 27 kDa protein disappeared after immunoadsorbent chromatography. This 27 kDa protein was however detected by immunoblots (Fig. 2.7.). Unfortunately insufficient material was obtained from NSIAC peak 2 to make any conclusive SDS-PAGE comparisons.

The results of the immunoblots of crude extracts, NSIAC peak 1 and 2 are illustrated in Fig 2.7. and tabulated in Table 2.6. since not all antigens were clearly visible in the immunoblot figure. Two minor antigenic proteins, 118 and 58 kDa, disappear and 2 new minor antigenic proteins, 87 and 72 kDa appear after the chromatographic step. The immunoblots also reveal the presence of *Cowdria* proteins in NSIAC peak 2.

The LD_{50} of *Cowdria* organisms before and after NSIAC was determined in mice an found to be 1:66 for the crude extract and 1:50 for the NSIAC Peak I. Infectivity tests in mice therefore indicated that *Cowdria* organisms were viable after the purification procedure with 1.3 fold drop

in LD_{50} titer. This could however not be repeated due to problems experienced when injecting the mice.

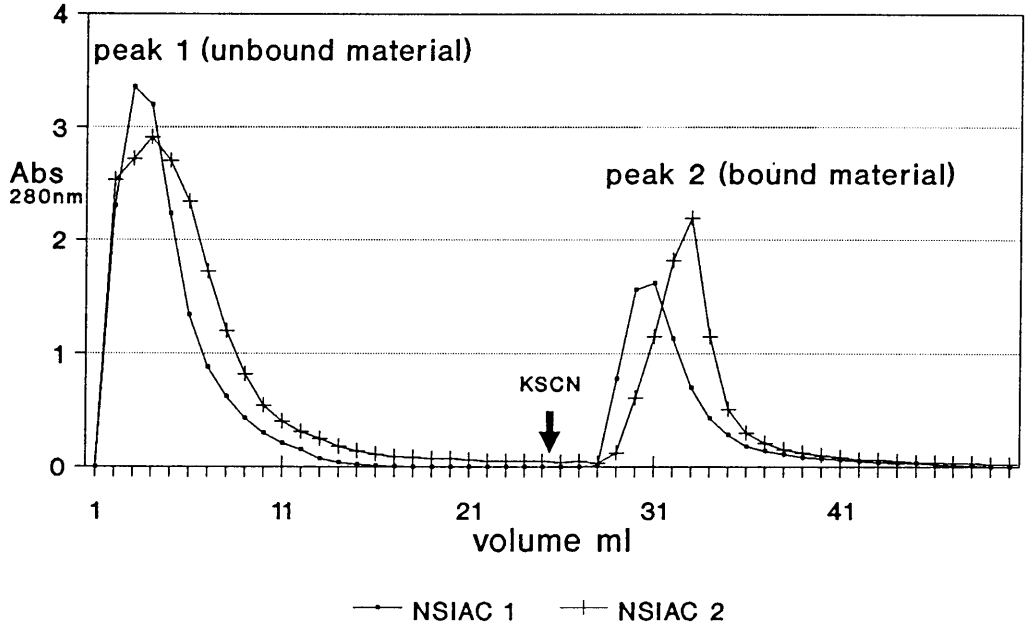
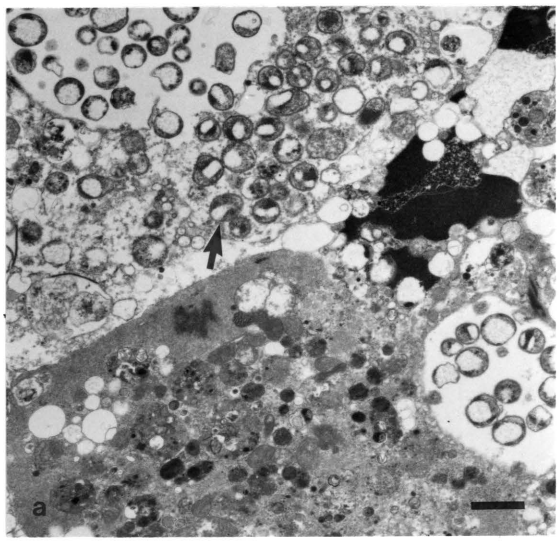


FIGURE 2.5. Chromatogram of NSIAC of *Cowdria* infected crude cell culture extracts. The unbound material was eluted with PBS pH 7,4 and the bound material with 50 mM Tris containing 3 M KSCN, pH 9. ----> = application of 3 M KSCN in Tris, pH 9,0.

TABLE 2.5. Protein content, antigen properties and infectivity of Welgevonden isolate infected crude extracts and immunoadsorbent chromatography fractions. Duplicate values are in parenthesis. # - ELISA I was only done on the duplicate sample. * - 50 % mortality endpoint titer as determined in mice.

Sample Fraction	Protein content mg	Reciprocal titers		
		ELISA II	ELISA I#	LD50*
10 000 xg crude cell culture extract pellet	4,2 (4,8)	100 (neg)	500	66
NSIAC peak 1	3,3 (5,1)			50
NSIAC peak 1 10 000 xg pellet	0,7 (2,4)	500 (neg)	500	nd
NSIAC peak 2	0,4 (0,9)			
NSIAC peak 2 10 000 xg pellet	0,1 (0,1)	2500 (neg)	neg	nd



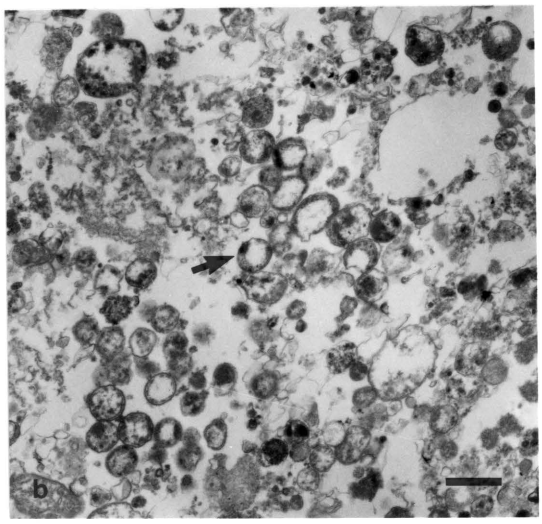


FIGURE 2.6. Electron micrographs of *Cowdria* infected crude E5 cell culture extracts a) before and b) after NSIAC. Bar = 1 µm.

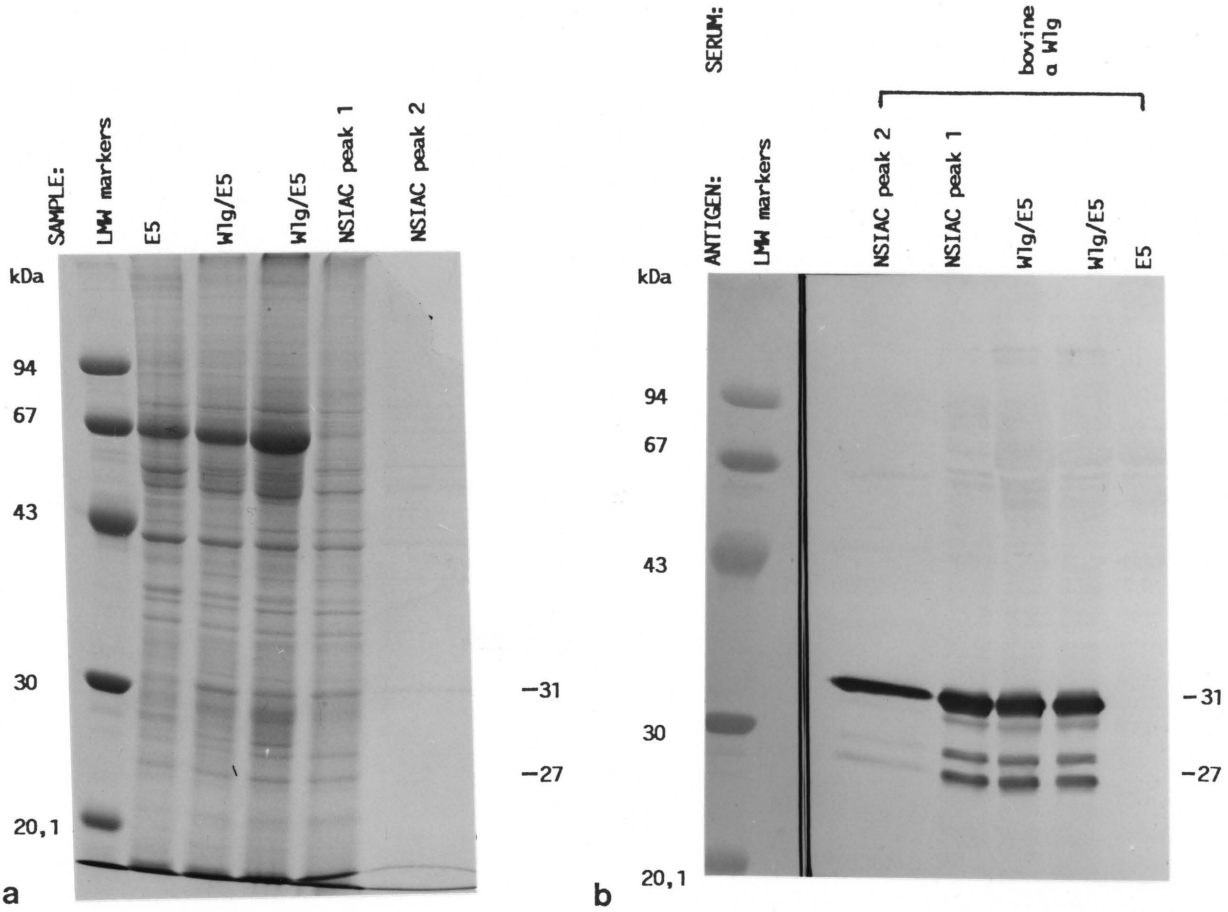


FIGURE 2.7. SDS-PAGE protein patterns of *Cowdria* infected crude E5 cell culture extracts before and after NSIAC. a) Coomassie stained gel and b) Immunoblot probed with bovine polyclonal anti-Welgevonden serum. All the samples were electrophoresed at 40 µg protein/sample except for NSIAC peak 2 (5 µg protein).

TABLE 2.6. Molecular weights of the antigenic proteins identified by immunoblots of *Cowdria* infected crude E5 cell extracts and NSIAC fractions.

Sample fraction	10000 xg crude cell culture extracts	NSIAC peak 1	NSIAC peak 2
Relative MW (kDa)	126	126	-
	118	-	-
	-	87	-
	-	72	-
	63	63	63
	58	-	-
	40	40	-
	38	38	-
	31	**31**	**31**
	29	**29**	**29**
	28	**28**	**28**
	27	**27**	**27**

2.4 DISCUSSION

Cultivation of *Cowdria* in cell culture, as a source of organisms for further investigations, has been a major breakthrough for heartwater researchers. It served as a continuous source of *Cowdria* as the cultures could be passaged and harvested weekly. Light microscopic monitoring of the infectious cycle ensured maximum infectivity and high concentration of the harvested organisms. Unfortunately, the release of *Cowdria* into the culture medium, during the infectious cycle, coincided with rupturing of the infected E5 cell. Thus pure organisms, free from cellular debri, could not be obtained by harvesting the mediums. Therefore rabbit anti-E5 cell serum was prepared for use in NSIAC in an attempt to remove the cellular debri.

Investigations into preparing polyclonal serum, against *Cowdria* or contaminating cell culture material, in a rabbit system had merit as it offered several advantages over the bovine or mouse system. Rabbits are not susceptible to heartwater, are cheaper experimental animals than are cattle and produce larger quantities of serum than mice. The polyclonal serum from rabbits compared well with the sera obtained from cattle or mice, with respect to ELISA titers. These results also indicate that repeated inoculations with the use of adjuvants are necessary when immunizing rabbits for polyclonal anti-serum production. Antibody titers of 1:2500 obtained for polyclonal rabbit anti-E5 cell culture serum, made the serum a good candidate for use in NSIAC.

In order to increase the number of extracellular organisms, the E5 cell membrane must be ruptured. Utilizing sonication for this purpose proved

to be disadvantageous. Sonication resulted not only in rupture of the E5 cell membrane but also of the *Cowdria* cell wall. Differential centrifugation investigations brought to light that a single freeze-thaw of infected cell cultures was effective in releasing whole undamaged *Cowdria* organisms from intracellular vacuoles.

Electron micrographs of pellets obtained from differential centrifugation investigations of heartwater infected cell cultures indicated that *Cowdria* can be quantitatively sedimented between 200 xg ≤ 10 000 xg together with cellular debri. This contradicts the expected behaviour of *Cowdria* organisms, obtained from infected tick or animal tissue during centrifugation, considering it's calculated sedimentation coefficient of 10^4 necessitating sedimentation at 30 000 xg (Neitz *et al.*, 1987). A possible explanation for this could be that certain components are present within the culture media, probably introduced with the serum, resulting in clumping together of the organisms and thus sedimenting at lower relative centrifugal force. Because of the rapid sedimentation of the organism even at low rotor speed differential centrifugation cannot be employed for separating *Cowdria* from cellular debri. When using heartwater infected cell cultures as a source of organisms, centrifugation should be carried out at 10 000 xg for 30 min at 4°C to concentrate the organisms for further purification.

NSIAC of heartwater infected bovine endothelial cell cultures was investigated for the purification of *Cowdria* organisms. NSIAC removed 10-19% protein from crude extracts ie. between 0,4 and 0,9 mg protein. The presence of *Cowdria* antigens in NSIAC peak 1 and 2 fractions were confirmed by ELISA and immunoblots. Electronmicrographs revealed

partially purified *Cowdria* organisms in peak 1. Because of insufficient material no electronmicrographs could be obtained for peak 2. The unexpected presence of *Cowdria* antigen in the bound fraction could be explained by the fact that *Cowdria* colonies occur within membrane bound vacuoles within the cytoplasm of the host cell. The possibility for the presence of antibodies on the immunoadsorbent with affinity to E5 vacuole membrane components exists. Alternatively *Cowdria* may incorporate host proteins in its cell wall or be closely associated with host material, resulting in the organisms being retained on the immunoadsorbent column.

The presence of E5 cell material in the unbound peak indicates that the capacity of the NSIAC column was exceeded. Microzone electrophoresis, of fractions obtained during purification of rabbit anti-E5 cell IgG by DEAE Cibacron blue 3-G-Agarose chromatography, indicated the presence of beta globulin in the IgG fraction. This contamination, which was unknown at the time of anti-E5 cell IgG coupling to Sepharose 6MB, therefore led to incorrect IgG quantitation and a corresponding lower IgG binding to the Sepharose beads and lower column capacity. In addition to this, all the cellular debri may not be removed by the immunoadsorbent as there may not be antibodies present with affinities to all contaminating antigens.

Cowdria retained infectivity after the NSIAC procedure with a 1,3 fold decrease in 50% mortality endpoint titers. However the low LD_{50} titer of $10^{1.8}$ for cell cultures used in NSIAC was very low when compared to LD_{50} titer of $10^{5,4}$ for mice organs (Du Plessis, 1985a). This may be as a result of cell cultures that were harvested when all organisms were extracellular. For greater viability but lower yield, the infected

cultures should be harvested immediately when maximum organisms are extracellular and not left until all are released.

Protein assays and electronmicrographs revealed a 60-75% reduction in protein content and a loss in *Cowdria* organisms in the pellet obtained from NSIAC fractions centrifuged at 10 000 xg. This centrifugation step was employed to concentrate the material for ELISA, SDS-PAGE/immunoblot assays and electronmicroscopic investigations. A possible explanation for this could be that the immunoadsorbent purification step removed the factor responsible for *Cowdria* sedimenting at unexpected low centrifugal forces. SDS-PAGE protein profiles revealed little differences between the crude extracts and NSIAC fractions. However albumin was almost completely absent in the bound peak fractions. There could therefore possibly be a link between albumin and the differences observed in the sedimentation rate of *Cowdria* derived from cell cultures and other sources. It should therefore be investigated whether centrifugation at 30 000 xg after NSIAC would not result in a higher yield. If so, differential centrifugation could be used to further purify the organisms.

Utilization of ELISA I or II as an assay to determine either the presence of *Cowdria* antibodies or organisms in samples in the early stages of the investigations was effective. Later, however, ELISA II gave false negative results and did not correlate with results obtained by ELISA I. When immunoblotting techniques were introduced to monitor the purification procedures, it was clear that the IB was more suitable with regard to sensitivity and reliability than the ELISA. Until the ELISA is

optimized either the IFA assay or IB techniques should rather be used when samples are to be screened for antibodies or antigens.

When the antigenic proteins, identified by immunoblots, of crude cell culture extract, NSIAC peak 1 and NSIAC peak 2 were compared, minimal differences were observed. Therefore, for the identification of the antigenic proteins of *Cowdria*, when appropriate controls are used, partially purified organisms by NSIAC are not necessary. Crude infected and uninfected cell culture extracts should be sufficient for the purpose of identifying the antigenic differences between the different stocks of *Cowdria*, by immunoblotting and will be investigated in the next chapter.

Chapter 3

IDENTIFICATION OF ANTIGENIC PROTEINS OF *COWDRIA*

3.1 INTRODUCTION

Identification and characterization of antigenic and immunogenic proteins of *Cowdria* could lead to clarification of differences observed between different stocks regarding pathogenicity, immunogenicity and virulence. It may also lead to the development of a sensitive, specific diagnostic assay which could be used to identify *Cowdria* or distinguish between the different stocks. Development of a subunit vaccine protecting against serologically different stocks may also be achieved. This is of importance, because the only commercially available vaccine against heartwater is a live, blood vaccine which has various disadvantages (Oberem & Bezuidenhout, 1987).

To date, stocks of *Cowdria* from geographically widely separated areas have shown either complete, partial or no cross-immunity *inter se* or against the reference Ball3 stock, when tested in goats (Jongejan *et al.*, 1988), sheep (Du Plessis *et al.*, 1989) or mice (Stewart, 1989). Du Plessis, using an indirect fluorescent antibody technique, demonstrated cross-reacting antibodies between the Kümm and Ball3 stocks (Du Plessis, 1981). These 2 stocks, however, differed with respect to 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 *Cowdria* that may even occur for the same stock during serial passage in sheep.

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 *et al.*, 1986). Antigenic heterogenicity and the presence of common antigens have been demonstrated among isolates of *Rickettsiae* (Tamura *et al.*, 1985), *Chlamydia* (Caldwell & Judd, 1982) and *Anaplasma* (Adams *et al.*, 1986) by immunoblotting techniques. 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 antigenic differences to geographical distribution. It may also be used to identify organisms in cross-immunity experiments as being a primary persistent or a secondary heterogeneous challenge (McGuire *et al.*, 1984). Monoclonal antibodies recognizing common determinants can be used to identify organisms in vertebrate and invertebrate host tissues and cell cultures. In addition, common determinants can form the basis for specific serological tests and used for the synthesis of probes to screen genomic libraries and for diagnostic assays.

Various serological tests for the detection of antibodies to *Cowdria* have been reported, each utilizing a different source of antigen. These sources include infected brain (Du Plessis, 1981; Ilemobade & Blotkamp,

1976), *Amblyomma* nymph extracts (Neitz *et al.*, 1986b), mouse peritoneal cells (Du Plessis & Malan, 1987), and neutrophils (Holland *et al.*, 1987). The success with the cultivation of *Cowdria* in cell cultures (Bezuidenhout *et al.*, 1985) offers a possible alternative source of suitable specific antigens with which to increase the sensitivity and specificity of existing diagnostic assays. *Cowdria* antigen obtained from infected cell culture may be used in an immunoblot and probed with antisera, to the different stocks of *Cowdria*, for the identification of specific antigenic proteins.

At the time our investigations were in progress, a 32 kDa immunodominant, antigenically conserved, *Cowdria* protein was identified, by immunoblotting techniques (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, Ball3, Kümm, Kwanyanga and Welgevonden stocks. Two years later Jongejan and coworkers developed a competitive ELISA using cell culture antigen and a monoclonal antibody which reacts with the 32 kDa protein of *Cowdria* (Jongejan *et al.*, 1991a).

In this chapter a study is described to identify the antigenic proteins of *Cowdria*. Parts of the investigation have been published (Rossouw *et al.*, 1990).

3.2 MATERIALS AND METHODS

3.2.1 Stocks of *Cowdria*

Nine stocks of *Cowdria*, differing in antigenic composition, virulence, pathogenicity, serotype and origin were used in these investigations (Table 3.1.). Polyclonal serum was produced against all 9 stocks and only the Welgevonden, Ball3 and Kwanyanga stock were used as antigen in the immunoblots.

TABLE 3.1. Stocks of *Cowdria* and their origin (Du Plessis *et al.*, 1989).

Stock (abbreviation)	Origin	Year of isolation	Source from which stock was originally obtained
Ball3 (B3)	N-Transvaal, RSA	1952	bovine
Breed (Bre)	N-Transvaal, RSA	1983	goat
Comoro (Com)	Comoro islands	1987	*A. variegatum*
Germishuys (Grms)	NE-Transvaal, RSA	1984	sheep
Kümm	NE-Transvaal, RSA	1971	goat
Kwanyanga (Kwg)	E-Cape Province, RSA	1981	sheep
Mali	Moribabougou, Mali	1985	*A. variegatum*
Mara	N-Transvaal, RSA	1987	*A. variegatum*
Welgevonden (Wlg)	N-Transvaal, RSA	1985	*A. variegatum*

3.2.2 Production of polyclonal anti-*Cowdria* sera in sheep, bovine and a goat

Polyclonal sheep, bovine and goat anti-*Cowdria* serum, produced as described below, was used to probe western blots of *Cowdria* proteins. Two different approaches were taken to produce bovine anti-serum as not all bovine were heartwater susceptible subsequent to inoculation, resulting in low serum antibodies.

