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Diagnosis, pathogenesis and epidemiology of Crimean-Congo haemorrhagic fever

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found in 3/72 CCHF patients from locations within the distribution range of *A. hebraeum*, although there was no evidence to confirm that they had undergone recent infection with the virus. Rising titres and IgM antibody activity to Dugbe virus were detected in the serum of 1/172 patients with suspected VHF and a history of known or possible exposure to ticks, in whom no evidence of CCHF infection was detected. The patient concerned suffered from febrile illness with prolonged thrombocytopenia. It was concluded that systematic attempts should be made to confirm the presence of Dugbe virus in South Africa, and that the infection should be borne in mind in the differential diagnosis of haemorrhagic fevers in Africa.

antibodies to other agents, such as Ebola and Marburg viruses, for which the natural hosts are unknown.

In the course of routine investigation of suspected cases of VHF in South Africa, patients were encountered who had a history of being bitten by ticks, but who lacked evidence of infection with CCHF virus or non-viral tick-borne agents, such as rickettsias. Hence, the possibility that tick-associated viruses other than CCHF were involved was investigated. As a preliminary, ELISA were developed to test for antibodies to selected tick-borne viruses, and a survey was conducted on cattle sera to seek information on the occurrence of the viruses within the country. The prevalence of antibody activity in cattle sera from 46 herds from various locations in the country was found to be 905/2116 (42.8%) for CCHF virus antigen, 70/1358 (5.2%) for Dugbe, 21/1358 (1.5%) for louping ill, 6/450 (1.3%) for West Nile, 7/1358 (0.5%) for Nairobi sheep disease, 3/625 (0.5%) for Kadam and 2/450 (0.4%) for Chenuda, while no reactions were recorded with Hazara, Bahig, Bhanja, Thogoto and Dhori virus antigens.

The findings for CCHF confirmed previous observations that the virus is widely distributed within South Africa, and West Nile virus was also well known to be present in the country, but the low prevalence and low titres of antibody activity recorded with many of the other antigens suggested that most of the reactions may have been non-specific. Antibody to Dugbe virus, however, was found only within the distribution range of *Amlyomma hebraeum*, and it is notable that ticks of the genus *Amblyomma* are known to transmit the virus elsewhere in Africa. Moreover, antibody activity to Dugbe virus was

reproducible technique for detecting antibody to the virus in the sera of domestic animals and wild vertebrates. An ELISA procedure for the detection of IgG antibody was developed using serial serum samples obtained from experimentally infected sheep and cattle, and commercially available anti-sheep and cattle immunoglobulin peroxidase conjugates. The serum samples from the experimentally infected animals were also used to develop an IgM-capture ELISA with commercially available anti-sheep IgM as capture antibody; the same capture antibody could be used for testing both sheep and cattle sera. It was found that total antibody activity in sheep sera could also be demonstrated by means of the CCHF ELISA with rabbit anti-CCHF peroxidase conjugate as was used on human sera (see above), and this implied that the technique was suitable for use on the sera of wild vertebrates for which no species-specific anti-immunoglobulin conjugates exist.

The CCHF ELISA was applied to serum samples of animals from the Kruger National Park, and it was found that the prevalence of antibody to CCHF virus was lower than detected previously in wild vertebrates from other locations in southern Africa, but this may be related to the fact that *Hyalomma truncatum* is the only member of this genus of ticks known to occur in the Park (*Hyalommas* are believed to be the principal vectors of the virus). Nevertheless, the fact that the highest prevalences of antibody occurred in the sera of animals such as the rhinoceros, giraffe and buffalo in the Park is in accordance with previous observations that CCHF infection occurs most frequently in larger mammals which are the preferred hosts of adult *Hyalomma* tick vectors of the virus. The CCHF ELISA could find wide application in surveys on the sera of small and large wild vertebrates for

From the results of IHC and ISH examination of tissues it appears that the reticuloendothelial system and hepatocytes are the main targets of infection. The widespread infection of endothelium probably plays a major role in the pathogenesis of the bleeding tendency and disseminated intravascular coagulopathy, which commonly characterize the disease. The association of necrotic areas in liver with the presence of viral antigen suggests that the hepatocyte damage may be mediated by a direct cytopathic effect.

The genetic heterogeneity of 57 southern African CCHF isolates was investigated using restriction fragment length polymorphism (RFLP), and nucleic acid sequencing of a region of the S segment of the genome. The 57 isolates could be assigned to 10 RFLP groups on the basis of the fragment lengths obtained from digestion of RT-PCR products with eight restriction endonucleases. Fifteen isolates, selected to include representatives of all 10 RFLP groups, were subjected to nucleic acid sequencing. There was 2-18% variation in nucleotide sequences, but the majority of changes were conservative, with 94-100% similarity existing between the predicted amino acid sequences. No correlation could be made between differences in CCHF isolates and source of infection, year of infection or pathogenicity of the virus. Examination of the relationship among southern African and other isolates (using sequence data available from other sources) showed that African isolates were more closely related to each other phylogenetically than to Asian isolates.

Epidemiological studies on CCHF have been hampered by the lack of a convenient and

were compared as methods for demonstrating antibody to CCHF virus on serum samples taken from 101 patients during the acute stage of the disease and at intervals up to 59 months after the onset of illness, with emphasis on early detection of the immune response. It was found that IF tests detected IgM and/or IgG antibodies at the earliest on day 4 of illness in about 10% of patients who survived the disease, and by day 9 all survivors had antibodies demonstrable by IF. Patients who died generally failed to develop a demonstrable antibody response, or had a delayed and weak response. The IgM and IgG antibody responses were detected at an earlier stage of infection by ELISA than by IF tests in 10 and 15 patients respectively, but the reverse was true in similar numbers of patients (11 and 14 respectively). A competitive ELISA (CELISA), which detects total antibody activity, was developed using an anti-CCHF peroxidase conjugate prepared from immune rabbit serum. The CELISA yielded results which were in close agreement with findings in IF tests. It was concluded that the IF tests are most convenient for use in making a rapid serodiagnosis of the disease.

The utility of IHC as a sensitive and rapid diagnostic procedure was investigated by examining stored tissues from 12 deceased CCHF patients. It was found that the histopathologic lesions of CCHF were similar to those seen in other viral haemorrhagic fevers, and it was established that in the absence of pathognomonic features the findings could be rendered diagnostic through demonstrating the presence of viral antigen by means of IHC. Detection of viral antigen in fixed tissues correlated well with isolation of infectious virus from fresh tissues.

21 samples from a further 19 patients in whom alternative diagnoses were established. No virus was isolated from nor viral RNA detected in the 21 serum samples derived from the 19 non-CCHF patients. Positive RT-PCR results were recorded in 15/80 of the stored serum samples when analysed on ethidium bromide-stained agarose gels. The use of Southern blots with a radio-labelled probe increased the sensitivity of the assay and resulted in the detection of viral RNA in 52/80 of the stored sera. However, in the tests performed prospectively on fresh serum samples from CCHF patients, positive RT-PCR results were recorded in 14/26 samples on ethidium bromide stained gels, and the use of Southern blots with a labelled probe resulted in a positive result being recorded in only one extra sample: this marginal increase in sensitivity was gained at the cost of increasing the time required for the test from 8 hours to 2 days.