Protocol 1: Polyclonal sheep serum against the Welgevonden, Ball3, Kwanyanga, Mali, Comoro, Breed, Germishuys, Kümm and Mara stocks were kindly donated by Dr J.L. du Plessis, OVI, Onderstepoort. The sera was prepared as described below.

Nine sheep, 9 bovine and 1 goat were infected with 5-10 ml heartwater infective sheep blood stabilate by intravenous injection (Oberem and Bezuidenhout, 1987). The sheep and bovine were infected with 1 of the following stocks of *Cowdria*: the Welgevonden, Ball3, Kwanyanga, Mali, Breed, Germishuys, Kümm, Comoro (only sheep) or Mara stock. The goat was infected with the Welgevonden stock stabilate. The animals were treated on the 3rd day of a rising febrile reaction (with exception of the goat which was treated on the first day of the reaction) by intravenous (i.v.) injection with oxytetracycline, Liquamycin 100, at a dosage rate of 10 mg/kg. All the animals were boosted 4 weeks after the initial inoculation, except the goat which was challenged as described in subsection 4.2.4. Blood was collected from the immunized animals 4 weeks after final challenge and processed as described in subsection 2.2.2.

Alternatively, in some cases the conglutinin titers of bovine were determined prior to immunization as described by Du Plessis, 1985b and kindly carried out by Dr J.L. du Plessis, OVI, Onderstepoort. Low conglutinin titers are reported to coincide with high heartwater susceptibility. Three bovine with titers of ≤1:320, were infected with the Mali, Mara and Germishuys stocks (Protocol 1*) as described above.

Protocol 2: Sheep were immunized individually with 7 different stocks of *Cowdria* for the purpose of obtaining fresh infective blood stabilate in large quantities with which to infect bovine. Seven sheep were individually infected, as described in protocol 1, with either of the following stocks: Kwanyanga, Ball3, Kümm, Mali, Mara, Breed and Germishuys. The sheep were bled (300 ml), in heparin (10 IU/ml blood), on the third day of a febrile reaction and 7 bovine were infused with 200 ml of the blood. Ten ml of sheep blood stabilate prepared as described above was used to boost the bovine four weeks after the initial inoculation. Blood was collected from the immunized cattle 4 weeks after the final challenge and prosessed as described in subsection 2.2.2.

Polyclonal sera produced as described above were screened for antibody titers by ELISA I (subsection 2.2.7.) and/or IFA assay (subsection 2.2.8.).

3.2.3 Preparation of *Cowdria* antigen

Preparation of crude Cowdria ***antigen from cell cultures:*** Three different stocks of *Cowdria* were used as antigen in Immunoblots. The Welgevonden and Ball3 stocks were cultured in a calf endothelial cell line (E5 cell line) and the Kwanyanga stock in a bovine aorta cell line (BA cell line), as described in subsection 2.2.1.

Cowdria-infected cell cultures (80-100 % infected with intracellular and maximum extracellular organisms) were harvested with a cell scraper into the culture media. Clumps of cells were disintegrated by drawing the mixture 3 times through a 19 G needle. After centrifugation for 30 min at 10 000 xg, the resultant pellet was resuspended in PBS, pH 7,4 and stored at -70 °C. The same procedure was also followed for the preparation of uninfected, crude culture extracts.

Protein assay: The protein content of the resuspended, crude, heartwater infected and uninfected culture extracts were determined by the Bio-Rad protein assay as described in subsection 2.2.6.

3.2.4 Immunoblotting

SDS-PAGE was performed on crude, Welgevonden, Kwanyanga or Ball3 infected or uninfected cell cultures (subsection 2.2.11.), western blotted onto PVDF membranes (subsection 2.2.12.) and immunostained with the polyclonal anti-*Cowdria* serum (subsection 2.2.13.). The sheep sera, donated by Dr Du Plessis, were used to probe Welgevonden, Kwanyanga or Ball3 stock samples while the Welgevonden stock was also probed by polyclonal bovine and goat serum. The only modification to the immunostaining protocol was the addition of a blocking step (5% Elite milk powder, 20 mM Tris-HCL and 0,09% NaCl, pH 7,4 for 2 h) when goat serum was used.

3.3 RESULTS

3.3.1 Production of polyclonal anti-*Cowdria* sera in sheep, bovine, and a goat

The polyclonal sheep serum kindly donated by Dr J.L. du Plessis, showed the following IFA assay titers: Welgevonden (1:1280), Ball3 (neg), Kwanyanga (1:20), Mali (1:80), Comoro (>1:320), Breed (not determined), Germishuys (1:80), Kümm (1:1280) and Mara (1:320).

Preparation of polyclonal serum in bovine against 8 different stocks of *Cowdria* according to protocol 1 was not totally successful. The anti-Welgevonden and anti-Kwanyanga bovine serum show ELISA titers of 1:2500 and 1:20, respectively (Table 3.2.). The anti-Kwanyanga bovine serum showed an IFA assay titer of 1:320. Negative ELISA and IFA assay results were obtained for anti-serum against the Ball3, Mali, Kümm, Mara, Germishuys and Breed stocks. The bovine that were infected with the Welgevonden and Kwanyanga stocks were the only ones that developed a febrile response. Oxytetracycline was administered to the bovine that were infected with the Welgevonden stock (on day 11 and 12 post infection) and the Kwanyanga stock (on day 13 post infection).

Sheep were immunized individually with 7 different stocks of *Cowdria* for the purpose of obtaining fresh inoculum in larger quantities with which to infect bovine (Protocol 2). Polyclonal sheep anti-serum was subsequently successfully prepared against the following stocks (IFA titers are shown in parenthesis): Ball3 (1:320), Mara (1:1280), Mali (1:320), Breed (1:80), Kümm (1:1280), Kwanyanga (1:1280) and Welgevonden (1:1280). All the sheep infected with these stocks developed a febrile response. No success was obtained in preparing polyclonal sheep anti-serum against the Germishuys stock. The subsequent trial, using 200 ml fresh heartwater infective sheep blood as primary inoculum for bovine, resulted in anti-Kwanyanga and anti-Breed IFA assay titers of 1:320 and 1:20 respectively (Table 3.2.). Negative IFA assay results were once again obtained for bovine anti-serum against Germishuys, Kümm, Mara and Mali. Negative results obtained in bovine for the Kümm stock are not unexpected as the Kümm stock is not pathogenic to bovine. The bovine infected with the Kwanyanga and Breed stocks were the only ones that developed a febrile response and were treated on day 14 and days 15 and 17 respectively.

Bovine with known conglutinin values of ≤1:320 (therefore low resistance to heartwater) did not lead to increased susceptibility or immunoblotting sensitivity. Neither of these bovine developed a febrile response.

Preparation of polyclonal serum in a goat against the Welgevonden stock of *Cowdria* resulted in an IFA assay titer of 1:1280. The goat developed a febrile response and was treated on days 8, 9, 10 and 18.

TABLE 3.2. Reciprocal ELISA and IFA assay titers for 3 trials of bovine anti-serum raised against 8 different stocks of *C. ruminantium*. * - conglutinin titers of the bovine were determined; # - serum not prepared against stock; IB - immunoblot (discussed in subsection 3.3.2.).

C. ruminantium stock	Protocol 1			Protocol 2		Protocol 1*	
	ELISA titer	IFA titer	IB	IFA titer	IB	IB	K titer
Welgevonden	2500	nd	pos	#	#	#	#
Kwanyanga	20	320	pos	320	pos	#	#
Ball3	neg	neg	pos	#	#	#	#
Mali	neg	neg	neg	neg	pos	pos	160
Kümm	neg	neg	neg	neg	nd	#	#
Mara	neg	neg	neg	neg	pos	nd	320
Germishuys	neg	neg	neg	neg	neg	neg	80
Breed	nd	neg	neg	20	pos	#	#

3.3.2 Identification of the antigenic proteins of *Cowdria*

The immuno blots with sheep polyclonal serum to the 9 stocks showed both similarities and differences between the 3 stock antigens and the 9 sheep anti-sera (Figs. 3.1. & 3.2. and Table's 3.3. - 3.5.). 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, Ball3 and Kwanyanga stocks and are recognized by sheep polyclonal serum to the Welgevonden, Ball3, Kwanyanga, Mali, Comoro, Breed, Germishuys, Kümm and Mara stocks (Figs 3.1. & 3.2. and Table's 3.3. - 3.5.), bovine polyclonal serum to the Welgevonden, Kwanyanga, Breed, Mali, Mara and Ball3 stocks (Figs 3.2. and Table 3.6.) and goat polyclonal serum to the Welgevonden stock (Fig 3.2. and Table 3.7.). 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, Ball3 and Kwanyanga stocks by the 9 sheep anti-sera are 20, 12 and 9, respectively. Presently no 2 stocks show identical or unique antigenic properties. As only 6 different bovine antisera were available for investigation, no conclusion could be made concerning the unique proteins recognised in Table 3.6.

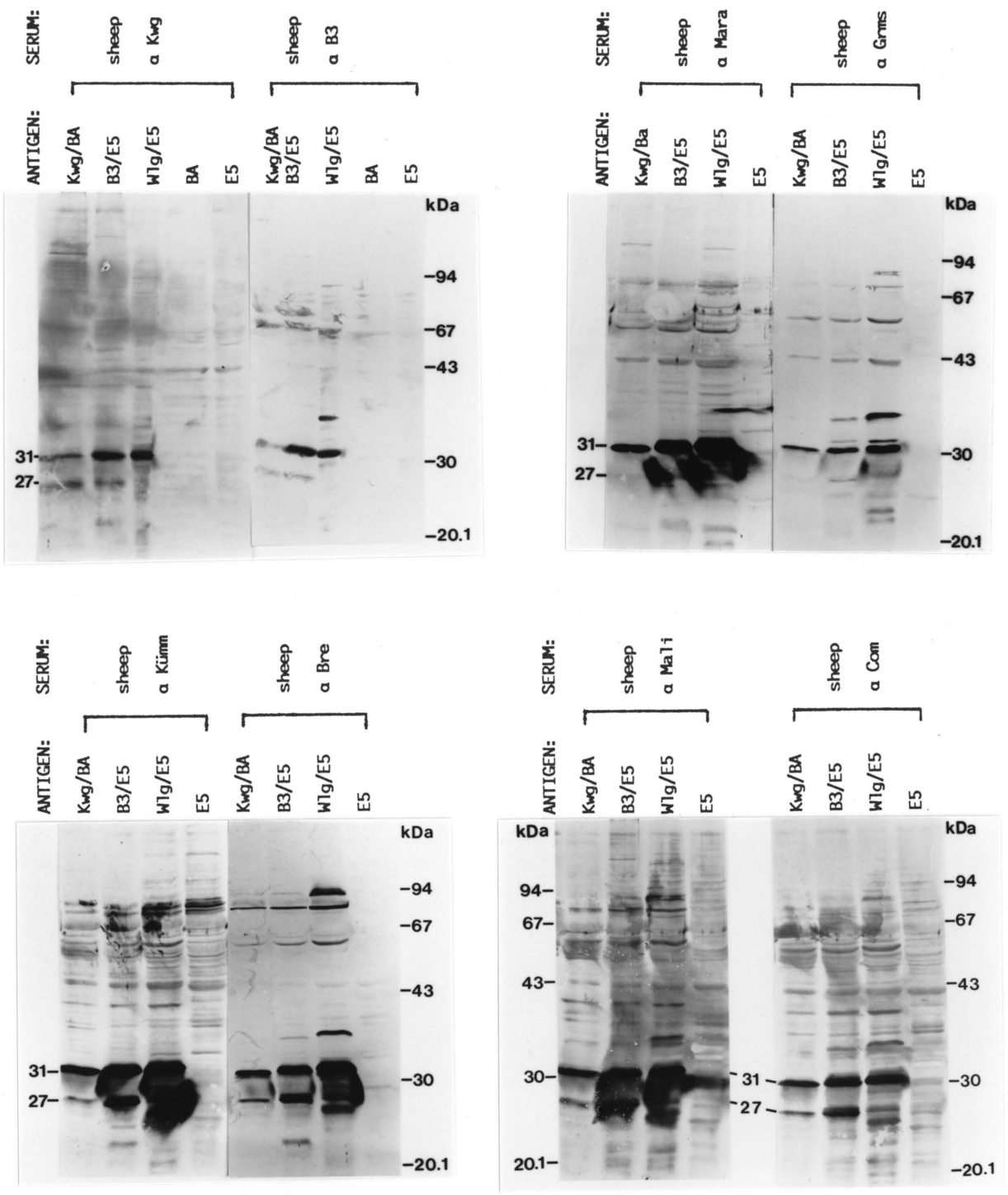


FIGURE 3.1. Western blot analysis of *Cowdria*-infected and uninfected crude cell culture extracts probed with polyclonal sheep serum.

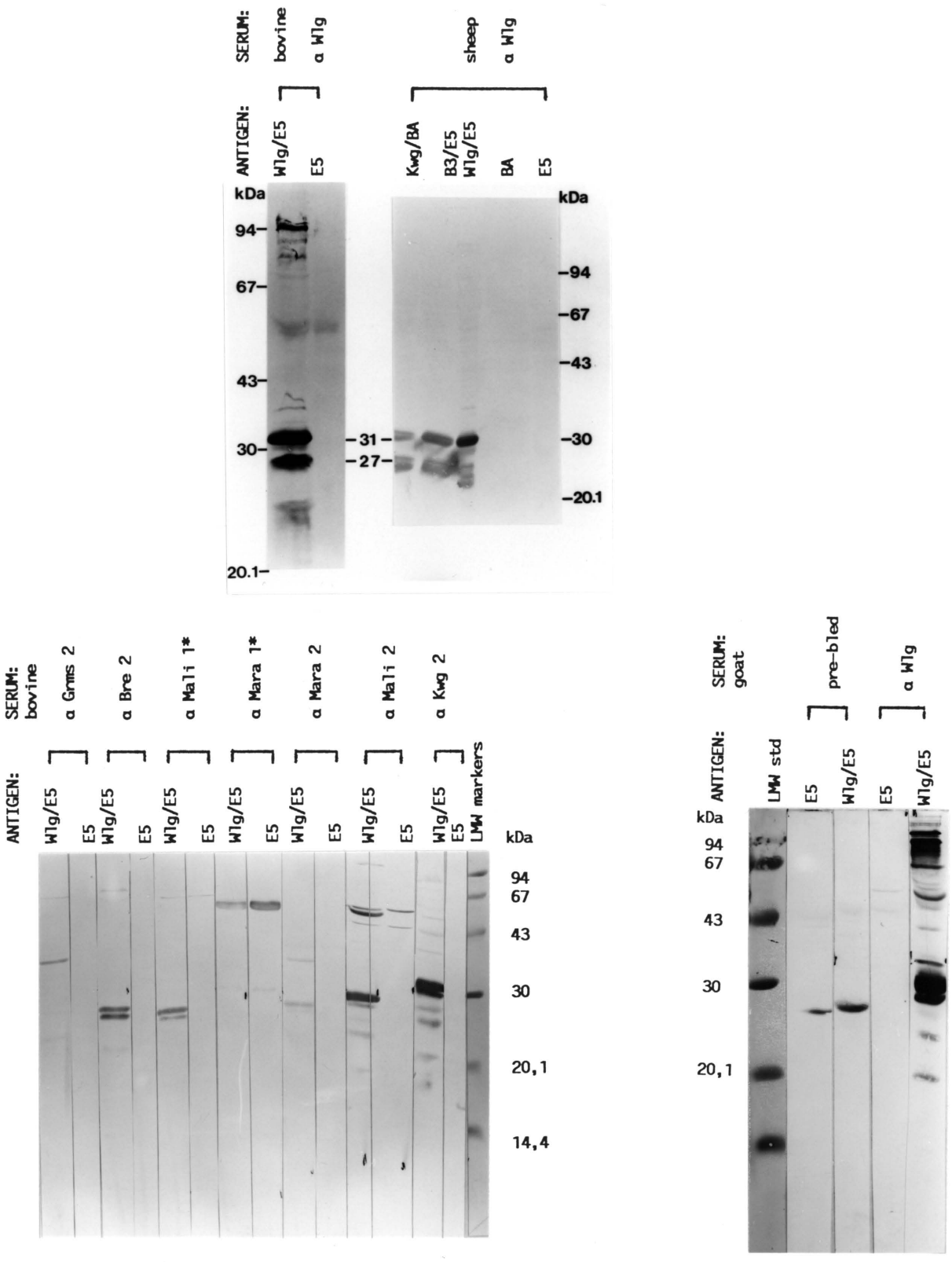


FIGURE 3.2. Western blot analysis of *Cowdria*-infected and uninfected crude cell culture extracts probed with polyclonal bovine, sheep or goat serum.

TABLE 3.3. Molecular weights of the antigenic proteins of the Welgevonden stock of *Cowdria*, identified by probing western blots with polyclonal sheep serum against 9 stocks.

Antigen	Welgevonden								
Serum	Kwg.	B3.	Wlg.	Mali	Com.	Bre.	Grms.	Kümm	Mara
MW	-	-	-	-	-	138	-	-	138
kDa	-	126	126	-	-	-	-	-	126
	-	-	-	120	-	-	-	-	-
	-	-	-	-	-	-	-	-	118
	-	-	-	115	-	-	-	-	-
	-	-	-	-	112	112	-	-	-
	-	-	-	-	-	-	-	-	107
	-	-	100	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	98
	-	96	96	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	93
	-	-	92	-	-	-	-	-	-
	-	-	91	-	-	-	-	-	-
	-	89	89	-	-	-	-	-	-
	87	87	87	-	-	87	87	87	-
	-	85	-	85	85	85	85	-	85
	-	-	83	-	-	-	-	-	-
	-	-	80	-	-	-	-	-	-
	79	-	-	-	-	-	79	-	-
	-	-	-	-	-	78	78	-	78
	76	-	-	76	-	-	76	-	76
	-	-	-	-	-	74	-	-	-
	-	-	-	71	-	71	71	-	-
	-	-	-	-	-	-	-	-	69
	-	-	68	-	-	68	-	-	-
	-	-	65	-	-	-	-	-	-
	-	-	63	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	62
	-	-	60	-	-	60	-	-	-
	-	56	-	56	-	-	-	-	-
	-	-	-	-	55	55	55	55	-
	-	54	-	-	-	-	-	-	-
	53	-	-	-	-	-	53	-	-
	-	-	-	-	-	-	-	52	-
	-	-	-	-	-	-	-	-	51
	-	-	50	-	-	50	-	-	-
	-	-	49	-	-	-	49	-	-
	-	-	-	-	48	48	-	-	-
	46	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	43	-	43
	-	-	41	-	41	-	41	-	-
	-	-	-	-	-	-	-	-	38
	37	37	-	-	-	37	-	-	37
	-	-	36	36	36	-	-	-	-
	-	35	-	-	-	35	35	-	35
	34	-	-	34	34	-	-	-	-
	-	-	-	-	33	33	33	33	33
	32	32	32	-	32	32	32	32	32
	31	31	31	31	31	31	31	31	31
	-	-	30	-	30	30	30	30	30
	-	-	-	-	29	29	29	29	29
	-	28	28	-	28	28	28	28	28
	27	27	27	27	27	27	27	27	27
	-	-	26	-	-	26	26	26	26
	-	-	25	25	25	25	25	-	-
	-	-	-	-	24	24	24	-	24
	-	-	23	-	-	-	23	-	-
	-	-	-	-	22	22	-	22	-
	-	-	-	21	21	-	-	-	21
	-	-	-	-	-	-	-	20	-
	-	19	-	-	19	-	-	19	-
	-	-	-	18	18	-	-	-	18
	-	-	-	-	-	-	-	-	16

TABLE 3.4. Molecular weights of the antigenic proteins of the Ball3 stock of *Cowdria*, identified by probing western blots with polyclonal sheep sera to 9 stocks.

Antigen	Ball3								
Serum	Kwg.	B3.	Wlg.	Mali	Com.	Bre.	Grms.	Kümm	Mara
MW	135	-	-	-	-	-	-	-	-
kDa	-	-	-	-	-	-	-	-	132
	-	126	-	-	-	-	-	-	126
	-	-	-	123	-	-	-	-	-
	-	-	-	120	-	-	-	-	120
	-	-	-	-	112	-	-	-	112
	100	-	-	-	-	-	-	-	-
	-	96	-	-	-	-	-	-	-
	-	-	-	89	-	-	-	-	-
	85	-	-	85	85	85	-	-	-
	-	-	-	-	83	-	-	-	-
	-	-	-	-	79	-	-	-	-
	-	-	-	78	-	-	-	-	-
	-	-	-	76	-	76	76	-	76
	-	-	-	-	-	74	-	-	-
	-	-	-	72	-	-	72	-	-
	-	-	-	-	-	71	-	-	-
	68	-	-	-	-	68	-	-	-
	-	-	65	-	-	-	-	-	-
	60	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	59
	-	-	-	-	-	-	58	-	-
	-	56	-	56	-	-	-	-	-
	-	-	-	-	55	55	55	55	-
	-	54	-	-	-	-	-	-	-
	-	-	-	-	-	-	53	-	-
	-	-	-	-	-	-	-	-	51
	-	-	50	-	-	-	-	-	-
	-	-	-	-	48	48	-	-	-
	46	-	-	-	-	-	-	-	-
	-	-	45	-	-	-	-	-	-
	-	-	-	-	-	-	43	-	43
	-	-	-	-	41	-	41	-	-
	39	-	-	39	39	-	-	39	-
	-	-	-	-	-	-	-	-	-
	37	-	-	-	37	37	-	-	37
	-	-	-	-	-	-	35	-	-
	34	-	-	-	-	-	-	34	34
	-	33	-	-	33	33	33	33	-
	-	-	32	32	-	-	-	32	-
	31	31	31	31	31	31	31	31	31
	-	-	-	-	30	30	-	-	30
	-	-	28	-	28	28	-	28	28
	27	27	27	27	27	27	27	27	27
	-	-	26	-	-	26	26	26	26
	-	-	-	-	-	-	-	25	-
	-	-	-	-	24	-	-	24	24
	-	-	-	-	-	-	-	22	22
	21	-	-	21	21	21	-	-	21
	-	19	-	-	19	-	-	19	-
	-	-	18	-	-	-	-	-	-

TABLE 3.5. Molecular weights of the antigenic proteins of the Kwanyanga stock of *Cowdria*, identified by probing western blots with polyclonal sheep sera against 9 stocks.

Antigen	Kwanyanga								
Serum	Kwg.	B3.	Wlg.	Mali	Com.	Bre.	Grms.	Kümm	Mara
MW	155	-	-	-	-	-	-	-	-
kDa	-	126	126	-	-	126	-	-	-
	118	-	-	-	-	-	-	-	-
	110	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	107	107
	105	-	-	-	-	-	-	-	105
	102	-	-	-	-	-	-	-	-
	100	-	-	-	-	-	-	-	-
	98	-	-	-	-	-	-	-	98
	93	-	-	-	-	-	-	-	-
	-	-	-	85	-	85	-	-	-
	-	-	-	-	83	-	-	-	-
	-	-	-	78	-	-	-	-	-
	-	-	-	76	-	76	76	-	76
	-	-	-	-	-	74	-	-	-
	-	-	-	-	-	72	72	-	-
	-	-	-	-	59	-	-	-	-
	-	-	-	56	-	-	-	-	-
	-	-	-	-	-	55	55	55	-
	-	-	-	-	-	-	-	-	51
	-	49	-	-	-	-	-	-	-
	-	-	-	-	48	48	-	-	-
	-	-	47	-	-	-	-	-	-
	46	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	43
	-	-	-	-	42	-	42	-	-
	39	-	-	39	39	-	-	39	-
	-	-	-	38	-	-	-	-	38
	37	-	-	-	37	-	-	-	37
	-	-	-	-	-	-	-	36	-
	34	-	-	-	-	-	-	34	-
	-	-	-	-	-	-	-	33	-
	-	-	32	-	32	-	-	32	-
	31	31	31	31	31	31	31	31	31
	-	-	-	-	30	30	-	-	-
	-	-	28	-	-	-	-	-	-
	27	27	27	27	27	27	27	27	27
	-	-	26	-	-	26	-	-	-
	-	-	-	-	24	-	-	-	-
	-	23	23	-	-	-	-	-	-
	-	-	-	-	21	-	-	-	21
	-	19	-	-	-	-	-	-	-

TABLE 3.6. Molecular weights of the antigenic proteins of the Welgevonden stock of *Cowdria*, identified by immunoblots with polyclonal bovine sera against 5 stocks.

Antigen	Welgevonden					
Serum	Welgevonden	Kwanyanga	Mali	Breed	Ball3	Mara
MW	100	-	-	-	-	-
kDa	91	-	-	-	-	-
	87	-	-	-	-	-
	-	83	-	-	-	-
	-	-	-	82	-	-
	80	-	-	-	-	-
	-	-	74	-	-	-
	72	-	-	-	-	-
	55	-	55	-	-	-
	54	-	-	-	-	-
	-	51	-	-	-	-
	38	38	-	-	-	38
	36	-	-	-	-	-
	35	-	-	-	-	-
	-	32	-	-	-	-
	31	31	31	31	31	31
	-	-	-	-	-	-
	-	29	29	29	-	29
	-	-	28	-	-	-
	27	27	27	27	27	27
	-	-	25	-	-	-
	24	-	-	-	-	-
	23	-	-	-	-	-
	-	22	-	-	-	-
	21	-	-	-	-	-
	20	20	20	-	-	-
	-	19	-	-	-	-

TABLE 3.7. Molecular weights of the antigenic proteins of the Welgevonden stock of *Cowdria*, identified by immunoblots with polyclonal sheep, bovine and goat serum against the Welgevonden stock.

Antigen	Welgevonden		
Serum source	Sheep	Bovine	Goat
MW kDa	-	129	-
	126	126	126
	-	123	123
	-	120	120
	-	118	-
	-	-	115
	-	112	112
	-	110	110
	-	105	105
	-	102	102
	100	100	-
	-	98	98
	96	-	-
	-	-	95
	-	93	93
	92	-	-
	91	91	-
	89	89	89
	87	87	-
	-	85	85
	83	83	83
	80	80	-
	-	79	79
	-	78	78
	-	-	76
	-	74	-
	-	72	72
	68	68	68
	65	-	65
	63	63	-
	-	62	62
	60	-	60
	-	-	59
	-	-	58
	-	56	-
	-	55	-
	-	54	54
	-	-	52
	50	-	50
	49	49	-
	-	-	48
	-	46	-
	-	-	45
	-	42	42
	41	-	-
	-	39	-
	-	38	-
	-	-	37
	36	36	-
	-	35	35
	-	34	34
	-	-	33
	32	-	32
	31	31	31
	30	-	-
	-	29	29
	28	-	-
	27	27	27
	26	-	-
	25	25	25
	-	24	24
	23	23	-
	-	21	21
	-	20	20
	-	-	18
	-	-	17
	-	-	14

3.4 DISCUSSION

The difficulties experienced in production of polyclonal serum in bovine against 8 stocks of *Cowdria* were reflected in the non-susceptibility of the animals to heartwater. Variation in the susceptibility to heartwater of cattle of different breeds, age and conditions is widely recognised. Furthermore adult cattle also vary markedly in their susceptibility to artificial infection (Du Plessis, 1985b). It was interesting to note that 3/8 of the bovine and 7/8 of the sheep, that gave positive (ELISA or IFA assay) polyclonal anti-serum, were the only animals that had temperatures of ≥ 40 °C for two or more consecutive days. Only the anti-Mali and anti-Ball3 serum from bovine which did not have temperatures of ≥ 40°C, and which were negative in the IFA assay, were positive in the immunoblots. The immunoblot was thus more sensitive than the IFA assay in detecting serum antibodies to *Cowdria*. Recently serum IFA assay titers have been reported to drop after homologous challenge of animals (Du Plessis *et al.*, 1989). The fact that the animals in the above trials were boosted may therefore also contribute to low or no IFA assay titers. A question therefore arises: Is a febrile reaction of ≥ 40 °C for more than one consecutive day and a single inoculation, a prerequisite for adequate polyclonal serum production against *Cowdria* for use in a serological assay?

The identification of *Cowdria* 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, Ball3 and Kwanyanga stocks and recognized by sheep, bovine and goat anti-serum to all the stocks tested. Recently, a 32 kDa immunodominant, antigenically conserved, *Cowdria* protein was identified (Jongejan & Thielemans, 1989). As molecular weight determination by SDS-PAGE may be determined with an accuracy of 10 % (Weber & Osborn, 1969), the probability exists that this 32 kDa protein is the same as the 31 kDa immunodominant protein identified in these investigations.

It was observed 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 Ball3 stock. The Welgevonden stock, therefore possesses a larger number of antigens recognized by the other 7 stocks than does Ball3. This is consistent with the observation that the Welgevonden stock elicits total immunity against 4 and partial immunity against 5 stocks, while Ball3 elicits total immunity only against 2 and partial immunity against 4 of the 8 stocks of *Cowdria* used in our investigations (Du Plessis *et al.*, 1989).

As complete, partial or no cross-protection is observed between the various stocks of *Cowdria* used in this investigation, 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 Ball3 and Comoro and the Ball3 and Germishuys stocks are totally cross-protective in sheep. Yet these 3 stocks do not possess any unique antigens to explain the cross-protection. However it has been demonstrated that immunization of cattle with a conserved 36 kDa protein of *Anaplasma marginale* induced protection against the homologous challenge, with the Florida isolate, and heterologous challenge, with the Washington O isolate (Palmer *et al.*, 1988). This potential cross-protective ability of a conserved surface protein which is common to antigenically different isolates of *A. marginale* suggests the possibility that the 31 kDa protein of *Cowdria* may have cross-protective capabilities and deserves further investigation.

Additionally, it was observed, by immunoblotting, that no stock possessed any unique antigen with which to distinguish it from the other *Cowdria* stocks. Du Plessis *et al*, (1989) was unable to find correlation between the antigenic diversity of several stocks of *Cowdria* and the antibody levels detected with the IFA assay, as the variations found in the titers were not stock related when the Kümm stock was used as antigen. Jongejan *et al.* (1989) on the other hand demonstrated the existence of distinct serotypes within *Cowdria*, using the IFA test, by observing that antibody titers on heterologous antigens were much lower than on homologous antigens. Jongejan *et al.* (1989) further commented that the existence of serotypes within the genus *Cowdria* complicates the serodiagnosis of

cowdriosis considerably and would require more than one antigen in the IFA assay. It still remains to be proven whether immunologically identical stocks of *Cowdria* belong consistently to the same serotype. Thus laborious cross-protection trials in experimental animals is still the only means whereby *Cowdria* stocks may be typed.

Jongejan *et al*, (1989) suggests that the use of an ELISA based on common antigenic determinants should improve the present situation regarding serological diagnosis of the heartwater disease. The use of the common 27 kDa and/or the 31 kDa proteins of *Cowdria* in a serological assay should therefore be investigated.

Purification and characterization of the 27 kDa and 31 kDa proteins of *Cowdria* is discussed in the next chapter.

Chapter 4

ISOLATION AND CHARACTERIZATION OF ANTIGENIC PROTEINS OF *COWDRIA*

4.1 INTRODUCTION

In the previous chapter, 2 antigenically conserved proteins, 1 an immunodominant 31 kDa and the other a minor 27 kDa protein, were described which are common amongst 9 stocks of *Cowdria*, differing in antigenicity, virulence, pathogenicity, serotype and origin. These proteins may be suitable for the development of nucleic acid probes, diagnostic assays and vaccines. They therefore merit characterization with respect to amino acid composition and sequence, immunogenicity and protection against heartwater, glycoprotein content and isoelectric point.

Amino acid analysis and partial sequencing of these proteins should make it possible to develop appropriate oligonucleotide probes either for screening *Cowdria* genomic libraries or for diagnosis of the disease. Development of a monospecific antisera against either the 27 kDa or 31 kDa proteins, provided that they are immunogenic, would allow an alternative means for identification of specific DNA clones coding for these proteins. Although polyclonal antisera will recognise a maximum number of epitopes presented by the recombinant antigen it often contains antibodies to contaminants and lacks specificity (Robinson *et al*, 1988). Monoclonal antibodies on the other hand recognise one epitope which may not be expressed by a fusion protein, or are directed against determinants sensitive to denaturing conditions (Knudsen, 1985). These disadvantages could be overcome by using monospecific antibodies.

The importance of characterization with respect to carbohydrate has become increasingly evident after their influence on biological functions such as antigenicity, turn over rate, stability or activity of a protein, was discovered. The valency of an antigen may have a strong effect on its immunogenicity. This is particularly true for large complex carbohydrates which are much more immunogenic than simple compounds (Harlow & Lane, 1988). As prokaryotes are not capable of glycosylating proteins, their presence would imply that these organisms incorporate host cell material. Determination of whether the 27 kDa or 31 kDa are glycoproteins or not is therefore of importance.

Characterization of a protein with respect to the isoelectric point defines a physical parameter of the protein and has great significance. For proteins it is the charged groups on the outside of the molecule that contribute mostly towards the pI of that particular protein. However if a protein is uncoiled, such as by urea, then a different charge structure, and consequently a different pI can be expected. The pH of a protein in urea usually cannot be correlated with that obtained in an aqueous environment and *vise versa*. Results obtained in urea have no physiological meaning although they are of use under denaturing experimental conditions which may be required for protein separation. Furthermore, pK values are influenced by the actual environment in the protein and, in extreme cases, may differ by several pH units from those of the free amino acid. It is for these reasons that the pI values of different proteins span a very wide range and may be used as a characteristic of a protein (Pharmacia, 1982).

Characterization of the 27 kDa and 31 kDa proteins requires purified proteins separated from other contaminants. For the purification of large quantities of proteins, gel permeation chromatography is more suitable than gel electrophoresis. However in the course of protein separation and purification by column chromatography, many problems arise regarding adequate resolution. Furthermore the use of gel filtration and ion-exchange column chromatography may not be the optimum choice when proteins of similar charge-to-mass ratios and/or sizes are to be separated.

Selective solubilization of *Cowdria* proteins should further simplify purification of specifically the 27 kDa and 31 kDa proteins. Partial and selective protein solubilization can be obtained by a number of methods involving chelating agents, manipulation of ionic strength and pH. Protein pertubants (ie. urea, guanidine and chaotropic agents) and enzymatic digestion have also been used. But the use of detergents appears to provide the most general extraction method presently available (Helenius & Simons, 1975). Sarkosyl, a mild anionic detergent, has been shown to selectively solubilize the cytoplasm membrane proteins of gram-negative bacteria (Ohashi *et al*, 1989). As *Cowdria* shows staining characteristics of gram-negative bacteria (Cowdry, 1926) the possibility of selectively solubilizing the 27 kDa and 31 kDa proteins of *Cowdria* was investigated.

The use of polyacrylamide gel electrophoresis systems for the separation of proteins via electrophoretic mobility in either the non-denatured or denatured state offers significant enhancement of protein separation over the above-mentioned methods. Purification of proteins may be complicated by insolubility in aqueous solutions and could be overcome by purification by SDS-PAGE. This method has been shown to offer high resolution and simplicity for the separation of small quantities of a specific protein from a crude extract. Two dimensional polyacrylamide gel electrophoresis may be used to determine whether the 27 kDa and 31 kDa proteins are homogeneous, when separated by SDS-PAGE. This method offers high resolution and sensitivity for the separation of proteins from a complex biological source since each dimension separates proteins according to independent parameters (O'Farrel, 1975).

Circumstances may require a protein to be extracted from the gel in which it was purified. Different methods for protein extraction from gels are based either on diffusion from crushed gel slices or on electrophoretic elution from the slices. Generally small proteins are eluted quantitavely by most of these methods. Gel elution can be time consuming, inefficient and limited by the difficulty in precisely excising the protein band from a complex mixture due to the elastic nature of preparative polyacrylamide gels. These problems may be circumvented by eluting intact proteins

quickly and precisely from membranes under mild conditions with detergents at high pH (Szewczyk & Summers, 1988).

If a protein is directly extracted or eluted from a SDS-PAGE gel for amino acid analysis and partial sequencing, SDS and free amino acids may be present contaminating the extract. This contamination will result in interference on further amino acid analysis. Additionally, detecting glycoproteins in polyacrylamide or agarose gels have several disadvantages such as low sensitivity, high background staining, time consuming protocols and SDS interference. These disadvantages are eliminated by transferring the protein, electrophoretically, onto a PVDF membrane for direct amino acid analysis, sequencing and glycoprotein determinations (Moos *et al.*, 1988; Nakagawa & Fukuda, 1989). The immobilized proteins cannot diffuse or be washed out, they are readily accessible to reagents or high-molecular ligands such as lectins, and incubation or washing steps can be performed easily. PVDF membranes are chemically inert and mechanically strong and therefore allow a broad range of analysis to be done on the transferred protein. There are two different methods to detect glycoproteins bound to immobilizing membranes. Either chemicals that react with aldehyde groups formed by periodate oxidation of vicinal hydroxyls of the carbohydrate moieties of glycoproteins are used or, a highly specific detection of certain sugar residues by means of lectins followed by a visualization step (Weiss *et al.*, 1991).

SDS-PAGE/electrophoretic transfer procedures have shown to give reproducible and reasonably accurate compositions and partial sequencing of several proteins. Only as little as 10 pmol of protein is required for the analysis provided an on line automatic amino acid sequencer is used (Tous *et al*, 1989; Matsudaira, 1989a). However, unacceptably low initial sequencing yields have been encountered. The most likely cause for this can be NH_2-terminal blockage occurring during electrophoresis. This may be due to reaction of primary amines with a substance resembling acrylamide monomer which is released during electrophoresis. As reactivity of these compounds increase with pH, operating at pH 7, as opposed to pH 8,3 with the Laemmli procedure, would much less likely

allow undesired modifications of the NH_2-termini. A buffer system has been developed which operates at pH 7,28 and does not contain glycine which might interfere with sequence analysis (Moos *et al.*, 1989).

However many proteins are not susceptible to the Edman degradation due to natural blockage of the α-amino groups of the proteins. The nature of the modified N-terminus cannot be easily determined and only a few of the modifications can be chemically or enzymatically reversed. In the case of a N-terminally blocked protein, the general way to obtain sequence information will be to sequence peptide fragments generated by cleavage of the polypeptide chain (Aebersold *et al.*, 1987).

SDS-PAGE separated proteins either directly excised or transferred to nitrocellulose membranes for use as immunogens has become increasingly popular (Chiles *et al*, 1987), and were investigated for the preparation of monospecific antisera against the 27 and 31 kDa proteins of *Cowdria*. For optimum monospecific anti-serum production in rabbits 50-1000 µg protein is generally required per immunization and in goats 500-2000 µg (Harlow & Lane, 1988). To ensure that the correct dosage of antigen is administered per immunization it is necessary to estimate the amount of immunogen, in the SDS-PAGE gel or on the nitrocellulose membrane by using standard proteins and appropriate staining and detection techniques (Chiles *et al*, 1987).

In this chapter the purification and certain characteristics of the 27 kDa and specifically the 31 kDa proteins of the *Cowdria* are described.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of crude *Cowdria* extracts from cell cultures

Welgevonden or Kwanyanga stock infected crude cell culture extracts were prepared as described in subsection 3.2.3. and used in purification and characterization investigations of the 27 kDa and 31 kDa proteins.

4.2.2 Solubilization of *Cowdria* proteins with detergents

Various detergents were investigated for their ability to selectively solubilize the 27 kDa and 31 kDa proteins from contaminating proteins.

Solubilization of the 27 and 31 kDa proteins of Cowdria with sarkosyl detergent: Solubilization of *Cowdria* proteins was performed as described by Ohashi *et al* (1989). A frozen Welgevonden stock infected crude cell culture sample was thawed and centrifuged at 10 000 xg for 30 min at 4 °C in a Beckman L7-55 centrifuge. The pellet was resuspended in 10 mM sodium phosphate buffer, pH 7,4. The protein content of the resuspended pellet was determined by the Bio-Rad protein assay (subsection 2.2.6.). Five volumes of aliquots of 400 µg protein each were treated with 1 ml of 0,05 %; 0,1 %; 0,5 %; 1 % or 2 % Sarkosyl detergent in 10 mM sodium phosphate buffer, pH 7,4 and one with water as a control. The samples were incubated at 37 °C for 30 min. Hereafter the samples were centrifuged at 16 000 xg for 30 min at 4 °C in a BGH Hermle Z230 M microfuge. The respective supernatants were collected and 300 µl of a buffer containing 0,06 M Tris-HCl (pH 6,8), 16 % glycerol, 2 % SDS, 2,5 % DTT and 0,001 % bromophenol blue (SDS-PAGE sample buffer) added to each. A volume of 200 µl SDS-PAGE sample buffer was also added to each pellet. Each sample was incubated at 100 °C for 10 min. The samples were subjected to SDS-PAGE (subsection 2.2.11.), one gel was stained with Coomassie stain and the other western blotted onto PVDF membranes (subsection 2.2.12.) and probed with anti-Welgevonden bovine serum (subsection 2.2.13.).

Solubilization of the 27 kDa and 31 kDa proteins of* Cowdria *with SDS or Tween 20: A frozen Kwanyanga stock infected crude cell culture extract was thawed and divided into six equal aliquots. The aliquots were centrifuged at 10 000 xg for 30 min. The supernatants were decanted and the pellets resuspended in 1 ml of one of the following solutions: a) water; b) 0,05 % Tween 20/water; c) 1 % SDS/water; d) 0,05 M HEPES containing 0,154 M NaCl, pH 7,4 (HEPES+); e) 0,05 % Tween 20, 0,05 M HEPES and 0,154 M NaCl, pH 7,4 (0,05 % Tween 20/HEPES+) and f) 1 % SDS, 0,05 M HEPES and 0,154 M NaCl, pH 7,4 (1 % SDS/HEPES+). The protein content of each solution was determined by the Bio-Rad protein assay (subsection 2.2.6.).

An aliquot of each sample representing 80 µg of protein was centrifuged at 16 000 xg for 30 min in a BHG Hermle Z230 M microfuge. The supernatants were collected individually and a 100 µl of SDS-PAGE sample buffer was added to each. A volume of 50 µl SDS-PAGE sample buffer was also added to each corresponding pellet. All the samples were then incubated at 100 °C for 10 min and subjected to SDS-PAGE (subsection 2.2.11.).

4.2.3 Two dimensional electrophoresis

Two dimensional electrophoresis (2DE) was performed as described by O'Farrel (1975) to determine the homogeneity of the 27 kDa and 31 kDa proteins, when crude infected cell culture material is subjected to SDS-PAGE.

Sample preparation: A volume of 541 µl of Welgevonden stock infected crude culture extract (prepared as described in subsection 3.2.3.), representing 2 mg protein, was centrifuged in a Hermle Z230 M microfuge at 16 000 xg, for 30 min at 4 °C. The pellet obtained was resuspended in 400 µl of a solution containing 10 mM CHAPS, 9,5 M urea and 2,5 % (w/v) DTT (CHAPS solution). This resuspended sample was incubated for 10 min at 37 °C. Hereafter 400 µl of a solution containing 4 % (w/v) Triton X-100, 9,5 M urea, 2,5 % (w/v) DTT and 4 % (v/v) ampholines (3 % pH 3,5-10; 1 % pH 5-7) (Triton X-100 solution) was added to the mixture and incubated for a further 10 min at 37 °C. The mixture was then centrifuged in a

Hermle Z230 M microfuge at 16 000 xg for 15 min at 4 °C. The supernatant, which will be referred to as 2DE soluble sample, and the corresponding pellet, which will be referred to as the 2DE insoluble sample, were aliquoted and stored at -20 °C.