Viral RNA was detected by RT-PCR in a proportion of serum samples taken up to day 16 of illness in CCHF patients, while infectious virus was isolated up to day 13 at the latest. Early in the disease there was relatively good correlation between the results obtained by RT-PCR and virus isolation, but it appears that after the first week infectious virus is progressively cleared from serum while nucleic acid remains demonstrable in a proportion of patients well into convalescence. The implication is that RT-PCR constitutes a sensitive and rapid means of diagnosing CCHF during the acute phase of illness, particularly before antibody becomes detectable, or in fatal infections where antibody response is generally not demonstrable.

Indirect immunofluorescence (IF) and enzyme-linked immunosorbent assays (ELISA)

Abstract

The objects of the study were:

a) to develop sensitive tests for the early diagnosis of Crimean-Congo haemorrhagic fever (CCHF) infection of humans, based on the detection of viral nucleic acid and antibody in serum;

b) to investigate the utility of histological assays such as immunohistochemistry (IHC) and *in situ* hybridization (ISH) as diagnostic tests on formalin-fixed paraffin-embedded tissues, and to apply them in studying the pathogenic implications of the cellular targets of CCHF virus;

c) to investigate the possibility that there is a correlation between genetic heterogeneity of CCHF virus and virulence, geographic distribution, year of isolation, or source of infection;

d) to develop assays for use in antibody surveys on small and large wild vertebrates for which no species-specific anti-immunoglobulin conjugates are available, in order to investigate distribution and natural reservoir hosts of the virus, and

e) to investigate the distribution and medical importance of other tick-borne viruses in South Africa and their role in the differential diagnosis of CCHF.

A reverse transcription-polymerase chain reaction (RT-PCR) was applied retrospectively to 80 serum samples which had been obtained from 45 patients with confirmed CCHF and stored at -70 °C for up to six years, and prospectively to 26 samples from 19 patients with suspected viral haemorrhagic fever (VHF) who proved to be cases of CCHF, plus

Publications

Burt, F.J., Swanepoel, R., Braack, L.E.O. (1993). Enzyme-linked immunosorbent assays for the detection of antibody to Crimean-Congo haemorrhagic fever virus in the sera of livestock and wild vertebrates. Epidemiol. Infect. **111**:547-557.

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Declaration

I declare that this thesis is my own work. It is being submitted to the University of the Witwatersrand, Johannesburg, for the degree Doctor of Philosophy. It has not previously been submitted for any degree or examination at this or any other University.

.....

Felicity Jane Burt

on this _____ day of _____, 1997

Diagnosis, pathogenesis and epidemiology of Crimean-Congo haemorrhagic fever

Felicity Jane Burt

A thesis submitted to the Faculty of Health Sciences
University of the Witwatersrand, Johannesburg
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suspensions prepared from ticks, which were suspected to be vectors of the agent. The causative virus was finally isolated in laborato. hosts, namely mice, in 1967 (Chumakov, 1974). The availability of a laboratory host made it possible to characterize the virus, and it was shown in 1969 that CHF isolates from European and Asian Republics of the USSR, and Bulgaria, were antigenically identical to an African virus named Congo which had been isolated in 1956 from a febrile patient in what was then the Belgian Congo (currently the Democratic Republic of Congo) (Woodall *et al.*, 1967; Casals, 1969; Chumakov *et al.*, 1970; Tignor *et al.*, 1980). Studies on the physicochemical characteristics, morphology and morphogenesis of CHF and Congo isolates confirmed that they were indistinguishable from each other and similar to other members of the family *Bunyaviridae*, and the combined name Crimean-Congo haemorrhagic fever was adopted for the virus (Koroley *et al.*, 1976; Donets *et al.*, 1977; Hoogstraal, 1979).

The disease was first recognized in South Africa in 1981, and since 1984 about 5-20 cases have been diagnosed in the country each year (Gear *et al.*, 1982; Swanepoel *et al.*, 1983a).

1.3 The virus

The CCHF virus is classified as a member of the genus *Nairovirus*, of the family *Bunyaviridae* (Karabatsos, 1985; Calisher, 1991; Peters and LeDuc, 1991). The genus is divided into 7 serogroups on the basis of serological analysis using complement-fixation, haemagglutination-inhibition, neutralization, immunoprecipitation, and IF

virus. ELISA were developed to test for antibody in sheep and cattle sera, using commercially available anti-species immunoglobulin peroxidase conjugates and serum samples from experimentally infected animals, while a competition ELISA (CELISA) using rabbit anti-CCHF peroxidase conjugate was developed for use in surveys on wild vertebrates for which no species-specific conjugates are available. The CELISA was applied to wild vertebrate sera obtained from a national park.

In the course of investigating suspected cases of VHF in the Unit, patients were encountered who had been bitten by ticks, but who lacked evidence of infection with CCHF virus or non-viral tick-borne agents, and this prompted an investigation to look for evidence that tick-borne viruses other than CCHF are present and causing human infections in South Africa. ELISA were developed to test for antibodies to selected tick-borne viruses and a survey was conducted on cattle sera before the tests were the applied to specimens from human patients.

1.2 History

A haemorrhagic disease with symptoms suggestive of CCHF infection was described in eastern Europe and Asia as far back as the twelfth century A.D. However, a disease given the name Crimean haemorrhagic fever (CHF) was first reported in 1944 in an outbreak involving about 200 soldiers and peasants on the Crimean peninsula in what was then the Soviet Union (Hoogstraal, 1979). In the following year, it was shown by inoculation of human subjects that the disease was caused by a filterable agent present in the blood of patients during the acute stage of illness, and that the agent was also present in

the treatment of patients, and for the protection of medical and laboratory staff. Conventional diagnostic techniques include isolation of infectious virus or demonstration of seroconversion, or a 4-fold rise in antibody titre and/or an IgM antibody response. Isolation of virus can take more than a week to achieve, while antibody may only become demonstrable on day 7-9 of illness, or not at all in fatal disease. It follows that a rapid laboratory diagnosis may be difficult to achieve during the first few days of illness.

The first object of the present study was to investigate the rapid detection of CCHF virus in blood through demonstrating the presence of viral RNA in serum by means of a reverse transcription-polymerase chain reaction (RT-PCR). Following this, enzyme-linked immunosorbent assays (ELISA) for demonstrating IgG and IgM antibodies to CCHF virus were compared with the indirect immunofluorescence (IF) test already in routine use in the laboratory for the rapid detection of antibody to CCHF virus. Next, the utility of immunohistochemistry (IHC) and *in situ* hybridization (ISH) techniques was investigated for diagnosing the disease on formalin-fixed paraffin-embedded tissues from fatal cases, and for identifying the cellular targets of the virus. Then 57 southern African isolates of CCHF virus were investigated using restriction fragment length polymorphism (RFLP) and 15 of the isolates were examined by nucleotide sequencing of a region of the S segment of the genome to determine if there was any correlation between genetic heterogeneity of CCHF virus and pathogenicity, year of isolation, geographic distribution or source of infection.

In order to facilitate the investigation of the distribution and natural reservoir hosts of the

1. Introduction and literature review

1.1 Introduction

Crimean-Congo haemorrhagic fever (CCHF) virus is a tick-borne viral zoonosis found in Africa, Asia and eastern Europe within the distribution range of ticks belonging to the genus *Hyalomma*. The disease is being recognized with increasing frequency as a cause of illness in Africa, and this is probably due to increased awareness, greater surveillance and the availability of specific diagnostic tests, which are provided in South Africa by the Special Pathogens Unit at the National Institute for Virology in Johannesburg (Swanepoel *et al.*, 1983a; 1985a; b; 1987; Shepherd, 1987). From January 1980-December 1996 inclusive the Unit examined 3,804 specimens from 2,108 patients in southern Africa suspected to be suffering from viral haemorrhagic fever (VHF) and diagnosed 141 cases of CCHF. The case fatality rate fluctuated around 30% for the first few years after CCHF was initially recognized in southern Africa but it has now declined to 19,9% (28/141), probably because of earlier recognition and better management of patients in most instances.

Preliminary diagnosis of CCHF can be based on a history of possible exposure to the virus, plus the occurrence of characteristic signs and symptoms of illness (Swanepoel *et al.*, 1989). However, CCHF may be confused with other African VHFs as well as with a number of common disease conditions, and hence laboratory confirmation of the diagnosis is necessary. Moreover, since CCHF has a mortality rate of approximately 30% and a propensity for nosocomial spread, rapid diagnosis of the disease is important for

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did not demonstrate antibody response sufficiently early in the course of the infection to be clinically useful (Zavadova, *et al.*, 1971; Buckley, 1974; Casals and Tignor, 1974; Gaidamovich, *et al.*, 1974; Hoogstraal, 1979; Swanepoel *et al.*, 1983b).

The use of ELISA for CCHF serology was first reported by Donets *et al.* (1982) who described a double sandwich technique for antigen detection, indirect ELISA for antigen and antibody detection and solid phase radioimmunoassays for demonstrating antibody and antigen. ELISA have since been described for antigen detection in tick suspensions, and for detection of IgG and IgM antibody in human sera (Smirnova *et al.*, 1985; Saluzzo *et al.*, 1987; Shepherd *et al.*, *1989a*; Logan *et al.*, 1993). Virus antigen can be detected in tissues of infected humans by passive haemagglutination or ELISA, however the sensitivity appears to be dependent on the intensity of viraemia and hence antigen detection has not been used routinely for diagnosis of CCHF (Shepherd *et al.*, 1988).

The IF antibody test is a rapid technique that is more sensitive than the fluorescent-focus reduction, complement fixation or immunodiffusion tests (Burney *et al.*, 1980; Swanepoel *et al.*, 1983b; Shepherd *et al.*, 1989a). ELISA and IF tests can be used to detect both IgG and IgM antibody responses, and hence these tests can be used to distinguish past from recent or current infection. The IF antibody test is currently the most frequently used method for antibody detection. In fatal cases of CCHF, however, an antibody response is not generally demonstrable and diagnosis is dependent on isolation of virus or demonstration of antigen (Shepherd *et al.*, 1989a). Virus can be isolated from serum, or from liver samples taken after death with a biopsy needle.

cellular targets and distribution of CCHF virus in human tissues.

1.10 Laboratory diagnosis

A diagnosis of CCHF is generally confirmed in the laboratory by isolation of virus, demonstration of seroconversion or a ≥4-fold increase in antibody titre, or detection of specific IgM antibody activity. Virus can be isolated in 1-6 days in cell cultures, but the method is less sensitive for the isolation of low concentrations of virus than the use of suckling mice which, however, takes 6-9 days (Shepherd *et al.*, 1986). Virus antigen can be detected by passive haemagglutination or ELISA in ticks and in tissues of infected mice and humans, but positive results were obtained in only about half of the patients from whom sera were tested during the first two weeks of illness, with greatest success being attained in severe and fatal infections with the most intense viraemias (Donets *et al.*, 1982; Shepherd *et al.*, 1985; Smirnova and Karavanov, 1985; Shepherd *et al.*, 1986; Saluzzo and Le Guenno, 1987; Logan *et al.*, 1993).

Among the serological techniques originally used for studying the disease, neutralization tests suffered from the defect that nairoviruses in general, including CCHF, induce weak neutralizing antibody response and, furthermore, serum samples frequently contain non-specific inhibitors of virus infectivity. Haemagglutination-inhibition and reversed passive haemagglutination-inhibition assays did not find wide application because of technical difficulties encountered in preparing antigen and sensitized cells, and lack of reproducibility. Consequently, complement fixation and agar gel immunodiffusion tests were used most commonly prior to the 1980s, but the techniques lacked sensitivity and

decline gradually and stabilize, while IgM antibody activity declines to low or undetectable levels in most patients after 4 months.

1.9 Pathogenesis

The pathogenesis of the disease is not fully understood largely due to the lack of a suitable animal model, and because of the difficulties of studying the biohazardous virus in the laboratory. However, clinical pathology findings recorded in patients contribute to some understanding of the pathogenesis of the disease, and inferences can be drawn from other VHFs. What has been established is that disseminated intravascular coagulopathy is an early feature of the disease (Joubert, *et al*., 1985; Swanepoel, *et al*., 1989). It has been suggested that endothelial damage caused either directly by the cytolytic effects of the virus, or indirectly by cytotoxic immune response, could explain several features that are observed in CCHF patients. The endothelial damage to the capillary bed would lead to increased permeability and to the occurrence of a petechial rash, and would contribute to haemostatic failure through activation of the intrinsic coagulation cascade. However, further investigations are required to substantiate these suggestions. Histopathologic studies have been relatively few and limited to examination of specimens from a small number of patients (Baskerville *et al*., 1981; Joubert *et al*., 1985; Swanepoel *et al*., 1987) or experimentally infected mice (Smirnova *et al*., 1973). Examination of human tissues has revealed evidence of haemorrhage in a number of organs, focal to massive necrosis in the liver, and lymphoid depletion with cellular necrosis in the spleen. It has been speculated that some of the lesions may be due to infarction resulting from vascular thrombosis, and little information is available on the

al., 1987; 1989).