First dimension: isoelectric focusing. Isoelectric focusing was performed as follows: The gel solutions contained 55 % (w/v) urea, 7 % (w/v) acrylamide, 0,1 % (w/v) bis acrylamide, 3,5 % (w/v) Triton X-100, 34 % (v/v) water, 6,5 % (v/v) Ampholines (pH range 3,5-10) and 2,2 % (v/v) Ampholines (pH range 5-7). The gels were degassed under vacuum and polymerized by the addition of 0,02 % (w/v) ammonium persulphate and 0,12 % (v/v) TEMED in 12 mm x 3 mm gel tubes. The gels were prefocused firstly for 15 min at 200 V followed by 30 min at 300 V and lastly for 30 min at 400 V with an electrophoresis constant power supply ECPS 2000/300 (Pharmacia). The anode and cathode solutions were 0,01 M H_3PO_4 0,02 M NaOH respectively. Hereafter each gel was overlayed with 15 µl 2DE soluble sample solution, corresponding to approximately 38 µg protein and the gels electrophoresed at 400 V for 15 h followed by 800 V for 60 min.

Processing of gel rods after IEF: The gels were removed from the tubes with the aid of a syringe and used for electrophoresis in the second dimension or stained with Coomassie.

Processing for the second dimension: The gel rods were immediately equilibrated after IEF for 30 min in SDS sample buffer. Hereafter the gels were ready for the second dimension.

Staining IEF gels: The gel rods were stained for 6 h in a solution containing 0,05 % (w/v) Coomassie brilliant blue, 0,09 % (w/v) $CuSO_4$, 17 % (v/v) acetic acid, 23 % (v/v) ethanol and 60 % water. Hereafter they were destained with a 10 % (v/v) acetic acid, 10 % (v/v) ethanol and 80 % water.

Second dimension: SDS-PAGE. The second dimension was performed by SDS-PAGE as described in subsection 2.2.11. The control sample (Welgevonden infected crude culture extract) and LMW standards were prepared for electrophoresis as described in 2.2.11. The top of the stacking gel was

covered with 1 ml molten 1 % (w/v) agarose. The equilibrated first dimension gel rod was immediately positioned on top of the molten agar and imbeded with more agarose. Two wells, for the control and LMW samples, were made on either side of the gel rod imbeded in the agarose. Electrophoresis was performed with an electrode buffer containing 0,02 M Tris-HCl, 0,1 M glycine and 0,06 % SDS, pH 8,3 at constant current of 20 mA for the stacking gel followed by 25 mA constant current for the separating gel. After SDS-PAGE, one gel was stained with Coomassie (subsection 2.2.11.) and the other gel western blotted onto PVDF membrane (subsection 2.2.12.) and immunostained with bovine anti-Welgevonden serum (subsection 2.2.13.).

4.2.4 Production of Monospecific anti-serum

The use of proteins electroblotted onto nitrocellulose membranes was first investigated for use as immunogen in production of monospecific anti-serum. This was found to be unsuitable due to protein losses. Alternatively proteins excised from SDS-PAGE gels were used as immunogen.

Western blotting, protein and immuno staining of Nitrocellulose membranes for use as immunogen: Welgevonden stock infected and uninfected crude cell culture extract proteins were separated by SDS-PAGE (subsection 2.2.11.). The separated proteins were western blotted onto NC membranes using a method similar to that used for PVDF membranes (subsection 2.2.12.). The only modification to the procedure was that 4 NC membranes, packed on top of one another, instead of 1 PVDF membrane, were used for each western blot. After the western blot, the membranes were either stained with dye or immunostained as described below for the detection of the 27 kDa and 31 kDa proteins.

Coomassie staining was performed as described in subsection 2.2.11. or alternatively the membranes were stained with 1 % Fast Green for 5 min and destained in a solution containing 50:40:10 v/v of methanol:water:acetic acid.

Immunostaining of *Cowdria* proteins on the NC membranes was done by modification of the Amersham protocol for hybond membranes (Amersham, 1990). The NC membranes were blocked by incubating them with 0,01 M sodium phosphate, 0,15 M NaCl, pH 7,5 (PBS) containing 0,1 % Tween 20 and 5 % Elite milk powder for 60 min or overnight (this and all subsequent steps were carried out at room temperature on an orbital shaker). Hereafter the membranes were incubated for 60 min with bovine anti-Welgevonden serum (prepared in subsection 3.2.2.) diluted 1:200 with PBS containing 1 % Elite milk powder. The membranes were washed 3 times for 5 min each with PBS containing 1 % Tween 20. This was followed by incubating the membranes for 60 min with peroxidase-conjugated rabbit anti-bovine IgG (Cappel) diluted 1:500 with PBS containing 1 % Elite milk powder. The membranes were washed 3 times for 5 min each with PBS containing 1 % Tween 20. The proteins were detected by immersing the membranes in 0,06 g chloronaphtol/20 ml cold methanol containing 0,06 ml 30 % H_2O_2/100 ml 20 mM Tris-HCl, pH 7,4 for 20 min. After the termination of substrate development by rinsing the membranes in distilled water, the membranes were dried and stored at room temperature.

Monospecific anti-serum production with proteins excised from SDS-PAGE gels: Preparative SDS-PAGE was performed with crude, Welgevonden stock infected E5 cell cultures (subsection 2.2.11.). An amount of 1900 µg crude protein was loaded per 1,5 x 120 x 160 mm gel, corresponding to 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 cell cultures and known amounts of BSA and chymotrypsinogen A. After preparative SDS-PAGE the gel was briefly rinsed in several changes of distilled water and stained with 0,3 M $CuCl_2$ for 5 min as described by Lee *et al.* 1987. Hereafter the gel was rinsed in several changes of water. The protein bands were viewed against a black background and the 27 kDa and 31 kDa protein bands excised with a scalpel. A volume of PBS was 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 between 2 syringes first connected by a 19 G needle followed by a 21 G

needle. The fragmented gel was stored at -70 °C until required for immunization.

Two rabbits were immunized with the 27 kDa protein and 2 rabbits and a goat with the 31 kDa protein. Each rabbit was immunized with either 133 µg of the 27 kDa protein or 114 µg of the 31 kDa protein per immunization and the goat was immunized with 266 µg 31 kDa protein per immunization according to Table 4.1. All inoculations were administered subcutaneous (s.c.) and intramuscular (i.m.). The serum collected at intervals indicated in Table 4.1 were evaluated and titers determined by immunostaining using Welgevonden stock infected crude cell culture extracts as antigen in the western blots (Subsection 2.2.12. and 2.2.13.).

TABLE 4.1. Protocol for production of monospecific anti-serum in rabbits and a goat.

Day	Inoculum/bleed
0	bleed antigen/FCA (1:1)
14	bleed
28	antigen/FIA (1:1)
42	bleed

Day	Inoculum/bleed
56	antigen
70	bleed
84	antigen
96	bleed

Determination of whether the 31 kDa protein of* Cowdria *is protective towards infection with heartwater: The goat that was immunized with the 31 kDa protein of *Cowdria* as described above, was challenged 48 weeks after the first inoculation with 5 ml Welgevonden stock blood stabilate. A heartwater naive goat and a goat that was inoculated with 5 ml Welgevonden stock blood stabilate (subsection 3.2.2.), was likewise challenged. Daily rectal temperatures of the goats were monitored. They were not treated when heartwater symptoms developed and the cause of death was determined by post mortem investigations.

4.2.5 Amino acid analysis

Welgevonden stock infected crude cell culture extract was subjected to SDS-PAGE (subsection 2.2.11.), blotted onto a PVDF membrane and stained with Coomassie (subsection 2.2.12.). A strip of approximately 10 x 1 mm was excised from the membrane at the position of the 31 kDa protein. A piece of membrane of equal dimension was cut from the same sheet of membrane directly next to the protein, to serve as a blank control.

Amino acid analysis by HCl hydrolysis: Amino acid analysis was performed according to the PICO-TAG method of Immobilon Tech, 1989. The data obtained was statistically analysed according to a Parameters of Varieties program written by Dr Van Ark, computer section, O.V.I., Onderstepoort. The bands were placed in pyrolyzed hydrolysis tubes of 6 x 50 mm. The sample tubes were placed into a larger glass vessel containing 200 µl of 6 N HCl containing 7 % thioglycolic acid. The container was sealed and evacuated after flushing with N_2 gas and placed in an oven at 110 °C for 24 h.

Extraction: After hydrolysis, 50 µl of 30 % methanol in 0,1 N HCl was added to the tubes containing the samples and vortexed repeatedly over a period of 30 min. The extraction was repeated twice. Hereafter all the samples were dryed by adding 10 µl of a solution consisting of 2:2:1 (ethanol:water:triethylamine) to each. After mixing gently the samples were evaporated under vacuum and derivatized.

Derivatization: Standard PITC derivatization and reverse phase HPLC analysis were performed according to the Waters Product Bulletin (1984). A volume of 20 µl derivatization reagent consisting of 7:1:1:1 (v/v) ethanol:triethylamine:water:PITC was added to each dried sample. The mixture was mixed by vortexing for a few seconds. The tubes were placed in a reaction vial and left at room temperature for 20 min thereafter it was placed under vacuum and allowed to dry thoroughly. The samples were removed and subjected to HPLC.

Separation of amino acid derivatives: A volume of 200 µl and 100 µl of the PICO-TAG diluent was added to the amino acid standard mixture and

samples respectively and 8 µl of the standard mixture and 20 µl of each sample was injected for HPLC analysis. A 3,9 mm x 15 cm Pico-Tag column was used to separate the PTC amino acid derivatives. The following buffer system was used: buffer A - 0,14 M sodium acetate pH 5,7 and buffer B - 60 % acetonitrile. The HPLC was performed with the buffer gradient as set out in Table 4.2. and the amino acid derivatives monitored at 254 nm.

TABLE 4.2. Amino acid analysis HPLC gradient profile.

min.	flow ml/min	% A	% B
0	1	90	10
10	1	49	51
10,5	1	0	100
12,7	1,5	0	100
13,2	1,5	90	10
20,7	1,5	90	10
21	1	injection of sample	
repeat			

Tryptophan analysis by MSA hydrolysis: MSA hydrolysis was performed according to the Millipore instruction manual on duplicate samples to determine the tryptophan content of the 31 kDa protein. Duplicate bands were placed in pyrolyzed hydrolysis tubes (6 x 50 mm) and 20 µl of 4 M MSA containing 0,2 % (w/v) tryptamine HCl was added to each sample. The sample tubes were placed into a larger reaction vial containing 100 µl of water and evacuated. Hydrolysis was then carried out at 110°C for 24 h. After completion of the hydrolysis, the vial was cooled to room temperature and 22 µl of 4 M KOH added to each sample tube and dried under vacuum. Extraction, derivatization and identification of the amino acids were performed as described above for HCl hydrolysis.

Cysteine analysis by performic acid oxidation: Performic acid hydrolysis was performed according to the Millipore instruction manual on duplicate samples to obtain the cysteine composition of the 31 kDa protein. A solution of 1:9 (30 % H_2O_2: 88 % HCOOH) was vortexed for 30 min at room temperature before being placed together with the samples, on ice for 30 min. Thereafter 25 µl of the solution was placed into each sample tube,

sealed and incubated at 4°C for 16 h. Hereafter 5 µl octanol was added to each sample tube and, whilst mixing, 8 µl of 48 % HBr was added slowly. The samples were then incubated at 0°C for 30 min and evaporated under nitrogen gas. Hydrolysis, extraction, derivatization and identification were performed as described above for HCl hydrolysis.

4.2.6 Amino acid sequencing

Manual gas phase amino acid sequencing.

The 31 kDa protein obtained as described below was subjected to manual gas phase amino acid sequencing (Brandt & Frank, 1988). horse apomyoglobin, spotted onto PVDF membrane, was sequenced beforehand to test the method.

Purification of the 31 kDa protein by SDS-PAGE for amino acid sequencing: SDS-PAGE was performed with the pH 7,28 MZE 3328.IV buffer system (Moos *et al.*, 1988). Gels of 1,5 x 120 x 160 mm were prepared. The stacking gel contained 4% acrylamide, 0,1% bisacrylamide, 0,1% SDS and 25% gel buffer (gel buffer: 0,5 M BIS-TRIS, 0,1 M thioglycolic acid and 0,2 M HCl, pH 6,61). The separating gel contained 12% acrylamide, 0,3% bisacrylamide, 0,1% SDS and 25% gel buffer. Each gel was polymerized by the addition of ammonium persulphate and TEMED at final concentrations of 0,05% and 0,1% respectively in the stacking gel and 0,05% each in the separating gel. Crude resuspended Welgevonden stock infected cell cultures and low molecular weight standard samples were dissolved in 12,5% gel buffer, 16% glycerol, 2% SDS, 2,5% DTT and 0,001% bromophenol blue by heating at 100°C for 10 min. Electrophoresis was performed with the following electrode buffers: cathode electrode buffer (upper chamber) = 0,04 M TES, 0,1 M BIS-TRIS and 0,1% SDS, pH 7,25 and the anode electrode buffer (lower chamber) = 0.06 M BIS-TRIS, 0,05 M HCl, pH 5,9. The gels were western blotted onto PVDF membranes (subsection 2.2.12.) and stained with Coomassie (subsection 2.2.11.).

Manual amino acid sequencing: Glass filters (Whatman GF/C) were prepared by soaking in TFA for 2 h and washing 4 times with butylchloride. The filters were dried *in vacuo* and placed at the bottom of a conical glass

column as a basis for the PVDF membrane. Alternatively a scintered conical glass column was used. A volume of 15 µl 5 % PITC in heptane was added evenly to completely wet the PVDF membrane. The glass column and a tube containing 1 ml 5 % TEA was placed into a small desiccator, purged with N_2 gas, evacuated for 1 sec and incubated at 50°C for 30 min. The glass column containing the membrane was dried under vacuum for 5 min and then washed twice with 200 µl heptane, twice with 200 µl ethyl acetate and once with 200 µl butylchloride before being dried under vacuum for 5 min. The glass column was placed in the small desiccator together with a tube containing 0,5 ml TFA, purged with N_2 gas, evacuated for 30 sec and incubated for 15 min at 50°C. The tube containing the TFA was removed before evacuating for 5 min. The ATZ derivative was extracted twice from the PVDF membrane with 200 µl butylchloride, into an Eppendorf tube. Hereafter the PVDF membrane was dried *in vacuo* stored at 4°C or the next coupling step performed immediately. The ATZ extract was evaporated under a stream of N_2, 50 µl 20 % TFA added, purged with N_2 gas and incubated for 10 min at 80°C. The TFA was evaporated under a stream of N_2 gas and stored at 4°C until the next day for identification.

The PTH-amino acids were dissolved in 20 µl methanol and identified by HPLC on a C_{18}-PTH ultrasphere 4,6 mm x 25 cm (Beckman) column using the following buffers (Column Data Sheet, Beckman): Buffer A = 5,8 mM sodium acetate, pH 5,1 and Buffer B = 10 % tetrahydrofuran in acetonitrile with a gradient of 0-40 % Buffer A over 20 min followed by 40 % Buffer A for 10 min and 40-0 % Buffer A for 7 min at a flow speed 1,3 ml/min in a Beckman System Gold.

Automated amino acid sequencing.

Automated amino acid sequencing was performed on the following samples: 31 kDa protein eluted from electroblotted PVDF membranes or SDS-PAGE gels, CNBr peptide fragments of electroeluted 31 kDa protein and horse apomyoglobin electroeluted from SDS-PAGE gels.

Elution of the 31 kDa protein electroblotted onto PVDF membranes: The elution was performed as described by Szewczyk and Summers, 1988. SDS-PAGE separated (subsection 4.2.6.) crude, Welgevonden stock infected cell

cultures were electroblotted onto PVDF membranes (subsection 2.2.12.). The membranes were either stained with Coomassie (subsection 2.2.11.) or 0,2 % (w/v) amido black 10B for 20 min. The band containing the 31 kDa protein was excised from the membrane and 200 µl per cm^2 membrane of 50 mM Tris-HCl, pH 9,0 containing 2 % SDS and 1 % Triton X-100 was added, vortexed and centrifuged for 20 min at 20 000 xg. The supernatant was collected and 4 times the volume of acetone added and incubated overnight at -20°C. The protein was pelleted by centrifuging at 20 000 xg for 20 min thereafter the supernatant was removed and the pellet stored at -20°C.

A portion of the pellet was checked for purity by SDS-PAGE (subsection 2.2.11.). The gel was stained with Coomassie or western blotted onto PVDF membranes (subsection 2.2.12.) and immunostained (subsection 2.2.13.) with goat anti-Welgevonden serum prepared as described in subsection 3.2.2. The rest of the sample was used for automated amino acid sequencing.

Electroelution of the 31 kDa protein from SDS-PAGE gels: The 31 kDa protein was alternatively electroeluted from a Coomassie stained SDS-PAGE gel. Horse apomyoglobin was also electroeluted from the same Commassie stained SDS-PAGE gel and used to determine whether the purification procedure leads to N-terminaly blocked proteins.

The protein bands were excised from the Coomassie stained SDS-PAGE gel (subsection 4.2.6.), cut into small pieces and soaked in 0,5 % (w/v) C-TAB containing 10 % 2-merkaptoethylammonium and 0,45 M acetic acid for 60 min at room temperature. The equilibrated gel pieces were 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. After the current was reversed for 30 sec the eluted sample was removed from the chamber and freeze dried. Electroelution was continued for a further 120 min and the eluted sample collected into a clean Eppendorf tube and freeze dried. A volume of 200 µl methanol was added to each freeze dried sample followed by 800 µl cold acetone. This solution was incubated at

-20°C for 30 min and centrifuged at 11 000 xg for 6 min. The supernatant was removed and the pellet washed with 800 µl cold acetone and centrifuged at 10 000 xg for 6 min. The supernatant was removed and the pellet dried under vacuum. The purity of the sample was determined by SDS-PAGE (subsection 2.2.11.) before amino acid sequencing or CNBr cleavage.

CNBr cleavage of the 31 kDa protein and high performance liquid chromatography of the CNBr cleaved peptides: The 31 kDa protein was electroeluted from a Coomassie stained gel as described above and CNBr cleavage performed as described by Matsudaira, 1989b. A volume of 50 µl 70 % formic acid was added to the electroeluted and dried 31 kDa protein and 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 (subsection 2.2.11.) and the rest subjected to HPLC.

The CNBr cleaved peptides were separated on a narrow-bore VIDAC C4 column 2,5 x 400 mm, reverse-phase HPLC system (Millipore). The sample was dissolved in 100 µl TFA and HPLC was performed 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. Automated amino acid sequencing was performed on selected peptides.

Automated amino acid sequencing: Sequence analysis was performed in a gas phase sequencer constructed as outlined by Hewick *et al.*, 1981, and slightly modified as described by Brandt *et al.*, 1984. The converted PTH amino acids were identified by isocratic HPLC system in 3 x 250 mm 3 µ Lichrospher C_{18} (Bishoff) column as described by Lottspeich, 1985.

4.2.7 Glycan assay

Welgevonden stock infected and uninfected cell cultures were subjected to SDS-PAGE (subsection 2.2.11.), western blotted onto PVDF membranes (subsection 2.2.12.) and assayed for carbohydrates by an enzyme

immunoassay according to the protocol described in the Glycan kit (Boehringer Mannheim).

All incubations were performed by gentle agitation at room temperature, except for colour development which was done without shaking. The membranes were wetted with methanol, washed in 50 mM KH_2PO_4 containing 150 mM NaCl, pH 6,5 (buffer A) and incubated in 100 mM sodium acetate buffer, pH 5,5 containing 10 mM sodium metaperiodate, for 20 min. The membranes were then washed 3 times for 10 min with buffer A and incubated with 25 µl digoxigenin-succinyl-ε-amidocaproic acid hydrazide dissolved in 25 ml 100 mM sodium acetate buffer pH 5,5, for 60 min. After washing 3 times for 10 min with buffer A and thereafter incubated for at least 30 min in 0,5 % blocking solution dissolved in 0,05 M Tris-HCl; 0,15 M NaCl, pH 7,5. Hereafter the membranes were washed 3 times for 10 min each with buffer A and incubated in 10 µl sheep anti-digoxigenin alkaline phosphatase conjugate per 10 ml buffer A for 60 min. After washing 3 times 10 min with buffer A they were stained with staining solution: 10 ml 0,1 M Tris-HCl, pH 9,5; 0,05 M $MgCl_2$; 0,1 M NaCl; 37,5 µl 5-bromo-4-chloro-3-indolyl- phosphate; 50 µl nitroblue tetrazolium chloride solution. The staining solution was mixed just before use and the membranes immersed into the staining solution without shaking. The colour development was stopped after 15 min by rinsing the membranes in several changes of distilled water, air dried and stored at -20°C.

4.2.8 Isoelectric focusing

Denaturing IEF: Denaturing IEF was performed according to Giulian *et al.*, 1984. Welgevonden stock infected crude cell culture extract, the 31 kDa protein PVDF eluted (subsection 4.2.6.) 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) TEMED and 0.04 % (w/v) ammonium persulphate were cast at 37°C. Prefocusing was performed using 0,02 M

NaOH at the cathode (upper chamber) and 0,02 M CH_3COOH at the anode (lower chamber), at 200 V for 15 min followed by 300 V for 30 min and 400 V for 30 min, at 10°C. The samples were then loaded into preformed wells and electrophoresis carried out at 400 V for 16 h followed by 800 V for 1 h. The gel was either stained with Coomassie or western blotted onto PVDF membranes and immunostained as described below.