Treatment generally consists of supportive and replacement therapy based on clinical pathology findings. Immune plasma has been administered to CCHF patients, but there is no conclusive evidence to indicate that immunotherapy is beneficial (van Eeden *et al.*, 1985; Burt *et al.*, 1994). Viraemia has remained demonstrable in patients after intravenous administration of immune plasma with proven neutralizing ability (Swanepoel *et al.*, 1987). Ribavirin, an anti-viral agent, has been shown to inhibit CCHF virus replication *in vitro* in cell cultures, and *in vivo* using suckling mice as an animal model (Watts *et al.*, 1989b; Tignor and Hanham, 1993), and it has been administered to limited numbers of patients in South Africa and Pakistan, with some apparent success when used before day 5 of illness (Swanepoel *et al.*, 1990; Fisher-Hoch *et al.*, 1995).

1.8 Viraemia and antibody response

Virus has been isolated from the blood of human patients from day 1 to 12 of illness in fatal infections, with a maximum recorded intensity of $10^{8.7}$ mouse intracerebral 50% lethal doses ($MICLD_{50}$)/ml, but neither viraemia nor antigenemia has been demonstrated after day 9 in surviving patients (Shepherd *et al.*, 1986; Swanepoel *et al.*, 1987; Butenko and Chumakov, 1990). The antibody response of surviving patients differs markedly from that of non-survivors (Shepherd *et al.*, 1989a). An IgG and/or IgM response is generally demonstrable from days 7-9 onwards in non-fatal infections, whereas an antibody response is often not demonstrable in fatal infections. Both IgG and IgM antibody titres increase until the second or third week of illness, after which the IgG titres

develop a haemorrhagic tendency on day 4-5 of illness with ecchymoses, and episodes of epistaxis, haematuria, melaena and haematemesis, plus bleeding from injection sites, vagina and gums. Patients may also develop internal bleeding, including intracranial, gastrointestinal and retroperitoneal haemorrhages. The illness has a fatality rate of approximately 30% with deaths usually occurring 6-14 days after onset, generally as a result of multiple organ failure. Recovery usually begins on day 9-10 of illness but patients may remain asthenic for a month or longer (Hoogstraal, 1979; van Eeden *et al.*, 1985; Joubert *et al.*, 1985; Swanepoel *et al.*, 1987; 1989).

Patients generally have elevated levels of serum aspartate transaminase, alanine transaminase, creatine kinase, alkaline phosphatase, and lactate dehydrogenase early in the disease. There is also early leukopenia or leukocytosis and thrombocytopenia, particularly in severe infections, and by day 7 most patients have low platelet counts, which begin to recover during the second week of illness. Markedly abnormal values for prothrombin ratio, activated partial thromboplastin time, thrombin time, fibrinogen and fibrin degradation products may be recorded from an early stage of the disease, particularly in severe infections, suggesting the occurrence of disseminated intravascular coagulopathy. Haemoglobin levels decline early and may remain low during the second week of illness even in patients where overt haemorrhage is not a significant feature of the disease. Levels of creatinine, bilirubin and urea generally become elevated from the end of the first week of illness onwards. The occurrence of certain markedly abnormal clinical pathology values during the first 5 days of illness are indicative of a poor prognosis (Hoogstraal, 1979; van Eeden *et al.*, 1985; Joubert *et al.*, 1985; Swanepoel *et*

susceptible to infection. Guinea fowl were shown to undergo low-titred viraemia followed by a short-lived antibody response after experimental infection (Shepherd *et al.*, 1987b). Antibody to the virus has been detected in ostriches from farming areas in South Africa, Namibia and Zimbabwe, and viraemia has recently been demonstrated in ostriches after experimental infection (Shepherd *et al.*, 1987b; Swanepoel *et al.*, 1997; unpublished laboratory data). Most recently, the phenomenon of non-viraemic transmission of CCHF virus to *H. m. rufipes* ticks while attached to ground feeding birds has been demonstrated in West Africa (Zeller *et al.*, 1994), suggesting that birds may well play a role in the amplification of infection, and that even non-migratory species may disseminate infected ticks locally.

1.7 Clinical features and treatment

The virus of CCHF is transmitted to humans through tick-bite and from contact with viraemic blood or other tissues of infected livestock or patients (Hoogstraal, 1979; Swanepoel *et al.*, 1987). Short incubation periods of 2-3 days (up to 7 days) have been recorded after exposure to tick bite, whereas infection through contact with viraemic blood or other infected tissues is associated with slightly longer incubation periods of 5-9 days (Swanepoel *et al.*, 1987). Onset of illness is sudden, with severe headache often accompanied by fever, myalgia, lower backache, dizziness, tachycardia, neck pain and stiffness, sore eyes and photophobia. Nausea, sore throat, vomiting, abdominal pain and diarrhoea are usually present early in the disease and there is a tendency for patients to experience sudden changes of mood. After 3-6 days a petechial rash may appear on the trunk and limbs, and on the throat, tonsils and buccal mucosa. Patients frequently

mammals such as hedgehogs, little susliks and hares have been implicated as potential reservoir vertebrate hosts of CCHF virus in laboratory studies (Hoogstraal, 1979; Reehav, 1986; Shepherd *et al.* 1989c). Sheep and cattle have been shown to develop a low grade viraemia that is short-lived, but can be a source of infection for ticks, and for humans exposed to fresh blood and other infected tissues (Hoogstraal, 1979; Watts *et al.*, 1989a; Shepherd *et al.*, 1991). The viraemia is followed by a specific antibody response, and seroepidemiological surveys of domestic animals have been useful in establishing the distribution and prevalence of CCHF infection (Hoogstraal, 1979; Watts *et al.*,1989a; Shepherd *et al.*, 1991; Wilson *et al.*, 1991). The role of wild mammals as reservoir hosts is unknown, although Shepherd *et al.*(1987a) did show that antibody was most prevalent in large mammals in the orders Artiodactyla and Perissodactlya, which probably reflects the feeding preferences of adult *Hyalomma* tick species. It has been suggested that the amplification of virus circulation in the environment is largely due to the infection of immature ticks while feeding on small mammals; the virus persists transstadially and is subsequently transmitted to the hosts of adult ticks. The role of large mammals as amplifying hosts is probably less significant because transovarial transmission rates appear to be low (Watts *et al.*,1989a).

Although birds are important hosts of immature ticks, and migratory species may serve to disseminate the virus through the translocation of transovarially infected immature ticks (Hoogstraal, 1979), their role as amplifying agents in nature is uncertain. It was initially believed that birds were refractory to infection with CCHF virus (Hoogstraal, 1979; Watts et al., 1989a). However, there is evidence that some species may be

marginatum rufipes, *H. m. marginatum*, *H. truncatum*, *H. dromedarii*, *H. impeltatum*, *H. rufipes*), *Rhipicephalus* (*R. evertsi mimeticus*, *R. appendiculatus*, *R. rossicus*), *Amblyomma* (*A. hebraeum*, *A. variegatum*), and *Dermacentor marginatum* (Levi and Vasilenko, 1972; Zgurskaya *et al.*, 1971; 1975; Kondratenko, 1976; Shepherd *et al.*, 1989b; Logan *et al.*, 1989; 1990; Wilson *et al.*, 1991; Okorie *et al.*, 1991; Gonzalez *et al.*, 1991; 1992; Gordon *et al.*, 1993). However, higher viral titres and longer persistence of the virus were obtained in experimentally infected ticks of the genera *Hyalomma* and *Rhipicephalus* than in *Amblyomma* (Shepherd *et al.*, 1989b). The geographic distribution of CCHF virus and the evidence from vector competence studies suggest that ticks belonging to the genus *Hyalomma* are the principal vectors. Transovarial transmission rates appear to be low (Watts *et al.*, 1989a). Haematophagus arthropods other than ticks have not been implicated as vectors of CCHF virus (Hoogstraal, 1979).

1.6 Vertebrate hosts

The majority of ticks that have been associated with CCHF infection are 2 or 3 host ticks and the prevalence of CCHF antibody in mammals reflects the feeding preferences of different stages of the life cycle of known vector species (Hoogstraal, 1979; Watts *et al.*, 1989a; Shepherd *et al.*, 1987a). The larvae and nymphs of the principal vectors, *Hyalommas*, feed on small mammals and ground feeding birds, and the adult ticks feed on larger mammals, including domestic animals and man. The role of small and large mammals as possible reservoir hosts has not been studied extensively. To establish that an animal can act as a reservoir host it must be shown to undergo viraemia of sufficient intensity to exceed the threshold level required for infecting ticks. Certain small

al., 1984; 1985; Butenko and Chumakov, 1990; Schwartz *et al*., 1995, Scrimgeour, 1996). Virus has been isolated from ticks or non-human mammals in Madagascar, Senegal, Nigeria, Central African Republic, Kenya, Ethiopia, and Greece (Woodall *et al*.,1967; Causey *et al*.,1969; Wood *et al*.,1978; Hoogstraal, 1979; Fontenille *et al* .1988; Mathiot *et al*., 1988; Camicas *et al*., 1991; Morrill *et al*.,1991). Serological evidence alone has been reported from Hungary, Portugal, Turkey, Egypt, India, Afghanistan, and Iran (Saidi *et al*.,1975; Casals, 1978; Hoogstraal, 1979; Morrill *et al*.,1990). Apart from a focus in Moldavia, and Madagascar, the known distribution of the virus coincides with that of ticks of the genus *Hyalomma* (Hoogstraal, 1979), and the implication is that it may yet be found in further countries within this range.

1.5 Vectors

The virus has been isolated from 31 species of ticks, including 29 ixodids (11 *Hyalomma*, 9 *Rhipicephalus*, 4 *Boophilus*, 2 *Dermacentor*, 1 *Amblyomma*, 1 *Haemaphysalis* and 1 *Ixodes*) and 2 argasids (belonging to the genera *Argas* and *Alveonasus*) (Hoogstraal, 1979; Swanepoel *et al*., 1983a; Watts *et al*., 1989a; Shepherd *et al*., 1989b; Camicas *et al*., 1990). However the importance of the majority of these ticks in the maintenance and transmission of the virus is uncertain. Experimental studies suggest that the virus is primarily transmitted by hard ticks (ixodids), and that isolations from soft ticks (argasids) are possibly due to the presence of virus in the blood meal after feeding on viraemic hosts; the virus does not appear to replicate in argasids (Shepherd *et al*., 1989b; Durden *et al*.,1993). There is convincing evidence from replication and transmission studies of the vector potential of various species of *Hyalomma* (*H*

and the L segment encodes the viral transcriptase, as is the case with other members of the *Bunyaviridae* family (Ward *et al.*, 1990; Marriott *et al.*, 1992; Marriott and Nuttall, 1992; 1996). Alignment of the amino acid sequences predicted from the nucleotide sequences of the S segments of CCHF, Hazara and Dugbe shows that 39.5% of the amino acids are conserved throughout the 3 viruses, and this suggests that the S segments of CCHF and Hazara also encode the N protein. The G1 and G2 proteins are believed to be associated with haemagglutinin activity and to serve as targets for neutralizing antibody, and the G1 protein of Dugbe virus has been shown to be involved in the process of infection of vertebrate host cells (Buckley *et al.*, 1990). The N protein induces production of complement-fixing antibody (Bishop and Shope, 1979).

CCHF virus has been shown to be highly labile. It is sensitive to lipid solvents, has a half-life of 2-3 h at 37 °C and less than 1 min at 56 °C, and rapidly loses infectivity outside the pH range 6-9.5 (Donets *et al.*, 1977; Smirnova, 1979).

1.4 Distribution

The geographic distribution of CCHF virus has been extensively reviewed by Hoogstraal (1979). The virus is widely distributed in Asia, eastern Europe and Africa. Cases of naturally acquired human infection have been documented in the former Soviet Union, Bulgaria, Yugoslavia, Pakistan, Iraq, United Arab Emirates, Oman, Tanzania , Zaire, Uganda, Mauritania, Burkina Faso, South Africa, and Namibia (Simpson *et al.*, 1967; Chumakov *et al.*, 1970; Hoogstraal, 1979; Burney *et al.*, 1980; Suleiman, 1980; Al-Tikriti *et al.*, 1981; Gear *et al.*, 1983; Swanepoel *et al.*, 1983a; 1987; 1989; Saluzzo *et*

respectively, and the nucleocapsid protein, N, with a molecular weight of 48-54 x10 daltons. A fourth protein, with a molecular weight of 200 x10^3 daltons, can sometimes be detected and is thought to be associated with the polymerase activity (Clerx *et al.*, 1981). The genome of the nairoviruses consists of 3 segments of single-stranded, negative sense RNA, which are designated L (large), M (medium) and S (small), and are ·ed approximately 4.1-4.9 x10^6, 1.5-1.9 x10^6 and 0.6-0.7 x10^6 daltons respectively. Each segment has conserved sequences at the 3' terminal with complementary (palindromic) sequences at the 5' end (Clerx *et al.*, 1981; Clerx-van Haaster *et al.*, 1982).