Coomassie staining was carried out as follows: The gel was submerged into 20 % (w/v) trichloroacetic acid solution and allowed to fix for 30 min and then incubated in ethanol:acetic acid:water (33:10:57) with 0,25 % (w/v) SDS for 30 min and rinsed 4 times with ethanol:acetic acid:water (33:10:57) for 15 min. The gel was stained for 120 min in ethanol:acetic acid:water (33:10:57) containing 0,05 % (w/v) Coomassie R-250 followed by destaining in several changes of ethanol:acetic acid: water (33:10:57). The gel was then dried in a Hoefer scientific instrument slab gel drier. The isoelectric points of the separated samples were determined from a standard curve of pI versus distance of the zones, measured from the cathode.

Western blotting of the IEF gel was performed as followes: A piece of Whatman filter paper was placed on a glass plate allowing both ends of the filter paper to be submerged in 10 mM CAPS buffer, pH 9, blotting buffer. This was sequentially overlayed with the IEF gel, PVDF membrane, 3 layers of Whatman filter paper, several layers of tissue paper and a weight. After incubating for 20 h at room temperature the membrane was subjected to immunostaining as described in subsection 2.2.13. with goat anti-*Cowdria* 31 kDa protein monospecific serum diluted 1:5000 (prepared as described in subsection 4.2.4.).

Native IEF: The 31 kDa protein was eluted from electroblotted PVDF membranes as described in subsection 4.2.6. This 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 was performed in a PhastSystem™ using PhastGel IEF 3-9, as described by the Pharmacia instruction manual (Pharmacia).

Staining was performed in the PhastSystem as followes: The gel was fixed with 20 % (w/v) trichloroacetic acid for 5 min at 20 °C and washed 2 times 2 min each at 20 °C with 30 % methanol and 10 % acetic acid in distilled water (3:1:6). Hereafter the gel was stained in a solution containing 0,02 % Coomassie R 250, 30 % methanol, 10 % acetic acid in distilled water and 0,1 % (w/v) $CuSO_4$ for 10 min at 50 °C. This was followed by destaining with 30 % methanol and 10 % acetic acid in distilled water (3:1:6) for 10 min at 50°C.

4.3 RESULTS

4.3.1 Solubilization of *Cowdria* proteins with detergents

Solubilization of the 27 kDa and 31 kDa proteins of Cowdria with Sarkosyl detergent: Treatment of Welgevonden stock infected crude cell culture extracts with various percentages of Sarkosyl detergent did not result in selective solubilization of the 27 kDa and 31 kDa proteins of *Cowdria*. This conclusion could be made as both the Coomassie stained SDS-PAGE gel and the immunoblot show that the 27 kDa and 31 kDa proteins appear together with the majority of proteins, either in the pellet or soluble supernatant (Fig 4.1.).

Welgevonden stock infected crude cell culture extracts were also treated with SDS-PAGE sample buffer (subsection 2.2.11.) which did not contain DTT (-DTT), to determine whether the 27 kDa and the 31 kDa proteins are single polypeptide chains or not. The 27 and 31 kDa proteins of *Cowdria* were detected in the immunoblot irrespective of whether DTT was present or not (Fig 4.1.).

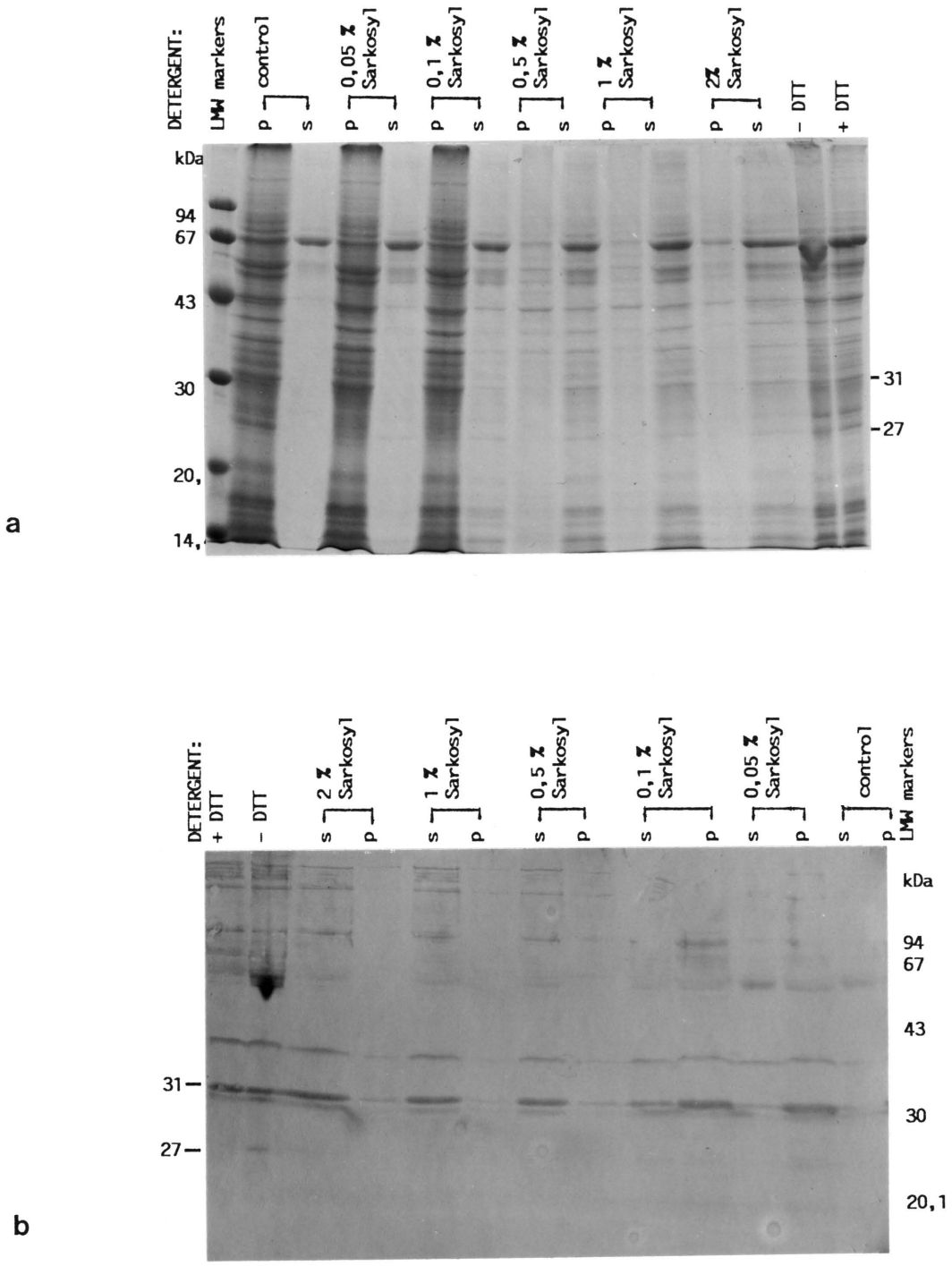


FIGURE 4.1. SDS-PAGE protein patterns of Welgevonden stock infected crude cell culture extracts after Sarkosyl detergent treatment. a) Coomassie stained gel and b) Western blot probed with anti-Welgevonden bovine serum. p = pellet; s = supernatant.

Selective solubilization of the 27 kDa and 31 kDa proteins of Cowdria with SDS or Tween 20: The 27 kDa and 31 kDa protein could also not be selectively solubilized by either SDS or Tween 20. *Cowdria* infected crude cell culture extracts were solubilized 93 % with 1 % SDS/Water and 73 % with 1 % SDS/HEPES+. Treatment with Water, HEPES+, Tween 20/Water or Tween 20/HEPES+ only resulted in an average of 45 % solubilization of the proteins (Fig 4.2. and Table 4.3.). The percentage of proteins solubilized was determined by dividing the number of protein bands in the Coomassie stained SDS-PAGE gel of the supernatant by the number of protein bands in the pellet and multiplying by 100.

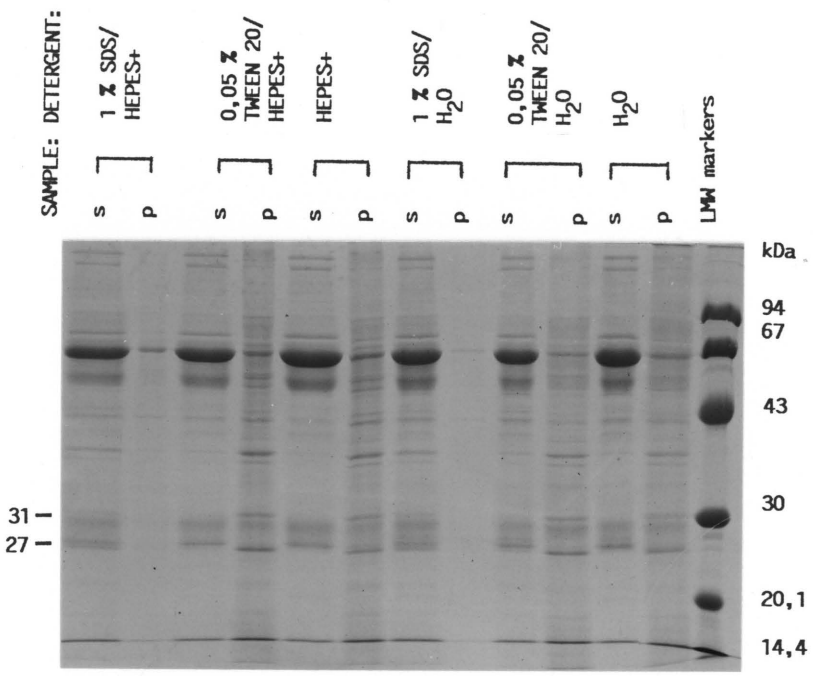


FIGURE 4.2. Coomassie stained SDS-PAGE protein patterns of various detergent treatments of Kwanyanga stock infected crude cell culture extracts. s = supernatant. p = pellet.

TABLE 4.3. Solutions tested for solubilization of Kwanyanga stock infected crude cell culture extracts and the percentage of proteins solubilized.

Solubilization solution	% protein solubilized
Water	45
0,05 % Tween 20/water	49
1 % SDS/water	93
HEPES+	42
0,05 % Tween 20/HEPES+	44
1 % SDS/HEPES+	71

4.3.2 Two dimensional electrophoresis

When Welgevonden stock infected crude cell cultures were subjected to 2D electrophoresis, Coomassie staining of the IEF gel revealed 20 protein bands while Coomassie staining of the SDS-PAGE gel showed 15 light spots and only one, very prominent, spot at 31 kDa (Fig 4.3.). The 20,1 kDa LMW marker and therefore other proteins in this molecular weight range, migrated in the dye front. Immunostaining of the second dimension gel detected only one very prominent spot at 31 kDa and nothing at the 27 kDa position (Fig 4.3.).

Immunoblot analysis of SDS-PAGE separated 2DE insoluble and soluble samples detected the 31 kDa protein in the pellet, supernatant and control sample while the 27 kDa protein was only detected in the supernatant and control (results not shown).

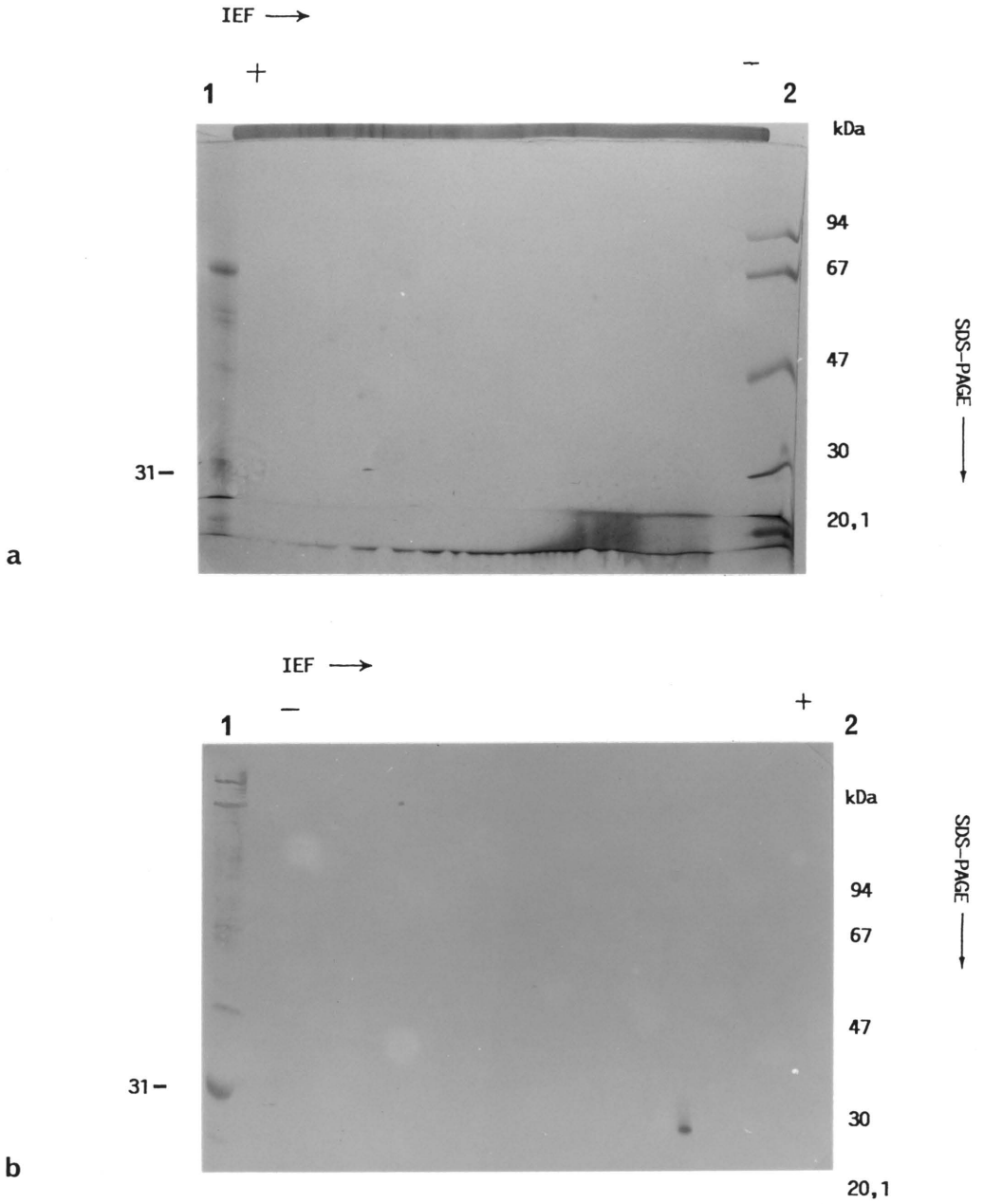


FIGURE 4.3. Two dimensional electrophoresis (IEF first dimension and SDS-PAGE second dimension) protein profiles of Welgevonden infected crude cell culture extracts. a) Coomassie stained gel and b) Western blot probed with bovine anti-Welgevonden serum. Lane 1 and 2, Low molecular weight standard and Welgevonden infected crude culture extracts respectively subjected to one dimension SDS-PAGE.

4.3.3 Production of monospecific anti-serum

For production of monospecific anti-serum against the 27 kDa and 31 kDa proteins electroblotting SDS-PAGE separated proteins onto nitrocellulose membranes was initially investigated. Coomassie staining of the electroblotted NC membranes (four consecutive membranes) detected the highest concentration of proteins on the first membrane, next to the gel, with a gradual decrease in protein content from the second to the fourth membrane. When the fourth membrane was coloured with Fast Green better distinction of bands with little background was observed. Coomassie staining resulted in high background and therefore difficulty in detecting bands. Immunoblotting of membranes 1-4 detected only light bands in the high molecular weight region on membrane 1 (results not shown).

After western blotting, Coomassie staining of the SDS-PAGE gel showed that a large quantity of proteins were still in the gel. This method therefore resulted in large losses as protein either remained in the gel or passed through at least four NC membranes during electroblotting. Therefore it was decided to excise the required protein band directly out of the SDS-PAGE gel for use as immunogen.

To ensure that the correct dosage of protein was administered to the rabbits and goat a standard curve for Coomassie stained proteins in a SDS-PAGE gel was prepared. The protein content of the 27 kDa and 31 kDa bands in the SDS-PAGE gel were 4 and 3,5 µg, respectively, when determined from a BSA standard curve and 10 and 6 µg, respectively with chymotripsinogen A as standard. The the average protein content of the 27 kDa and 31 kDa bands, when 100 µg of *Cowdria* infected crude culture extract was subjected to SDS-PAGE, was determined to be 7 and 6 µg, respectively.

Two rabbits were inoculated with the 27 kDa protein. Serum from one of these rabbits remained negative even after three inoculations and only recognised 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 *Cowdria* after the second

administration. After the third immunization the 27 kDa and the 31 kDa proteins were recognized (Table 4.4. & Fig. 4.4.).

Two rabbits were inoculated with the 31 kDa protein. Serum obtained from both rabbits recognised the 27 kDa and the 31 kDa proteins after the second, third and fourth inoculation (Table 4.4. & Fig. 4.4.).

One goat was inoculated with the 31 kDa protein. The serum recognised 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 4.4. & Fig. 4.5.). Other *Cowdria* proteins were also recognized by the serum and only a cell culture protein in the region just above 31 kDa was recognised at a dilution of ≤ 1:200. The IFA assay titer of the goat serum collected on day 98 was determined to be 1:5100.

TABLE 4.4. Immunoblot titers obtained from monospecific anti-serum prepared in rabbits and a goat. Only serum from one of the duplicate animals was tested. * - inoculated with the 27 kDa protein and # - inoculated with the 31 kDa protein.

Day Immunized	Day bled	Reciprocal of immunoblot titer					
		Rabbit 27 kDa *		Rabbit 31 kDa #		Goat 31 kDa #	
		27 kDa	31 kDa	27 kDa	31 kDa	27 kDa	31 kDa
0	14	neg	neg	neg	neg	1000	125 000
28	42	neg	40	200	1000	5000	125 000
56	70	40	200	200	1000	5000	125 000
84	98	200	1000	200	1000	1000	125 000

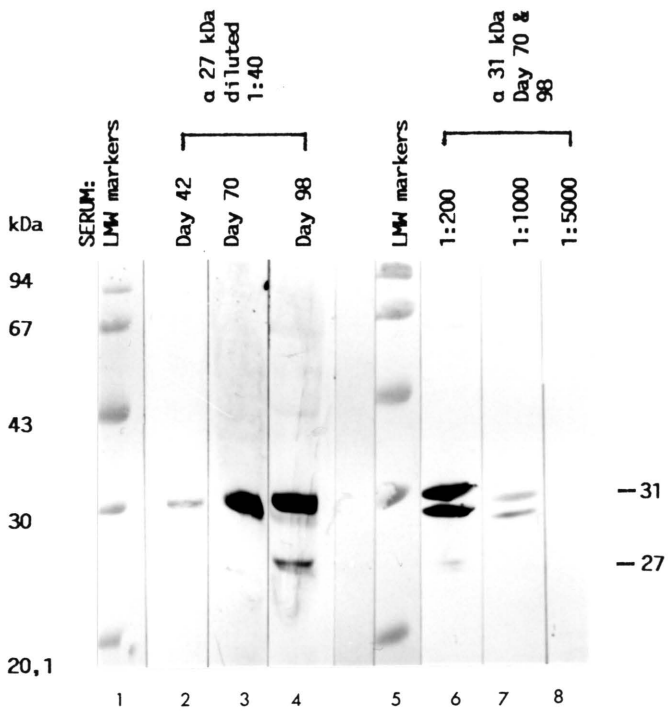


FIGURE 4.4. Western blot analysis of resuspended, crude, *Cowdria* infected culture extracts probed with rabbit anti-serum raised against the 27 kDa or 31 kDa proteins of *Cowdria*. Lanes 6, 7 & 8, also representative of days 70 and 98 anti-31 kDa serum.

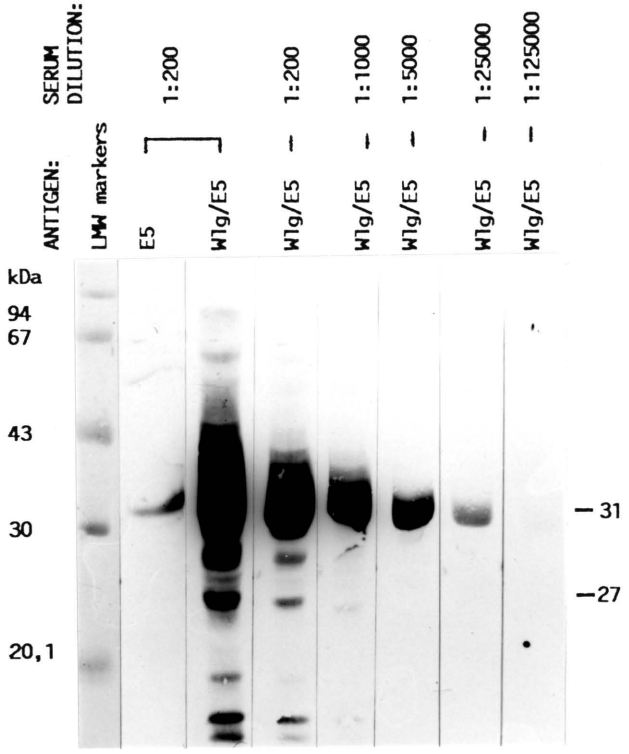


FIGURE 4.5. Western blot analysis of resuspended, crude, *Cowdria* infected and uninfected cell culture extracts probed with goat anti-serum raised against the 31 kDa protein of *Cowdria*.

Determination of whether the 31 kDa protein of* Cowdria *is protective towards infection with heartwater: Both the goat that was immunized with the 31 kDa protein of *Cowdria* and the naive control goat developed a febrile response after challenge lasting for 5 and 6 days respectively and they subsequently died (Fig. 4.6.). Post mortem investigations confirmed heartwater as the cause of death. The goat previously immunized with Welgevonden stock blood stabilate did not develop a febrile response after challenge and was immune to heartwater.