The nairoviruses constitute the least well characterised genus of the *Bunyaviridae*. However, there have been some recent studies on the replication and coding strategy of Dugbe virus, a member of the Nairobi sheep disease (NSD) serogroup (Ward *et al.*, 1990a). The NSD serogroup includes NSD virus, a pathogen of livestock in eastern and central Africa, and Dugbe, a tick-borne virus isolated from cattle sera and ticks in Nigeria, Ethiopia and Central African Republic. Dugbe virus has been associated with human illness resulting from naturally acquired infection in Nigeria and Central African Republic (Karabatsos, 1985). Serological studies have shown that members of the NSD serogroup cross-react only with members of the CCHF serogroup, and s le homologies between the S segments of Dugbe, CCHF and Hazara viruses confirm that the two serogroups are more closely related than other serogroups of the genus (Davies *et al.*, 1978; Casals and Tignor, 1980; Marriott *et al.*, 1990; Marriott and Nuttall, 1992; Ward *et al.*, 1992). It has been shown that the S RNA segment of the genome of Dugbe virus encodes the nucleoprotein, the M segment encodes the G1 and G2 glycoproteins

techniques (Casals and Tignor, 1980; Calisher and Karabatsos, 1988; Zeller *et al.*, 1989). CCHF belongs to a serogroup of the same name and is the only member of the group that is known to cause human illness. The two other members of the serogroup are Hazara and Khasan. Hazara is the closest known antigenic relative of CCHF virus, and was isolated from *Ixodes redikorzevi* ticks in Pakistan (Begum *et al.*, 1970; Smirnova, 1979). Khasan was isolated from *Haemaphysalis longicornis* ticks in the former USSR (Lvov *et al.*, 1978). The classification of the bunyaviruses was originally based on the results of serological investigations; however the groupings have subsequently been substantiated by comparison of morphological characteristics and molecular analyses (Casals and Tignor, 1980).

The nairoviruses have properties similar to other members of the family *Bunyaviridae* (Donets *et al.*, 1977; Bishop *et al.*, 1980; Clerx *et al.*, 1981; Schmaljohn, 1996). Members of the family are lipid-enveloped, spherical structures, 90-120 nm in diameter with glycoproteins projecting from the surface of the envelope. Electron microscopy studies have shown that the viruses replicate on the endoplasmic reticulum of the cytoplasm of infected cells and mature by budding into smooth-surfaced vesicles in or near the region of the Golgi apparatus. Mature virions are released at the plasma membrane by exocytosis (Murphy *et al.*, 1973, Korolev *et al.*, 1976; Donets *et al.*, 1977; Ellis *et al.*, 1981).

The three major structural proteins of nairoviruses are two surface glycoproteins, designated G1 and G2, with molecular weights of 72-84 $x10^3$ and 30-40 $x10^3$ daltons

2.2.5 RT-PCR

For cDNA synthesis, 2 pmol of primer F2 was added to RNA in a total volume of 10 µl, heated for 3 min at 95 °C and cooled for 3 min at 42 °C. To each reaction tube 10µl of a stock solution was added so that each tube received 1 µl RNase inhibitor (40 U/µl) (Boehringer Mannheim, Mannheim, Germany), 2 µl dNTP stock(10mM each dATP, dCTP, dGTP, dTTP) (Boehringer Mannheim), 4 µl 5x reaction buffer (250mM Tris-HCl pH 8.3, 375mM potassium chloride, 15mM magnesium chloride), 2 µl 0.1M dithiothreitol and 1 µl Moloney Murine Leukemia Virus Reverse Transcriptase (200 U/µl), (Gibco BRL). The tubes were incubated for 30 min at 42 °C, heated at 95 °C for 3 min, transferred to ice for 3 min, spun briefly in a microfuge and returned to ice.

For DNA amplification, 5 µl of first-strand cDNA synthesis product was added to a reaction mixture containing 2 µl primer F2 (100 pmol/µl), 2 µl primer R3 (100 pmol/µl), 10 µl 10x reaction buffer (500mM potassium chloride, 100mM Tris-HCl pH 9.0, 1% Triton X-100), 1 µl Taq DNA polymerase (5 U/µl), (Promega Corporation, Madison, WI, USA), 6 µl 25mM magnesium chloride, 2 µl dNTP stock (10mM each dATP, dTTP, dCTP, dGTP) and 72 µl nuclease free water. The reaction mixture was overlayed with 75 µl mineral oil and amplified on a Biometra thermal cycler for 30 cycles of 30 sec at 95 °C, 30 sec at 47 °C, and 30 sec at 72 °C followed by a 5 min incubation at 72 °C.

2.2.6 Radio-labelling of CCHF specific probe

A CCHF specific probe was prepared using a pBluebac plasmid containing a 1510 base pair (bp) insert of the S segment of CCHF virus (supplied by the Virology Division,

2. Detection of CCHF viral nucleic acid using a reverse transcription-polymerase chain reaction

2.1 Introduction

The IF test can generally detect antibody to CCHF virus by day 7-9 of illness in non-fatal infections, and occasionally as early as day 4 (Swanepoel *et al.*, 1987; Shepherd *et al.*, 1989a). However, in fatal cases antibody is often not demonstrable. Virus can be isolated in 1-6 days in cell cultures, but the method is less sensitive for the isolation of low concentrations of virus than the use of suckling mice which, however, takes 6-9 days to achieve (Shepherd *et al.*, 1986). Viral antigenaemia can be detected by ELISA in a high proportion of fatal cases, but antigen may be undetectable in the serum of about half of patients with non-fatal infection (Shepherd *et al.*, 1988). Thus, there are residual difficulties in making a rapid diagnosis during the acute phase of illness, and it was decided to evaluate the use of an RT-PCR procedure, which amplifies viral nucleic acid present in low concentrations.

The pair of oligonucleotide primers to be used in the RT-PCR procedure were selected from conserved regions of the S RNA segment of 7 geographically distinct CCHF isolates, and had previously been used to amplify viral RNA extracted from infected cells (Lofts *et al.*, 1994; Schwarz *et al.*, 1996; Rodriguez *et al.*, 1997). The primers target a region of the S segment of the viral genome which encodes the nucleocapsid protein, which has been shown to be the most type-specific protein of the virus in monoclonal antibody studies (Smith *et al.*, 1991).

diluted 1/1,000 was added to the wells and incubated for 1 h at 37 °C. The enzyme activity was reacted with azino-di-(3-ethyl-benzthiazoline-6-sulfonate) (ABTS) (Kirkgaard & Perry Laboratories Gaithersburg, Md. USA) substrate for 30 min in the dark at room temperature. The results were determined by reading the optical density at 402 nm on a Multiscan spectrophotometer (Flow Laboratories Inc., Mclean, Va., USA). Specimens were considered positive if the absorbance was at least twice that of the negative control serum.

2.2.9 Isolation of CCHF virus

Infectious virus was isolated from the sera of human patients by inoculation of Vero 76 cell cultures, plus intracerebral inoculation of 0.02 ml of serum in 1 day-old mice (Swanepoel *et al.*, 1983a). Duplicate cell cultures were examined on days 3 and 6 of incubation by immunofluorescence with reference CCHF antiserum. Mice were observed for 14 days and brain smears from dead mice were examined by immunofluorescence. Serum from the experimentally infected mice was inoculated into infant mice only, and brain material from mice that died was homogenised as 10% weight/volume suspension in Liebowitz L-15 media, centrifuged at 10,000xg for 10 min, and the supernatant fluids were tested for CCHF antigen using complement fixation tests.

2.2.10 Complement fixation tests

Complement fixation tests were performed by a modification of the 4-volume method of Bradstreet and Taylor (1962). The tests were performed in round-bottomed 96-well microplates (Bibby Sterilin, Staffordshire, UK) and test volumes of 25 µl were used.