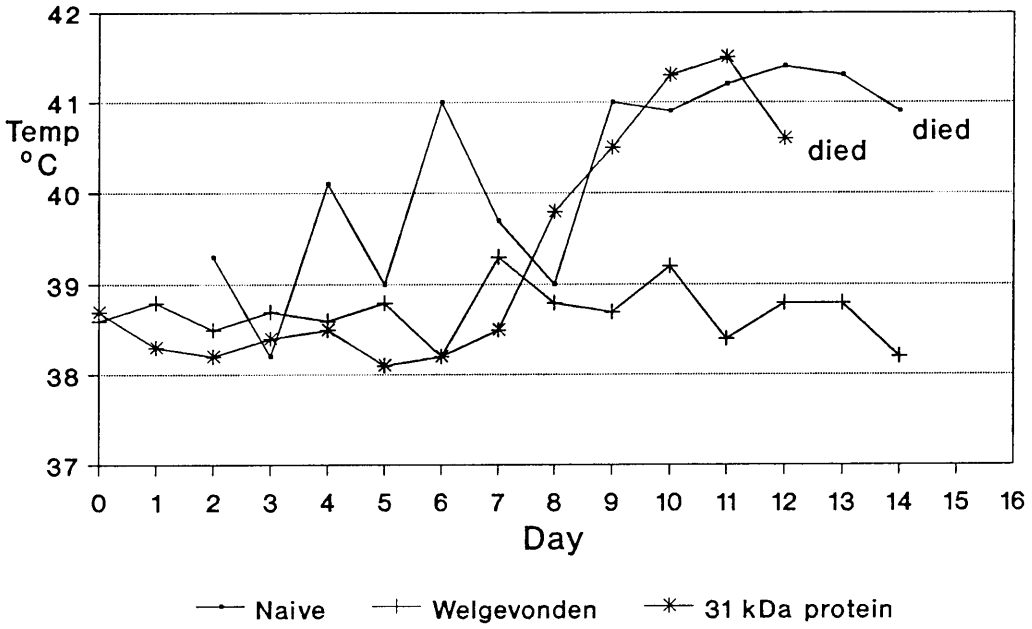


FIGURE 4.6. Daily rectal temperatures recorded of goats challenged (previously exposed to Welgevonden stock or the 31 kDa protein) or primarily infected (naive) with Welgevonden stock infective blood stabilate.

4.3.4 Amino acid analysis

The amino acid composition of western blotted 31 kDa protein of *Cowdria* is tabulated below (Table 4.5.). 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.

TABLE 4.5. Amino acid composition (residues per mole) of the 31 kDa protein of *Cowdria* 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

4.3.5 Amino acid sequencing

Manual amino acid sequencing was first performed on 6000 pmol horse apomyoglobin spotted onto a PVDF membrane. Despite background peaks and low amino acid recoveries the first 12 amino acids were correctly identified (Table 4.6.). As the amount of the gly and leu (1st and 2nd amino acid of apomyoglobin) was not known neither the initial yield or repetitive yield could be determined. Since preliminary amino acid analysis trials revealed that electroblotting of the PVDF membrane removed background amino acids the 31 kDa protein was electroblotted onto PVDF membranes and sequenced manually. With either approximately 100 pmol or 1000 pmol of the 31 kDa protein no sequence concensus could be found (Table 4.6.). Two factors were thought to contribute to this; either the concentration of the 31 kDa protein was too low for manual amino acid sequencing or the N-terminal of the protein was blocked.

TABLE 4.6. Manual amino acid sequencing of apomyoglobin and the 31 kDa protein of *Cowdria*.

Amino acid residue no.	apomyoglobin		31 kDa protein of *C. ruminantium*					
	6000 pmol spotted onto PVDF		100 pmol electro-blotted onto PVDF		1000 pmol electroblotted onto PVDF			
	a.a.	pmol	a.a.	pmol	a.a.	pmol	a.a.	pmol
1.	gly		asn	62	ser	226	asn	245
2.	leu		arg		met	1882	glu	260
3.	ser	173	?		gln	812	thr	109
4.	asp	438	asn pro		?		cys	
5.	gly	1873	met arg		tyr	196	gln	131
6.	glu	2606			pro	59	ser	85
7.	trp				ser	59		
8.	gln	90			arg	429		
9.	gln	175			asn gly tyr			
10.	val	233			arg	625		
11.	leu	144			met	381		
12.	asn				ser			

Automated amino acid sequencing is far more sensitive than manual sequencing and was done on approximately 300 pmol of the 31 kDa protein eluted from PVDF membranes. SDS-PAGE and immunoblot analysis, with goat anti-Welgevonden polyclonal serum, of the eluted 31 kDa protein showed a single polypeptide band at 31 kDa (Fig 4.7.). An inconsistent amino acid sequence with amino acid recoveries in the 10 pmol range was obtained.

To determine if N-terminal blockage occurred during the isolation, bands containing approximately 4000 pmol horse apomyoglobin or 2000 pmol of the 31 kDa protein, both excised from Coomassie stained SDS-PAGE gels, were electroeluted and subjected to on-line automated sequencing. The first 3 amino acids of horse apomyoglobin were identified with recoveries in the 200 pmol range. No clear amino acid sequence could be obtained for the 31 kDa protein. Therefore it was concluded that the protein was N-terminally blocked. Since the partial sequence of horse apomyoglobin could be

obtained after electrophoresis and electroelution, isolated identically to the 31 kDa protein, it can be reasoned that the 31 kDa protein did not become N-terminaly blocked during the isolation procedures.

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 excised from SDS-PAGE gels. Re-electrophoresis of the electroeluted protein showed a single polypeptide in the region of 31 kDa with relatively low protein recovery (Fig 4.7.). Purification of the resultant CNBr peptides by HPLC showed several peaks (Fig 4.8.). 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 very small in comparison to peak heights of 10 µg standard histone peptides. Automated amino acid sequencing was performed on the indicated selected purified peptide (Fig 4.8.). 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

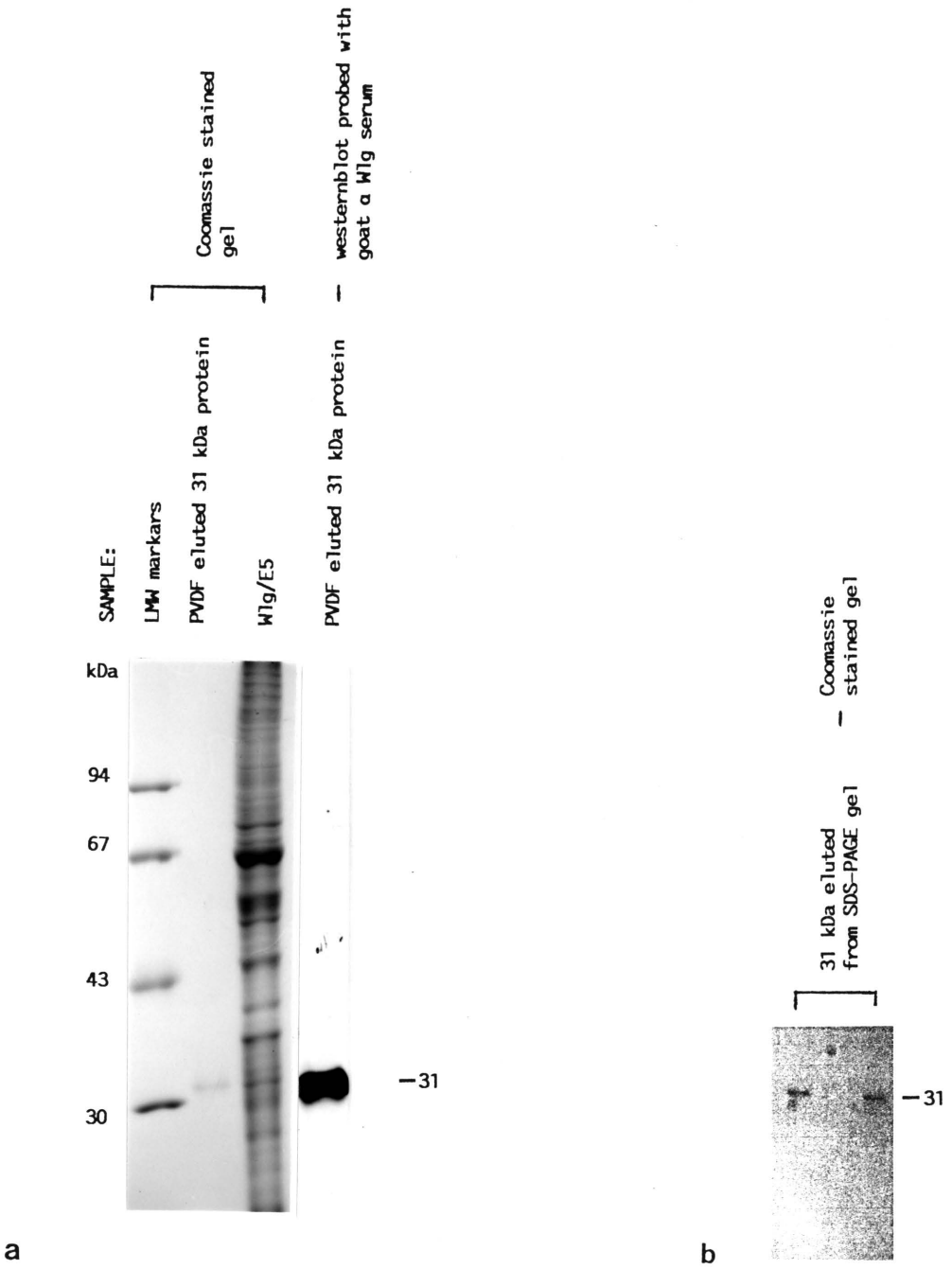


FIGURE 4.7. SDS-PAGE analysis of a) PVDF eluted and b) SDS-PAGE electroeluted 31 kDa protein of *Cowdria*. b) is a photo print.

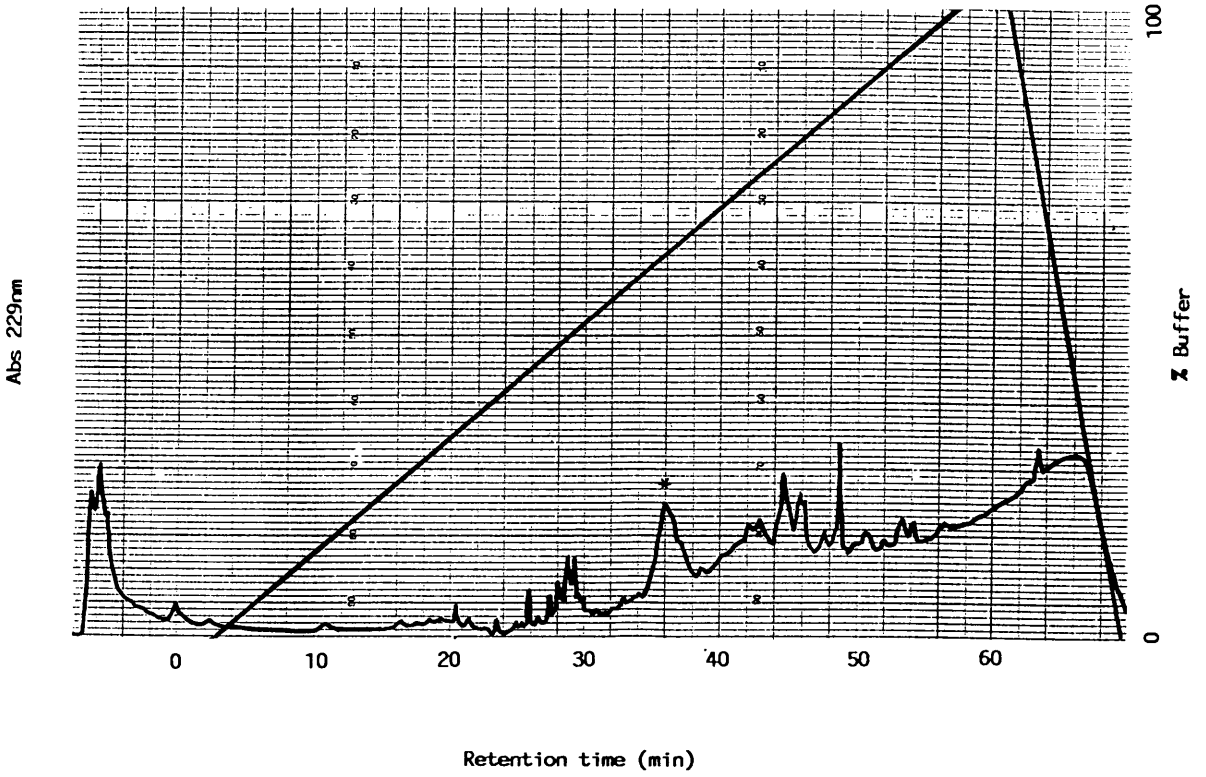


FIGURE 4.8. HPLC chromatogram of peptides released after CNBr cleavage of the 31 kDa protein of *Cowdria*. * - peptide sequenced. Buffer = 0,08% TFA in 70% acetonitrile.

4.3.6 Glycan assay

The glycan enzyme immunoassay revealed that the 29 kDa and 31 kDa proteins of *Cowdria* are not glycoconjugated proteins (Fig 4.9.). No conclusion could be made as to whether the 27 kDa or other proteins of *Cowdria* are glycoproteins or not as the patterns of infected and uninfected cell cultures were identical.

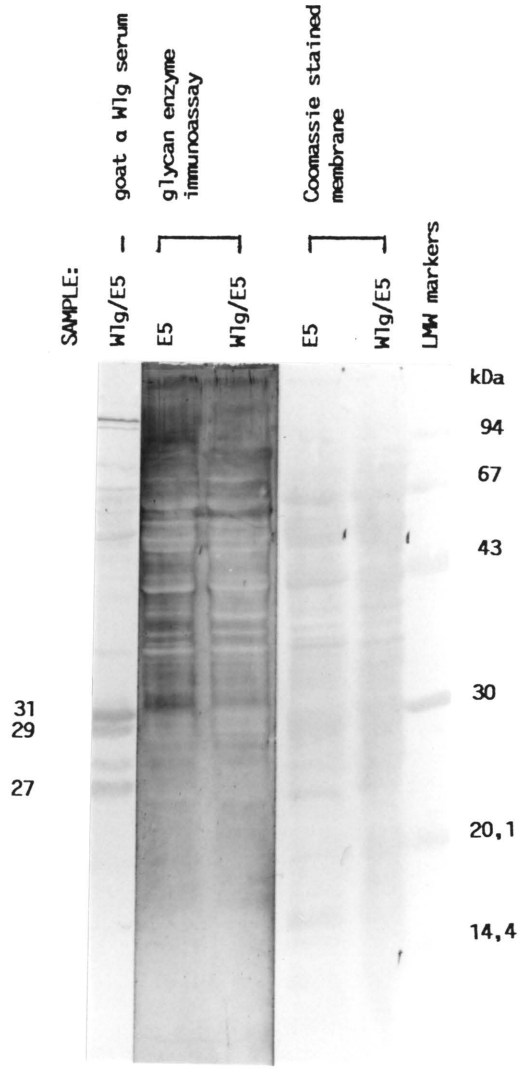


FIGURE 4.9. Glycan enzyme immunoassay, immunostaining and Coomassie staining of crude, Welgevonden stock infected and uninfected cell cultures.

4.3.7 Isoelectric focusing

Denaturing IEF: The PVDF eluted 31 kDa protein used in the IEF was not homogeneous. Two protein bands were observed when the eluted protein was run on SDS-PAGE and IEF. This was due to difficulties experienced when cutting out the protein from membranes. However, an immunoblot of the denaturing IEF gel, with the monospecific goat anti-31 kDa serum, detected two very light bands in the crude extract. One band corresponded to the PVDF eluted 31 kDa protein band position on the IEF gel. As the monospecific anti-serum detects both the 27 kDa and the 31 kDa proteins the other band must be the 27 kDa protein. Therefore the isoelectric points of the 27 kDa and 31 kDa proteins were determined to be 5,2 and 5,7 respectively, under denaturing conditions (Fig. 4.10.).

Native IEF: The isoelectric point of the 31 kDa protein, under native conditions, could not be determined because the protein precipitated regardless of the position of application.

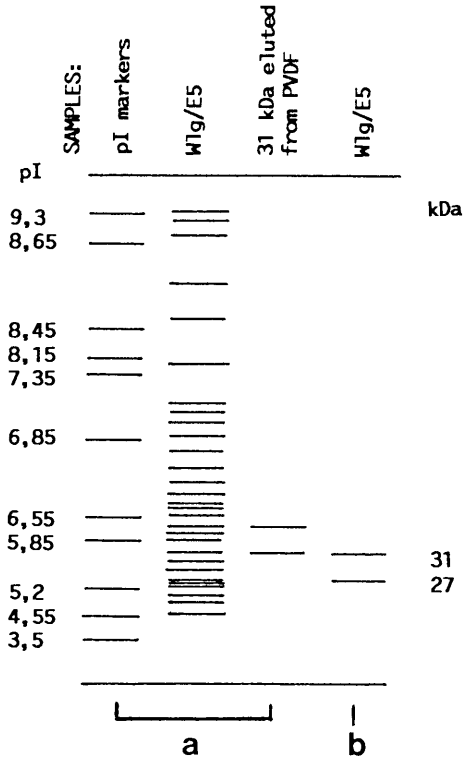


FIGURE 4.10. Representation of analytical denaturing isoelectric focusing of the 31 kDa protein of *Cowdria*. a) Coomassie stained gel and b) Western blot probed with anti-31 kDa protein goat serum.

4.4 DISCUSSION

Selective solubilization of specifically the 27 kDa and 31 kDa proteins of *Cowdria* could not be obtained by Sarkosyl, SDS or Tween 20 detergent treatment of heartwater infected crude cell culture extracts. However, 1 % SDS treatment of *Cowdria* infected crude cell culture extracts resulted in 93 % solubilization of all proteins. SDS-PAGE sample buffer should therefore sufficiently solubilize *Cowdria* proteins for further purification by SDS-PAGE, as 2 % SDS is used. Solubilization with SDS-PAGE sample buffer, with or without DTT, resulted in detection of both the 27 kDa and 31 kDa proteins by immunoblots. Therefore both these proteins are single polypeptide chains.

Two dimensional electrophoresis of Welgevonden infected crude cell culture extracts revealed that the 31 kDa protein is a major component and probably not contaminated with other proteins of different pI but the same molecular weight. The homogeneity of the 31 kDa protein is relative as the impurities may be present at too low concentrations to be detectable. However if that is the case they should not interfere with further characterization as the protein of interest is by far in abundance. The 27 kDa protein was not detected in either the Coomassie stained gel or immunoblot of the second dimension. This may be due to a number of proteins including the LMW standard protein of 20,1 kDa and the 27 kDa protein migrating at the dye front of the uniform 10 % acrylamide gel (used in the second dimension). An acrylamide gel of 12 % should be used which would not result in proteins with molecular weight ≥ 20 kDa migrating at the dye front. The only disadvantage of a 12 % acrylamide gel is that the distribution of proteins in 2DE is not uniform. The overall poor detection of *Cowdria* proteins in the second dimension may be due to separation of proteins of the same molecular weight but different pI, resulting in each individual protein being present in too low concentration to be detected by Coomassie. Additionally, low detection can also be as a result of protein loss due to equilibration of the IEF gel before SDS-PAGE. Coomassie detects 10 ng protein in comparison to autoradiography which can detect 10 pg protein, when 100 µg of protein is

loaded onto a gel. Sensitivity may also be increased when using the Coomassie stain by loading at least 100 µg of protein onto the IEF gel as opposed to 38 µg of protein which was loaded (O'Farrel, 1975). As the 31 kDa protein appears to be homogeneous it can either directly be excised from SDS-PAGE gels or be transferred onto PVDF membranes for further characterization.

The use of the 27 kDa and 31 kDa proteins blotted onto nitrocellulose membranes for the preparation of monospecific anti-serum was unsuitable as it resulted in large losses of protein. Since proteins excised from SDS-PAGE gels resulted in higher yields and the acrylamide polymers are chemically inert and do not induce an immune reaction in animals (Harlow & Lane, 1988) they were used as immunogen. The amounts of protein, 114 µg of 27 kDa and 133 µg of 31 kDa protein, used per immunization of the rabbits fell well in the margin of 50-1000 µg protein recommended (Harlow & Lane, 1988). The goat was however immunized with 266 µg of the 31 kDa protein, which was well below the margin of 500-2000 µg protein required per immunization (Harlow & Lane, 1988). Nevertheless the goat serum gave higher immunoblot titers (1:125 000) than the rabbit serum (1:1000), with respect to the 31 kDa protein. This emphasizes the individuality with which each immunogen is to be treated when immunization experiments are to be planned and carried out. Furthermore the response between two animals of the same or different species may differ. Harlow & Lane, (1988), states that even in genetically identical animals, a single preparation of antigen will elicit different antibody responses. When outbred animals are used, such as rabbits and goats, these differences become more apparent. Several animals should therefore be used for an immunization program and screened separately (Harlow & Lane, 1988).

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 kDa and 31 kDa proteins, suggest that the 27 kDa 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 (Retegui & Paladini, 1986). Although the isolated 27 kDa and 31 kDa proteins were in a denatured state they retained their immunogenicity which was also unaltered by the staining procedures. 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 does not react with cell culture proteins at high serum dilutions and are therefore specific for *Cowdria* proteins. They may therefore be used to screen *Cowdria* expression libraries.

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. 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) (Eckerskorn & Lottspiech, 1990). 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 (Righetti & Caravaggio, 1976).