DNA was transferred onto Hybond-N+ nylon filters (Amersham). The DNA was cross-linked by exposing the filters to ultra-violet light for 1 min. The Southern blots were prehybridized in hybridization buffer (6x SSC, 0.5% SDS, 5x Denhardts solution, 100 µg/ml denatured salmon sperm DNA) (Maniatis *et al.*, 1982) for 30 min at 68 °C and hybridized overnight at 68 °C in hybridization buffer containing 10 ng denatured [α-^{32}P]-dCTP labelled DNA/ml buffer (specific activity approximately 1.5×10^{9} cpm/µl). The filters were washed in 2x SSC and 0.5% SDS at room temperature for 5 min followed by a 15 min wash at room temperature in 2x SSC and 0.1% SDS. The filters were subsequently washed in 0.1x SSC and 0.5% SDS for 2 h at 68 °C, the buffer was replaced with fresh buffer and the filters washed for a further 30 min. The filters were then dried at room temperature and the bound probe was visualised by exposing the membranes to β-Max Hyperfilm (Amersham) overnight.

2.2.8 CCHF antigen detection ELISA

An indirect ELISA was used to detect CCHF viral antigen in the sera of infected mice. The 96 well immunoassay microplates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with a monoclonal antibody 6D5 diluted 1/1,000 in carbonate buffer pH 9.6 (Blackburn *et al.*, 1987). Volumes of 100 µl were used unless stated otherwise. The diluent was 10% foetal calf serum in PBS pH 7.4 and plates were washed thrice with PBS containing 0.1% Tween 20. The wells were post-coated with 200 µl 2% bovine serum albumin (Calbiochem, La Jolla, CA, USA) in PBS for 1 h at 37 °C. Mouse sera diluted 1/2 were added to the wells and incubated for 2.5 h at 37 °C. The plates were washed, rabbit anti-CCHF horse radish peroxidase conjugate (refer to Section 3.2.3)

magnesium chloride and 2-mercaptoethanol) in a total volume of 100 µl. The mixture was incubated at 15 °C for 2 h. The percentage of incorporated label was determined by trichloroacetic acid (TCA) precipitation. A 1 µl aliquot of labelled probe was blotted onto a GF/C glass microfibre filter (Whatman International Ltd., Maidstone, UK) and 1 µl of probe was added to a tube containing 5 ml cold 10% TCA, chilled for 15 min on ice and the precipitate collected by filtering through a glass microfibre filter. The filters were washed thrice under vacuum with 3 ml 5% TCA solution and 3 ml of 70% ethanol. The radioactivity was determined for each filter using a liquid scintillation counter and the amount of [α-^{32}P]-dCTP incorporated into nucleic acids was calculated.

2.2.7 Analysis and detection of PCR products

2.2.7.1 Ethidium bromide staining of agarose gels

Aliquots of 10 µl of PCR products were electrophoresed on 1.2% agarose gels (FMC BioProducts, Rockland, ME, USA), containing 1µg/ml ethidium bromide, in Tris-acetate buffer (TAE). The DNA bands were visualised on a UV-transilluminator and the size of the DNA fragments was estimated by comparison with molecular weight marker VI (pBR328 DNA cleaved with *Bgl* I and pBR328 DNA cleaved with *Hin*f I), (Boehringer Mannheim).

2.2.7.2 Southern blot analysis

PCR products were separated on agarose gels as described in Section 2.2.7.1. The gels were soaked in a solution of 1.5M sodium chloride and 0.5M sodium hydroxide for 1 h and then soaked in 1M Tris-HCl buffer pH 8.0 and 1.5M sodium chloride. The denatured

USAMRIID). The plasmid was amplified by growing the bacteria overnight at 37 °C in 150 ml Luria-Bertani broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% sodium chloride, pH 7.5) containing 100 µg/ml ampicillan. The cells were harvested and lysed in 8 ml of lysozyme solution (2 mg lysozyme/ml buffer containing 50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8.0) on ice for 30 min. Two volumes of 0.2M sodium hydroxide containing 1% sodium dodecyl sulphate (SDS) were added to the cell suspension which was kept for on ice for 5 min, and 12 ml 3M sodium acetate pH 4.8 was added and the mixture held for a further 30 min on ice. The cell suspension was centrifuged for 10 min at 15,300xg and the nucleic acids precipitated from the supernatant fluid using 2 volumes of 96% ethanol and incubation at -70 °C for 1 h. The precipitate was resuspended in 12 ml 1x Tris-EDTA (TE) buffer (10mM Tris, 1mM EDTA pH 8.0) and clarified at 500xg for 5 min. The RNA was precipitated by adding 5 ml 7.5M ammonium acetate and holding for 30 min on ice. The solution was centrifuged at 15,300xg for 10 min and the DNA precipitated from the supernatant fluid with 2 volumes of ethanol at -70 °C for 1 h. The precipitate was washed with 80% ethanol followed with a wash in absolute ethanol, dried and resuspended in 1 ml 1x TE buffer. The DNA was quantitated spectrophotometrically assuming that an optical density reading of 1 at a wavelength of 260nm corresponds to approximately 50 µg/ml for double-stranded DNA (Maniatis *et al.*, 1982). The probe was labelled with [α-^{32}P]-dCTP (Amersham International plc, Buckinghamshire, UK) using a nick translation kit according to the manufacturers instructions (Amersham). The reaction mixture contained 10 µg of DNA to be labelled, 20 µl [α-^{32}P]-dCTP (10 mCi/ml), 10 µl enzyme solution (5 units DNA polymerase I, 100 pg DNAse I in Tris-HCl buffer pH 7.5 containing

2.2.4 RNA extraction

Total RNA was extracted from specimens using the acid guanidium thiocyanate-phenol-chloroform method as described by Chomczynski and Sacchi (1987). Briefly, 100 µl aliquots of mouse or human sera were mixed sequentially with 500 µl of lysis buffer (4M guanidium thiocyanate, 25mM sodium citrate pH 7.0, 5% sarcosyl and 0.1M 2-mercaptoethanol), 1 µg yeast tRNA, 100 µl 2M sodium acetate pH 4.0, 500 µl water saturated phenol and 100 µl chloroform:isoamylalcohol (49:1) and incubated on ice for 15 min. The samples were centrifuged at 1,800xg for 20 min at 4° C, the aqueous phase was removed and the RNA was precipitated with an equal volume of isopropanol at -20° C for 2 h. The RNA was recovered by centrifuging the samples at 12,000xg for 20 min at 4° C and the pellet washed twice with chilled 70% alcohol, dried and resuspended in 9 µl nuclease free water.