If the partial amino acid sequence of a protein is known, it is possible to use the genetic code to predict the nucleotide sequence of the relevant gene. This prediction will always be an approximation, as only methionine and tryptophan can be unambiguously assigned to triplet codons. All other amino acids are coded by 2 or more codons each (Brown, 1986). From the partial amino acid sequence of the 31 kDa protein of *Cowdria* the following nucleotide sequence can be derived:

amino acid sequence: Met-Pro-Ile-Ala-Glu-Asp-Phe-Gly-Asp-Thr
nucleotide sequence: AUG-CCU-AUU-GCU-GAA-GAU-UUU-GGU-GAU-ACU

C	C	C	G	C	C	C	C	C
A	A	A				A		A
G		G				G		G

If all 10 amino acids are to be used to derive a nucleotide sequence, 21 of the 30 nucleotides can be predicted with certainty and a total

degeneracy of 12288 will exist. To decrease the total degeneracy of the nucleotide, and increase the specificity, a nucleotide sequence can be derived from a minimum of 6 amino acids. Therefore by choosing either the amino acid sequence of ala-glu-asp-phe-gly-asp or glu-asp-phe-gly-asp-thr, 12 out of the 18 nucleotides can be predicted with certainty with a total degeneracy of 256. An oligonucleotide probe can therefore be constructed synthetically and the probe may be used to screen *Cowdria* DNA libraries to find the gene coding for the 31 kDa protein. The selected gene may then be isolated, cloned and characterized.

CONCLUDING DISCUSSION

The aim of this investigation was to identify, purify and characterize antigenic proteins of *Cowdria*. It was hoped that data from these investigations might clarify and explain the differences observed between stocks of *Cowdria* regarding their pathogenicity and immunogenicity. This in turn could lead to the development of a specific diagnostic test and possibly the development of a subunit vaccine protecting against antigenically different stocks. A disadvantage of most available assays, for diagnosis in the live animal, is that they are unreliable, unspecific and insensitive. False positive and false negative results are frequently obtained. The identification of a major, common 31 kDa protein of *Cowdria* may be the breakthrough to a more specific and sensitive diagnostic assay for heartwater. The recent development of a CELISA specific for *Cowdria*, using a monoclonal antibody which reacts with an immunodominant, common 32 kDa protein of *Cowdria* confirms this (Jongejan *et al.*, 1991). The fact that the 31 kDa protein is a major, common protein holds promise for its use in diagnostic assays either in an ELISA or as an oligonucleotide probe. It is therefore important that the gene coding for the 31 kDa protein be identified and cloned. This may lead to a source of large quantities of pure DNA or the expressed protein for use in diagnostic assays.

There are 2 alternative pathways to follow in order to identify the gene of the 31 kDa protein: either screening of *Cowdria* genomic libraries with a synthetic oligonucleotide derived from the partial amino acid sequence of the 31 kDa protein or screening of *Cowdria* genomic expression libraries with the monospecific anti-serum. However it has been reported that the yield of *Cowdria* DNA, purified from cell cultures for the construction of a genomic library has consistantly been low (Wilkens & Ambrosio, 1989). Therefore for the isolation of *Cowdria* DNA, large

quantities of pure organisms free from cell culture DNA is required. Results obtained in the investigations described here indicate that the NSIAC column's capacity and specificity was very low and impractical for large scale purification. Unless a broader range of antibody specificity is obtained together with a greater column capacity the problem of insufficient quantity and partially pure *Cowdria* organisms shall remain. However NSIAC resulted in sufficient partially purified material for electron microscopic, SDS-PAGE, immunoblotting, ELISA and infectivity analysis.

One of the reasons for limited antibody diversity against antigen mixtures containing a variety of proteins is non-specific suppression due to intermolecular antigenic competition. That is inhibition of the immune response to one antigen caused by the administration of another. Passive immunization may be used to overcome this suppression, ie. application of passively administered antibodies raised against dominant antigen and the resulting decrease of antigenic competition will lead to the enhancement of the immune response to weak immunogens (Thalhamer & Freund, 1985). Passive immunization may improve antibody diversity and thus the diversity of the NSIAC, resulting in purer *Cowdria* organisms.

The possible link between albumin and the difference in the sedimentation rate of *Cowdria* before and after NSIAC should be further investigated. Removal of the factor responsible for sedimentation of the organism at lower centrifugal forces, when derived from cell cultures, should lead to partial purification by differential centrifugation. By combining NSIAC with differential centrifugation and including an additional purification step, such as density gradient centrifugation, purer organisms may be obtained. However, if pure *Cowdria* organisms, free of host DNA, are required for purification of *Cowdria* DNA, positive selection immunoadsorbent chromatography (PSIAC) may be a better choice. PSIAC using either the anti-31 kDa monospecific serum, prepared in these investigations, or monoclonal antibodies to surface epitopes of *Cowdria* should provide the specificity required and eliminate the problems encountered with NSIAC.

Amino acid sequence information of the 31 kDa protein may serve as a starting point for further pursuits such as the screening of DNA libraries to isolate, clone and characterize the gene. Obtaining a clone, in addition to providing further sequence information allows quantitative analysis of message expression and may be used to address cellular localization using in situ hybridization (Kennedy *et al.*, 1988). In the molecular cloning of genes with the use of synthetic oligonucleotides, using short stretches of amino acid sequence may give rise to false positives. Multiple independent probes or a single probe with minimum degeneracy will reduce the incidence of false positives (Aebersold *et al.*, 1987). Additionally two independent oligonucleotide probes derived from peptide sequences can be used to amplify the intervening segment of DNA by the polymerase chain reaction. This enzymatically synthesized segment is a faithful, nondegenerative copy of the template and therefore provides an ideal probe for the efficient screening of DNA libraries (Aebersold & Leavitt, 1990).

Alternatively, the monospecific anti-serum can be used as probes to identify recombinant clones producing either the 27 kDa or 31 kDa proteins of interest. Successful immunoscreening depends on the specificity of the antibody. Antibodies that produce good signals on immunoblots usually work well for expression library screening. Both monoclonal and polyclonal antibodies have been used successfully. The monospecific anti-serum produced in these investigations worked well on the immunoblots of *Cowdria* proteins and should therefore produce good results in screening expression libraries. The ability to detect a variety of epitopes on a protein is important since DNA inserts often will not be full length and hence only a portion of the polypeptide will be expressed. Since a single epitope can be common to multiple proteins, clones other than the desired recombinants may also be detected. Despite the fact that the monospecific anti-serum recognised epitopes on other proteins in immunoblots they were still specific for *Cowdria* proteins and hold promise for use in screening expression libraries.

Considering that complete, partial or no cross-protection is observed between various stocks of *Cowdria in vitro*, it seemed unlikely that the

common 27 kDa and/or the 31 kDa proteins play a role in cross-protection. This was confirmed by the observation that the goat that was immunized with the 31 kDa protein failed to survive a challenge with heartwater infective blood. This should be further investigated to determine whether perhaps the titer at the time of challenge should be higher, dose of antigen and immunization strategy be improved, before any conclusions may be made. At present it seems that although the 31 kDa protein is antigenically and immunogenically dominant it does not confer protection against heartwater. Additionally no correlation could be found between the serologically detectable antigens and cross-protection of certain stocks. As cellular immunity rather than humoral immunity has been suggested to play the dominant role in protection against heartwater (Du Plessis *et al.*, 1991), further investigation for proteins of *Cowdria* that stimulate cellular immunity should be undertaken. This would have to be investigated if a successful subunit vaccine is to be developed. A lymphocyte transformation test (LTT) using various proteins of *Cowdria* may reveal proteins which stimulate cellular immunity. However it should be noted that an antigen which generates the protective immunity *in vivo* might not necessarily be the dominating antigen in an antigen preparation for LTT *in vitro* (Kristensen *et al.*, 1982). Examining different stocks of *Cowdria* at the DNA level may reveal differences between them. Probes developed specifically for the different stocks may facilitate epidemiological studies and stock differentiation. Alternatively the 31 kDa protein may be used as antigen in a serodiagnostic assay for the detection of serum antibodies to *Cowdria*.

It is hoped that the diagnosis of heartwater disease may be improved with either a DNA probe or a suitable serological assay utilizing the 31 kDa protein. Tests, such as those involving a DNA probe or ELISA are suitable for large scale epidemiological studies that could lead to the detection of important carriers, improved control strategies for the disease and also assist in the evaluation of experiments planned to improve the present blood vaccine.

SUMMARY

Cowdria ruminantium organisms were successfully cultured in endothelial cells and used to investigate methods for purification of the organism and to identify, isolate and characterize its antigenic proteins.

For the liberation of organisms from the endothelial cells it was found that sonication was disadvantageous as it resulted in almost total rupture of the organism and cell culture material. Release of whole *Cowdria* organisms from cell cultures for further investigation was obtained by a single freeze/thaw step. Differential centrifugation as an initial step in the purification of the organisms from cell cultures revealed that *Cowdria* sediments at relatively low centrifugal forces of ≤ 10 000 xg. Negative selection immunoadsorbent chromatography of *Cowdria* infected cell cultures resulted in partial purification of viable organisms. However, the capacity of the column was found to be low.

Immunoblotting of *Cowdria* proteins with sheep, bovine or goat anti-*Cowdria* serum identified immunodominant antigenically conserved 31 kDa and 27 kDa proteins. These two proteins were present in the antigenic protein profiles of the Welgevonden, Ball3 and Kwanyanga stocks. They were also identified by sheep anti-serum to the Welgevonden, Ball3, Kwanyanga, Mali, Comoro, Breed, Germishuys, Kümm and Mara stocks; bovine anti-serum to the Welgevonden, Kwanyanga, Ball3, Mara, Breed and Mali stocks and goat anti-serum to the Welgevonden stock. No 2 stocks revealed identical or unique antigenic properties which could explain the existence of immunologically related or unrelated stocks.

Isolation of the 27 kDa and 31 kDa proteins was attempted by means of detergent solubilization. It was found that Sarkosyl, SDS or Tween 20 detergent treatment of heartwater infected cell culture extracts could not selectively solubilize these proteins.

However, sufficient quantities of the 27 kDa and 31 kDa proteins were obtained by SDS-PAGE for further characterization.

The 27 kDa and 31 kDa proteins were found to be single polypeptide chains. Two dimensional electrophoresis showed that the 31 kDa protein was not contaminated with other proteins of the same molecular weight. The amino acid composition of the 31 kDa protein indicated 23 % acidic and 12 % basic amino acids and the absence of tryptophan and cysteine. IEF analysis, under denaturing conditions, revealed that the pI of the 27 kDa and 31 kDa proteins are 5,2 and 5,7 respectively. An investigation involving an enzyme immunoassay for carbohydrates showed that the 31 kDa protein is not glycoconjugate. The partial, internal amino acid sequence of the 31 kDa protein was determined and found to be: met-pro-ile-ala-glu-asp-phe-gly-asp-thr.

Monospecific anti-serum was prepared against the 27 kDa and 31 kDa proteins in rabbits and a goat. Results indicated that these proteins share common epitopes and that those on the 31 kDa protein are immunologically and antigenically dominant in comparison to the 27 kDa protein.

Further investigations are necessary to determine if the 31 kDa protein is protective towards heartwater infection and if it would be suitable for use in serodiagnostics of the disease.

OPSOMMING

Cowdria ruminantium organismes is suksesvol in kulture gekweek en gebruik vir ondersoeke van suiwerings metodes vir die organismes en vir identifikasie, isolasie en karakterisering van hul antigeniese proteïene.

Vir die vrystelling van organismes vanuit endoteelselle is gevind dat sonikasie beide die organisme en weefselkultuur materiaal beskadig. 'n Vries/ontdooi stap was voldoende om organismes vanuit weefselkulture vry te stel vir verdere studies. Differensiële sentrifugasie, as 'n eerste stap in die suiwering van *Cowdria*, het getoon dat die organismes by lae relatiewe sentrifugale kragte van ≤ 10 000 xg sedimenteer. Negatiewe seleksie immunoadsorbent chromatografie van *Cowdria* geïnfekteerde weefsel kulture het tot gedeeltelike suiwering van lewendige organismes gelei. Die kolom se kapasiteit was egter baie laag bevind.

Immunoklad van *Cowdria* proteïene met skaap, bees of bok poliklonale anti-*Cowdria* serum het tot die identifisering van immuundominante, antigeniese gekonserveerde 31 kDa en 27 kDa proteïene gelei. Albei hierdie proteïene was teenwoordig in antigeniese profiele van Welgevonden, Kwanyanga en Ball3 stamme. Hulle was ook geidentifiseer deur skaap anti-serum teen die Welgevonden, Kwanyanga, Ball3, Mali, Comoro, Breed, Germishuys, Kümm en Mara stamme; bees anti-serum teen die Welgevonden, Kwanyanga, Ball3, Breed, Mara en Mali stamme en bok anti-serum teen die Welgevonden stam. Geen 2 stamme het identies of unieke antigeniese eienskappe getoon wat die bestaan van immunologiese verwante of onverwante stamme kon verklaar nie.

Daar is gepoog om die 27 kDa en 31 kDa proteïene met behulp van detergente te isoleer. Dit is gevind dat beide proteïene nie met Sarkosyl, SDS of Tween 20 detergent-behandeling selektief in oplossing gebring kon word nie. Daar kon egter genoegsame hoeveelhede van die 27

kDa en 31 kDa proteïene deurmiddel van SDS-PAGE verkry word vir verdere karakterisering.

Twee dimensionele electroforese het gewys dat die 31 kDa proteïen nie met ander proteïene van dieselfde molekulêre massa gekontamineer is nie. Beide proteïne is enkel-polipeptiedkettings. Die aminosuur-samestelling van die 31 kDa proteïen het aangetoon dat daar 23 % suur en 12 % basiese aminosure teenwoordig is en geen triptofaan of sisteïen residue nie. IEF analise, onder denaturerende kondisies, het gewys dat die pI van die 27 kDa en 31 kDa proteïene 5,2 en 5,7 onderskeidelik is. 'n Ondersoek met 'n ensiem immuno-essai vir koolhidrate het getoon dat die 31 kDa proteïen nie 'n glikokonjugaat is nie. Die gedeeltelike interne aminosuur-volgorde van die 31 kDa proteïen is bepaal en vasgestel as: met-pro-ile-ala-glu-asp-phe-gly-asp-thr.

Monospesifieke anti-serum teen die 27 kDa en 31 kDa proteïen is in konyne en 'n bok berei. Die resultate verkry dui daarop dat hierdie proteïene gemeenskaplike epitope deel en dat dié van die 31 kDa proteïen immunologies en antigenies dominant is in vergelyking met dié van die 27 kDa proteïen.

Verdere studies is nodig om vas te stel of die 31 kDa proteïen beskermend is teen hartwater infeksie en of die proteïen geskik is vir gebruik in serodiagnostiese toetse.

REFERENCES

1. Aebersold, R.H. and Leavitt, J., 1990. Sequence analysis of proteins separated by polyacrylamide gel electrophoresis: Towards an integrated protein database. *Electrophoresis,* 11, 517-527.

2. Aebersold, R.H., Leavitt, J., Saavedra, R.A., Hood, L.E. and Kent, S.B.H., 1987. Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis after *in situ* protease digestion on nitrocellulose. *Proc. Natl. Acad. Sci. USA.,* 84, 6970-6974.

3. Adams, J.H., Smith, R.D. and Kühlenschmidt, M.S., 1986. Identification of antigens of two isolates of *Anaplasma marginale,* using a western blot technique. *Am. J. Vet. Res.,* 47, 501-506.

4. Alexander, R.A., 1931. Heartwater. - The present state of our knowledge of the disease. *Rep. Dir. Vet. Serv. Anim. Ind., Onderstepoort,* 17, 89-150.

5. Ambrosio, R.E., Du Plessis, J.L. and Bezuidenhout, J.D., 1987. The construction of genomic libraries of *Cowdria ruminantium* in an expression vector, gt11. *Onderstepoort J. Vet. Res.,* 54, 255-256.

6. Andrew, H.R. and Norval, R.A.I., 1989. The carrier status of sheep, cattle and African buffalo recovered from heartwater. *Vet. Parasitol.*, 34, 261-266.

7. Barré, N. and Camus, E., 1987. The reservoir status of goats recovered from heartwater. *Onderstepoort J. Vet. Res.,* 54, 435-437.

8. Bezuidenhout, J.D., 1981. The development of a new heartwater vaccine using *Amblyomma hebraeum* nymphae infected with *Cowdria ruminantium*. In: WHITEHEAD, G.B. & GIBSON, J.D. (ed.). *Tick biology and control*, 41-54. Tick Research Unit, Rhodes University, Grahamstown, Republic of South Africa.

9. Bezuidenhout, J.D., 1987. Natural transmission of heartwater. *Onderstepoort J. Vet. Res.*, 54, 349-351.

10. Bezuidenhout, J.D., 1988. Sekere aspekte van hartwateroordraging, voorkoms van die organisme in bosluise en *in vitro* kweking. DVSc. Tesis, Fakulteit Veeartsenykunde Universiteit Pretoria.

11. Bezuidenhout, J.D., Camilla, L.P. and Barnard, B.J.H., 1985. *In vitro* cultivation of *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.*, 52, 113-120.

12. Bezuidenhout, J.D. and Jacobsz, C.J., 1986. Proof of transovarial transmission of *Cowdria ruminantium* by *Amblyomma hebraeum*. *Onderstepoort J. Vet. Res.*, 53, 31-34.

13. Bovarnick, M.R. and Snyder, J.C., 1949. Respiration of typhus rickettsiae. *J. Exper. Med.*, 89, 561-565.

14. Brandt, W.F., Alk, H., Chauhan, M. and Von Holt, C., 1984. A simple modification converts the spinning cup sequencer into a vapour phase sequencer. *FEBS Lett.*, 174, 228-232.

15. Brandt, W.F. and Frank, G., 1988. Manual gas-phase isothiocyanate degradation. *Anal. Biochem.*, 168, 314-323.

16. Brown, T.A., 1986. Gene cloning: An introduction. Thetford Press Ltd.

17. Caldwell, H.D. and Judd, R.C., 1982. Structural analysis of Chlymidial major outer membrane proteins. *Am. Soc. Microbiol.*, 38, 960-968.

18. Calton, G.J., 1984. Immunosorbent separations. *Meth. Enzym.*, 104, 381-387.

19. Camus, E. and Barré, N., 1987. Diagnosis of heartwater in the live animal. *Onderstepoort J. Vet. Res.*, 54, 291-294.

20. Chiles, T.C., O'Brien, T.W. and Kilberg, M.S., 1987. Production of monospecific antibodies to a low abundance hepatic membrane protein using nitrocellulose immobilized protein as antigen. *Anal. Biochem.*, 163, 136-142.

21. Cohn, E.J. and Ferry, J.D., 1943. The interaction of proteins with ions and dipolar ions. *In:* Cohn, E.J. and Edsall, J.T. (eds). Proteins, amino acids and peptides as ions and dipolar ions., 586-622. New York: Reinhold Publishing Corporation.

22. Cowdry, E.V., 1925a. Studies on the aetiology of heartwater. I. Observation of a rickettsia, *Rickettsia ruminantium* (n.sp.) in the tissues of infected animals. *J. Exp. Med.*, 42, 231-252.

23. Cowdry, E.V., 1925b. Studies on the aetiology of heartwater. I. Rickettsia ruminantium (N.Sp.) in the tissues of ticks transmitting the disease. *J. Exp. Med.*, 42, 253-274.

24. Cowdry, E.V., 1926. Studies on the aetiology of heartwater. III. The multiplication of *Rickettsia* ruminantium within the endothelial cells of infected animals and their discharge into the circulation. *J. Exp. Med.*, 44, 803-814.

25. Craigie, J., 1945. Application and control of ethyl-ether-water interface effects to the separation of Rickettsiae from yolk sac suspensions. *Canad. J. Res. E.*, 23, 104-114.

26. Dixon, R.W., 1898. Heartwater experiments. *Agric. J. Cape G. H.*, 12, 754-762.

27. Du Plessis, J.L., 1970a. Pathogenesis of heartwater: I *Cowdria ruminantium* in the lymph nodes of domestic ruminants. *Onderstepoort J. Vet. Res.*, 37, 89-96.

28. Du Plessis, J.L., 1970b. Immunity in heartwater. 1. A preliminary note on the role of serum antibodies. *Onderstepoort J. Vet. Res.*, 37, 147-150.

29. Du Plessis, J.L., 1975. Electron microscopy of *Cowdria ruminantium* infected reticulo endothelial cells of the mammalian host. *Onderstepoort J. Vet. Res.*, 42, 1-14.

30. Du Plessis, J.L., 1981. The application of the indirect fluorescent antibody test to the serology of heartwater. *Proceedings of an International Congress of Tick Biology and Control. Rhodes university, Grahamstown, South Africa.* 47-52.

31. Du Plessis, J.L., 1985a. A method for determining the *Cowdria ruminantium* infection rate of *Amblyomma Hebraeum*: effects in mice injected with tick homogenates. *Onderstepoort J. Vet. Res.*, 52, 55-61.

32. Du Plessis, J.L., 1985b. The natural resistance of cattle to artificial infection with *Cowdria ruminantium*: the role played by conglutinin. *Onderstepoort J. Vet. Res.*, 52, 273-277.

33. Du Plessis, J.L., Berche, P. and Van Gas, L., 1991. T cell-mediated immunity to *Cowdria ruminantium* in mice: The protective role of Lyt-2^+ T cells. *Onderstepoort J. Vet. Res.*, 58, 171-179.

34. Du Plessis, J.L., Camus, E., Oberem, P.T. and Malan, L., 1987. Heartwater serology: some problems with the interpretation of results. *Onderstepoort J. Vet. Res.*, 54, 327-329.

35. Du Plessis, J.L. and Kümm, N.L., 1971. The passage of *Cowdria ruminantium* in mice. *J. S. Afr. vet. med. Assoc.*, 42, 217-221.

36. Du Plessis, J.L. and Malan, M., 1987. The application of the indirect fluorescent antibody test in research on heartwater. *Onderstepoort J. Vet. Res.*, 54, 319-325.

37. Du Plessis, J.L., Van Gas, L., Olivier, J.A. and Bezuidenhout, J.D., 1989. The heterogenicity of *Cowdria ruminantium* stocks: Cross-immunity and serology in sheep and pathogenicity to mice. *Onderstepoort J. Vet. Res.*, 56, 195-202.

38. Eckerskorn, C. and Lottspeich, F., 1990. Combination of two-dimensional gel electrophoresis with microsequencing and amino acid composition analysis: Improvement of speed and sensitivity in protein characterization. *Electrophoresis*, 11, 554-561.

39. Edington, A., 1898. Heartwater. *Agric. J. Cape G. H.*, 12 , 749-754.

40. Eisen, H.N. and Siskind, G.W., 1964. Variations in affinities of antibodies during the immune response. *Biochemistry*, 3, 996-1008.

41. Giulian, G.G., Moss, R.L. and Greaser, M., 1984. Anylitical isoelectric focusing using a high-voltage vertical slab polyacrylamide gel system. *Anal. Biochem.*, 142, 421-436.

42. Hara, H., 1958. Partial purification of *Rickettsia mooseri* with cation exchange resin. *Japan J. Microb.*, 2, 69-77.

43. Harlow, E. and Lane, H.D., 1988. Antibodies: A laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y., U.S.A.

44. Hart, A., Kocan, K.M., Bezuidenhout, J.D. and Prozesky, L., 1991. Ultrastructural morphology of *Cowdria ruminantium* in midgut epithelial cells of adult *Amblyomma hebraeum* female ticks. *Onderstepoort J. Vet. Res.*, 58, 187-193.

45. Helenius, A. and Simons, K., 1975. Solubilization of membranes by detergents. *Biochem. Biophys. Acta.*, 415, 29-79.

46. Hewick, R.M., Hunkapiller, M.W., Hood, L.E. and Dreyer, W.J., 1981. A gas liquid solid phase peptide and protein sequencer. *J. Biol. Chem.*, 256, 7990-7997.

47. Hofstee, B.H.J., 1976. Hydrophobic adsorption of proteins. *In:* Catsimpoolas, N. (ed). Methods of protein separation., 2, 245-278. New York: Plenum Press.

48. Holland, C.J., Logan, L.L., Mebus, C.A. and Ristic, M., 1987. The serological relationship between *Cowdria ruminantium* and certain members of the genus *Ehrlichia*. *Onderstepoort J. Vet. Res.*, 54. 331.

49. Horak, I.G., MacIvor, K.M.DE F., Petney, T.N. and De Vos, V., 1987. Some avian and mammalian hosts of *Amblyomma hebraeum* and *Amblyomma marmoreum* (acari: ixodae). *Onderstepoort J. Vet. Res.*, 54, 397-403.

50. Hoyer, B.H., Bolton, E.T., Ormsbee, R.A., LeBouvier, G., Ritter, D.B. and Laeson, C.L., 1958. Mammalian viruses and rickettsiae. *Science,* 127, 859-863.

51. Ilemobade, A.A., 1976. Study of heartwater and the causative agent, *Cowdria ruminantium* (Cowdry, 1925), in Nigeria. PhD.

52. Ilemobade, A.A. and Blotkamp, C., 1976. Preliminary observations Thesis, Zaria. on the use of the capillary flocculation test for the diagnosis of heartwater (*Cowdria ruminantium* infection). *Res. Vet. Sci.*, 21, 370-372.

53. Ilemobade, A.A. and Blotkamp, C., 1978. Heartwater in Nigeria. II. The isolation of *Cowdria ruminantium* from live and dead animals and the importance of routes of inoculation. *Trop. Anim. Hlth. Prod.*, 10, 39-44.

54. Jongejan, F., 1990. Tick/host interactions and disease transmission with special reference to *Cowdria ruminantium*

(Ricketsiales). PHD Thesis, Rijks University, Utrecht, Netherlands.

55. Jongejan, F. and Thielemans, M.J.C. 1989. Identification of an immunodominant antigenically conserved 32-kilodalton protein from *Cowdria ruminantium*. *Infect. Immun.*, 57, 3243-3246.

56. Jongejan, F., Thielemans, M.J.C., De Groot, M., Van Kooten, P.J.S. and Van Der Zeijst, B.A.M., 1991a. Competitive enzyme-linked immunosorbent assay for heartwater using monoclonal antibodies to a *Cowdria ruminantium*-specific 32-kilodalton protein. *Vet. Microbiol.*, 28, 199-211.

57. Jongejan, F., Uilenberg, G. and Franssen, F.F.J., 1988. Antigenic differences between stocks of *Cowdria ruminantium*. *Res. Vet. Sci.*, 44, 186-189.

58. Jongejan, F., Wassink, L.A., Thielemans, M.J.C., Perié, N.M. and Uilenberg, G., 1989. Serotypes in *Cowdria ruminantium* and relationship with *Ehrlichia Phagocytophila* determined by immuno fluorescence. *Vet. Microbiol.*, 21, 31-40.

59. Jongejan, F., Zandbergen, T.A., Van De Wiel, P.A., De Groot, M. and Uilenberg, G., 1991b. The tick-borne rickettsia *Cowdria ruminantium* has a chlamydia-like developmental cycle. *Onderstepoort J. Vet. Res.*, 58, 227-237.

60. Kennedy, T.E., Gawinowicz, M.A., Barzilai, A., Kandel, E.R. and Sweatt, J.D., 1988. Sequencing of proteins from two-dimensional gels by using *in situ* digestion and transfer of peptides to polyvinylidine difluoride membranes: Application to proteins associated with sensitization in *Aplysia*. *Proc. Natl. Acad. Sci. USA.*, 85, 7008-7012.

61. Kiefer, H., 1975. Separation of antigen-specific lymphocytes. *Eur. J. Immunol.*, 5, 624-627.

62. Kristensen, F., Kristensen, B. and Lazary, S., 1982. The lymphocyte stimulation test in veterinary immunology. *Vet. Immunol. Immunopath.*, 3, 203-277.

63. Knudsen, K.A., 1985. Proteins transferred to nitrocellulose for use as immunogens. *Anal. Biochem.*, 147, 285-288.

64. Kocan, K.M. and Bezuidenhout, J.D., 1987. Morphology and development of *Cowdria ruminantium* in *Amblyomma* ticks. *Onderstepoort J. Vet. Res.*, 54, 177-182.

65. Kocan, K.M., Bezuidenhout, J.D. and Hart, A., 1987. Ultrastructural features of *Cowdria ruminantium* in midgut epithelial cells and salivary glands of nymphal *Amblyomma hebraeum*. *Onderstepoort J. Vet. Res.*, 54, 87-93.

66. Krieg, N.R. and Holt, J.G., 1984. Bergey's manual of systematic bacteriology, Vol. I. Baltimore: Williams and Wilkins.

67. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature.*, 227, 680-685.

68. Lee, C., Levin, A. and Branton, A., 1987. Copper staining: A five-minute protein stain for sodium dodecyl sulphate-polyacrylamide gels. *Anal. Biochem.*, 166, 308-312.

69. Logan, L.L., Whyard, T.C., Quintero, J.C. and Mebus, C.A., 1987. The development of *Cowdria ruminantium* in neutrophils. *Onderstepoort J. Vet. Res.*, 54, 197-204.

70. Lottspeich, F., 1985. Microscale isocratic separation of the phenylthiohydantoin amino acid derivatives. *J. of Chromatography.*, 326, 321-327.

71. Lounsbury, C.P., 1900. Tick heartwater experiments. *Agric. J. Cape G. H.*, 16, 682-687.

72. MacKenzie, P.K.I. and McHardy, N., 1987. *Cowdria ruminantium* infection in the mouse: A review. *Onderstepoort J. Vet. Res.*, 54, 267-269.

73. Matsudaira, P.T., 1989a. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.*, 262, 10035-10038.

74. Matsudaira, P.T., 1989b. A practical guide to protein and peptide purification for microsequencing. Academic Press Inc. pp 29.

75. McGuire, T.C., Palmer, G.H., Goff, W.L., Johnson, M.I. and Davis, W.C., 1984. Common and isolate-restricted antigens of *Anaplasma marginale* detected with monoclonal antibodies. *Infect. Immun.*, 45, 697-700.

76. Moos, M., Nguyen, N.Y. and Liu, T., 1988. Reproducible high yield sequencing of proteins electrophoretically separated and transferred to an inert support. *J. Biochem Chem.*, 263, 6005-6008.

77. Nakagawa, S. and Fukuda, T., 1989. Direct amino acid analysis of proteins electroblotted onto polyvinylidene difluoride membrane from sodium dodecyl sulphate-polyacrylamide gel. *Anal. Biochem.*, 181, 75-78.

78. Neitz, W.O., 1939. The immunity in heartwater. *Onderstepoort J. Vet. Sci. Anim. Ind.*, 13, 245-283.

79. Neitz, W.O., 1947. Die oordraging van hartwater deur *Ammblyomma pomposum*, Dönitz, 1909. *S.A. Sci.*, 1, 83.

80. Neitz, W.O., 1968. Heartwater. *Bull. Off. int. Epiz.*, 70, 329-336.

81. Neitz, A.W.H. and Vermeulen, N.M.J., 1987. Purification of *Cowdria ruminantium* by immunoadsorbent affinity chromatography. *Onderstepoort J. Vet. Res.*, 54, 247-253.

82. Neitz, A.W.H., Vermeulen, N.M.J. and Viljoen, G.J., 1987. Purification of *Cowdria ruminantium* by density gradient centrifugation. *Onderstepoort J. Vet. Res.*, 54, 223-231.

83. Neitz, A.W.H., Viljoen, G.J., Bezuidenhout, J.D., Oberem, P.T., Putterill, J.F., Verschoor, J.A., Visser, L. and Vermeulen, N.M.J., 1986a. Isolation of *Cowdria ruminantium* by means of Percoll density gradient centrifugation and detection by an enzyme-linked immunosorbent assay. *Onderstepoort J. Vet. Res.*, 53, 63-67.

84. Neitz, A.W.H., Viljoen, G.J., Bezuidenhout, J.D., Oberem, P.T., Van Wyngaardt, W and Vermeulen, N.M.J., 1986b. The detection of antigen to *Cowdria ruminantium* in serum and *Cowdria ruminantium* antigen in *Amblyomma hebraeum* by an enzyme-linked immunosorbent assay. *Onderstepoort J. Vet. Res.*, 53, 39-41.

85. Neitz, A.W.H., Viljoen, G.J., Bezuidenhout, J.D., Oberem, P.T., Visser, L. and Vermeulen, N.M.J., 1986c. The detection of *Cowdria ruminantium* antibodies during the course of heartwater disease in sheep as well as antigen in the blood by an enzyme-linked immunosorbent assay. *Onderstepoort J. Vet. Res.*, 53, 205-207.

86. Oberem, P.T. and Bezuidenhout, J.D., 1987. The production of heartwater vaccine. *Onderstepoort J. Vet. Res.*, 54, 485-488.

87. O'Farrel, P.H., 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.*, 250, 4007-4021.

88. Ohashi, N., Tamura, A., Ohta, M. and Hayashi, K., 1989. Purification and partial characterization of a type-specific antigen of *Rickettsia tsutsugamushi*. *Infect. Immun.*, 57, 1427-1431.

89. Palmer, G.H., Oberle, S.M., Barbet, A.F., Goff, W.L., Davis, W.C. and McGuire, T.C., 1988. Immunization of cattle with a 36-kilodalton surface protein induces protection against homologous

and heterologous *Anaplasma marginale* challenge. *Infect. Immun.* 56, 1526-1531.

90. Perreau, P., Morel, P.C., Barré, N. and Duranol, P., 1980. Existance de la cowdriose (heartwater) à *Cowdria ruminantium*, chez les petits ruminants des Antilles Françaises (la Guadeloupe) et des Mascareignes (la Réunion et Ile Maurice). *Revue Elev. Méd. vét. Pays trop.*, 33, 21-22.

91. Pharmacia. 1982. Isoelectric focusing principles and methods. Pharmacia Fine Chemicals.

92. Pharmacia. 1983. Affinity chromatography principles and methods. Pharmacia fine chemicals.

93. Pienaar, J.G., 1970. Electron microscopy of *Cowdria (Rickettsia) ruminantium* (Cowdry, 1926) in the endothelial cells of the vertebrate host. *Onderstepoort J. Vet. Res.*, 37, 67-78.

94. Provost, A. and Bezuidenhout, J.D., 1987. The historical background and global importance of heartwater. *Onderstepoort J. Vet. Res.*, 54, 165-169.

95. Prozesky, L., Bezuidenhout, J.D. and Patterson, C.L., 1986. Heartwater: An *in vitro* study of the ultrastructure of *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.* 53, 153-159.

96. Prozesky, L. and Du Plessis, J.L., 1985. The pathology of heartwater. I. A study of mice infected with the Welgevonden strain of *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.*, 52, 71-79.

97. Prozesky, L. and du Plessis, J.L., 1987. Heartwater. The development and lifecycle of *Cowdria ruminantium* in the vertebrate host, ticks and cultured endothelial cells. *Onderstepoort J. Vet. Res.*, 54, 193-196.

98. Reed, L.J. and Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *American J. Hyg.*, 27, 493-497.

99. Retegui, L.A. and Paladini, A.C., 1986. Heteroclitic behaviour of some monoclonal antibodies against bovine growth hormone. *Mol. Immunol.*, 23, 119-123.

100. Ribi, E. and Hoyer, B.H., 1960. Purification of Q fever rickettsiae by density-gradient sedimentation. *J. Immunol.*, 85, 314-318.

101. Righetti, P.G. and Caravaggio, T., 1976. Isoelectric points and molecular weights of proteins. *J. Chromatog.*, 127, 1-28.

102. Robinson, P.A., Anderton, B.H. and Loviny, T.L.F., 1988. Nitrocellulose-bound antigen repeatedly used for the affinity purification of specific polyclonal antibodies for screening DNA expression libraries. *J. Immunol. Methods.*, 108, 115-122.

103. Rossouw, M., Neitz, A.W.H., De Waal, D.T., Du Plessis, J.L., Van Gas, L. and Brett, S., 1990. Identification of the antigenic proteins of *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.*, 57, 215-221.

104. Robyt, J.F. and White, B.J., 1987. Biochemical techniques theory and practice. Brooks/Cole Publishing Company.

105. Scholossman, S.F. and Hudson, L., 1973. Specific purification of lymphocyte populations on a digestible immunoadsorbent. *J. Immunol.*, 110, 313-315.

106. Scott, G.R., 1987. The taxonomic status of the causative agent of heartwater. *Onderstepoort J. Vet. Res.*, 54, 257-260.

107. Shepard, C.C. and Topping, N.H., 1947. Preparation of suspensions of Rickettsiae from infected yolk sacs without the use of ether. *J. Immunology.*, 55, 97-102.

108. Stephan, J., Gallop, R.G.C. and Smith, H., 1966. Separation of antigens by immunological specificity. Use of disulphide-linked antibodies as immunosorbent. *Biochem. J.*, 101, 717-720.

109. Stewart, C.G., 1987. Specific immunity in farm animals to heartwater. *Onderstepoort J. Vet. Res.*, 54, 341-342.

110. Stewart, C.G., 1989. *Cowdria ruminantium* stocks: how significant are they? *Zimbabwe Vet. J,*. 20, 149-154.

111. Stewart, M.W., 1977. Affinity of antibody-antigen reaction and its biological significance. *In:* Glynn, L.E. and Steward, M.W. (eds). Immunochemistry: An advanced textbook., 233-262. New York: A Wiley Interscience Publications.

112. Szewczyk, B. and Summers, D.F., 1988. Preparative elution of proteins blotted to Immobilon membranes. *Anal. Biochem.*, 168, 48-53.

113. Tamura, A., Ohashi, N., Urakami, H., Takahashi, K. and Oyanagi, M., 1985. Analysis of polypeptide composition and antigenic components of *Rickettsia tsutsugamushi* by polyacrylamide gel electrophoresis and immunoblotting. *Infec. Immun.*, 48, 671-675.

114. Thalhamer, J. and Freund, J., 1985. Passive immunization: a method of enhancing the immune response against antigen mixtures. *J. Immunol. Methods.*, 80, 7-13.

115. Tous, G.I., Fausnaugh, J.L., Akinyosoye, O., Lackland, H., Winter-Cash, P., Victoria, F.J. and Stein, S., 1989. Amino acid analysis on polyvinylidene difluoride membranes. *Anal. Biochem.*, 179, 50-55.

116. Uilenberg, G., 1983. Heartwater (*Cowdria ruminantium* infection): Current status. *Adv. vet. Sci. Comp. Med.*, 27, 427-480.

117. Van Amstel, S.R. and Oberem, P.T., 1987. The treatment of heartwater. *Onderstepoort J. Vet. Res.*, 54, 475-479.

118. Van de Pypekamp, H.E. and Prozesky, L., 1987. Heartwater: An overview of the clinical signs, susceptibility and differential diagnosis of the disease in domestic ruminants. *Onderstepoort J. Vet. Res.*, 54, 263-266.

119. Vermeulen, N.M.J., Neitz, A.W.H. and Viljoen, G.J., 1987. Purification of *Cowdria ruminantium* by lectin cellular affinity chromatography. *Onderstepoort J. Vet. Res.*, 54, 233-246.

120. Verschoor, J.A., Coetzee, L. and Visser, L., 1989. Monoclonal antibody characterization of two field strains of *Haemophilus paragallinarium* isolated from vaccinated layer hens. *Avian Dis.*, 33, 219-225.

121. Viljoen, G.J., Neitz, A.W.H., Bezuidenhout, J.D., Oberem, P.T. and Vermeulen, N.M.J., 1988. The presence of *Cowdria ruminantium* antigen in various tissues of *Amblyomma hebraeum* imagines as detected by an enzyme-linked immunosorbent assay. *Onderstepoort J. Vet. Res.*, 55, 239-240.

122. Viljoen, G.J., Vermeulen, N.M.J., Oberem, P.T., Prozesky, L., Verschoor, J.A., Bezuidenhout, J.D., Putterill, J.F., Visser, L. and Neitz, A.W.H., 1985. Isolation of *Cowdria ruminantium* by cellular affinity chromatography and detection by an enzyme-linked immunosorbent assay. *Onderstepoort J. Vet. Res.*, 52, 227-232.

123. Walker, J.B., 1987. The tick vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, genus *Amblyomma*) and their distribution. *Onderstepoort J. Vet. Res.*, 54, 353-379.

124. Webb, C., Teitelbaum, D., Rauch, H., Maoz, A., Aron, R. and Fuchs, S., 1975. Fractionation of functional lymphocytes sensitized to basic encephalitogen on derivatized collagen and gelatin gels. *J. Immunol.*, 114, 1469-1472.

125. Weber, K. and Osborn, M., 1969. The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis. *J. Biol Chem.*, 244, 4406-4412.

126. Weiss, E., Coolbaugh, J.C. and Williams, J.C., 1975. Separation of viable *Rickettsia typhi* from yolk sac and L cell host components by renografin density gradient centrifugation. *Appl. Microbiol.*, 30, 456-463.

127. Weiss, W, Postel, W. and Görg, A., 1991. Barley cultivar discrimination: I. Sodium docecyl sulphate-polyacrylamide gel electrophoresis and glycoprotein blotting. *Electrophoresis.*, 12, 323-330.

128. Wilkins, S.C. and Ambrosio, R.E., 1989. The isolation of nucleic acid sequences specific for *Cowdria ruminantium. Onderstepoort J. Vet. Res.*, 56, 127-129.

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Appendix A

Abreviations:

a.a. - amino acid
Abs - absorbance
ATV - activated trypsin versene
ATZ - anilinothiazolinone
B3 - Ball3
BA - bovine aorta cell
BIS-TRIS - [bis(2-hydroxyethyl)imino-tris(hydroxy-methyl)methane
BLP - buffered lactose-peptone citrate
Bre - Breed
BSA - bovine serum albumin
CAPS - cyclohexylamine propane sulphonic acid
CELISA - competitive enzyme-linked immunosorbent assay
CHAPS - 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate
Com - Comoro
C-TAB - N-cetyl-N,N,N,-trimethylammonium bromid
Da - Dalton
2DE - 2 dimensional electrophoresis
DEAE - diethylaminoethyl
DMSO - dimethyl sulfoxide
DTT - dithiothreitol
E5 - calf endothelial cell
EB - elementary body
ELISA - enzyme-linked immunosorbent assay
EM - electron microscopic
FCA - Freunds complete adjuvant

FIA - Freunds incomplete adjuvant

G - gauge

Grms - Germishuys

HEPES - 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethane sulphonic acid

HPLC - high performance liquid chromatography

IB - immunoblot

IEF - isoelectric focusing

IFA - indirect fluorescent antibody

IgG - immunoglobulin G

i.m. - intramuscular

i.v. - intravenous

k - conglutinin

Kr - kilo rad

kDa - kilo-Dalton

Kwg - Kwanyanga

LMW - low molecular weight

LTT - lymphocyte transformation test

MSA - methane sulphonic acid

MW - molecular weight

n - number of variables

NC - nitrocellulose

nd - not done

neg - negative

no - number

NSIAC - negative selection immunoadsorbent chromatography

OPD - o-phenylene diamine

PBS - phosphate buffered saline

pI - isoelectric point

PITC - phenylisothiocyanate

pos - positive

prot - protein

PTC - phenylthiocarbamyl

PTH - phenylthiohydantoin

PVDF - polyvinylidene difluoride

RB - reticulate body

RBC - red blood cell

s.c. - subcutaneous

SD - standard deviation

SDS - sodiun dodecyl sulphate

SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis

TEA - triethylamine

TEMED - tetramethylenediamine

TES - N-[Tris(hydroxymethyl)methyl]-2-aminoethane-sulphonic acid

TFA - trifluoroacetic acid

Tris - tris(hydroxymethyl)aminomethane

Tween - polyoxyethylene sorbitan monolaurate

Wlg - Welgevonden

xg - centrifugal force calculated at a distance from axis of rotation to mid point of tube