RNA was extracted from cell cultures using the same method. The cells were washed once with PBS pH 7.4, harvested in 5 ml of lysis buffer and total RNA was extracted from the infected cells using the acid guanidium thiocyanate-phenol-chloroform, method. RNA was quantitated spectrophotometrically assuming that an optical density reading of 1 at a wavelength of 260 nm corresponds to approximately 40 µg/ml of single-stranded RNA (Maniatis *et al.*, 1982). RNA was aliquoted and stored in 2.5M ammonium acetate and 2.5 volumes of ethanol. Before use, the RNA was centrifuged for 10 min at 12,000xg, the pellet was washed once with chilled 70% ethanol and dried. The pellet was resuspended in RNase free water at a concentration of 1 µg/µl and 5 µg was used in the RT-PCR reaction.

essential amino acid medium with Earle's balanced salt solution (EMEM) (Gibco BRL, Life Technologies, Uxbridge, UK) supplemented with 10% foetal calf serum (State Vaccine Institute, South Africa), 0.5% gentamycin sulphate (Duchefa, Haarlem, Netherlands) and 0.25% fungizone (amphotericin B) (Squibb, Princeton, NJ, USA) at 37 °C. Infected cultures were maintained in EMEM containing 2% foetal calf serum. Vero cells were grown in 150cm² culture flasks and infected with CCHF isolate SPU 497/89 which had been isolated from a human patient. After incubation of the infected cultures at 37 °C for 4 days, the medium was discarded and the cells washed in phosphate buffered saline, pH 7.4 (PBS), harvested, and total RNA was extracted as described below (Section 2.2.4). Negative control RNA was prepared from uninfected Vero 76 cells by the same method. For each RT-PCR assay that was performed using mouse or human sera the controls included, a serum sample from an uninfected mouse or human, water to control for reagent contamination, RNA extracted from uninfected cells and RNA extracted from CCHF infected cells.

2.2.3 Oligonucleotide primers

The nucleotide sequences for the pair of primers used in the PCR were supplied by the Virology Division, US Army Medical Research Institute for Infectious Diseases (USAMRIID), Fort Detrick, Frederick, Md, USA (Loffs *et al.*, 1991; Smith *et al.*, 1991). The forward primer designated F2: 5' TGG ACA CCT TCA CAA ACT C 3' is complementary to S segment viral RNA between nucleotide positions 135-153 relative to the positive sense strand of CCHF virus reference strain 10200, and the reverse primer designated R3: 5' GAC AAA TTC CCT GCA CCA 3' is complementary to the message sense between nucleotide positions 670 and 653.

stock virus by subcutaneous inoculation of additional mice, and clarified 10^{9}% liver suspension prepared from these mice was stored in small volumes at -70 °C.

2.2.2 Specimens

Litters of day-old mice were inoculated subcutaneously with approximately 10^{7} mouse intraperitoneal 50% lethal doses of stock virus SPU 265/88. (Animal ethics clearance certificate 95/94/3). Five mice were sacrificed and bled each day from day 1 post-inoculation to day 8, after which all of the remaining mice had succumbed to the infection. Mouse serum samples were cultured for isolation of infectious virus as described below, and stored at -70° C until all were tested simultaneously for viral RNA by RT-PCR, and for antigen by ELISA after day 8.

Eighty serum samples received from 1987-1992 from 45 patients in whom a diagnosis of CCHF was confirmed by isolation of virus and/or demonstration of a specific antibody response, were tested retrospectively by RT-PCR after they had been stored at -70 °C for up to 6 years, while a further 47 specimens from 38 patients received between 1993 and 1996 were tested prospectively at the time they were submitted to the laboratory for the diagnosis of suspected VHF. The stored sera had been frozen and thawed on several occasions for use in unrelated studies over the years.

Positive control viral RNA for the RT-PCR procedure was prepared from monolayers of Vero 76 cell cultures (American Tissue Type Collection, [ATTC] CRL No. 1587). Vero cells were obtained as required from stock cultures maintained in Eagle's minimum

As a preliminary, the detection of viral RNA by RT-PCR was compared to isolation of virus and detection of viral antigen by ELISA in the serum of experimentally infected mice. It was known that infant mice develop viraemia following intracerebral inoculation with CCHF virus, and that they are less susceptible to infection by peripheral route (Shepherd *et al.*, 1988; Hoogstraal, 1979). Nevertheless, it was decided to infect the mice subcutaneously with a CCHF isolate selected to be pathogenic by this route.

Following the feasibility study in mice, the RT-PCR assay was applied retrospectively to stored serum samples from confirmed CCHF patients, and prospectively to samples submitted from suspected CHF patients, which were tested immediately on arrival at the laboratory.

2.2 Materials and methods

2.2.1 Mice and stock virus

Litters of day-old, specific pathogen free, BALB/c mice were obtained from the South African Institute for Medical Research, Johannesburg. (Animal ethics clearance certificate number 90/141/6). Six virus isolates from CCHF patients in South Africa, in third mouse pass, were inoculated subcutaneously into litters of day-old mice in ten-fold dilutions of 10% weight/volume mouse liver suspension prepared in Liebowitz L-15 medium (Gibco BRL), and clarified by centrifugation at 10,000xg for 30 min at 5 °C. Liver was harvested from mice that succumbed to the highest dilutions of virus that were lethal, and subinoculated in ten-fold dilutions into further mice. Isolate SPU 265/88, which killed infant mice uniformly at a dilution of 10^{-8}, was selected for preparation of

East and West Africa and it could spread to eastern parts of South Africa where suitable mosquito vector species occur. Chikungunya is a mosquito-borne virus which occurs in parts of Africa where non-human primates serve as reservoir hosts, and in urban situations in parts of Asia. It generally causes outbreaks of benign febrile illness with joint pains, and has been associated with haemorrhagic disease on isolated occasions in Asia only.

Since CCHF is a tick-borne virus, other febrile diseases associated with ticks should be excluded as possible causes of illness in patients with a history of known or potential exposure to tick bite. Diseases which could be considered include tick bite fever caused by *Rickettsia conorii* or *R. africae*, Q fever (*Coxiella burnetii* infection), ehrlichiosis (*Ehrlichia* spp infection), borreliosis (*Borrelia* spp infection), or infection with *Babesia* spp. Furthermore, there are a number of viruses in Africa apart from CCHF, which are known or suspected to be tick-borne, and some of these, including Dugbe and NSD, have been associated with human disease, while the pathogenicity of others remains unknown, and warrants further investigation.

It should be borne in mind that the vast majority of suspected cases of VHF prove to be severe infections with more common agents, including bacterial septicaemias, malaria and viral hepatitis (Swanepoel, 1987), and in arriving at a diagnosis it is important to take into account an accurate history of possible exposure to infection, signs and symptoms of illness, and clinical pathology findings.

1.11 Differential diagnosis

Infection with CC cll' virus must be distinguished from disease caused by the other so-called formidable haemorrhagic fever viruses of Africa, including the filoviruses Marburg and Ebola, Rift Valley fever virus, arenaviruses and hantaviruses (Swanepoel, 1987). Marburg and Ebola viruses have no known arthropod vectors or vertebrate reservoir hosts, but are sometimes associated with human disease acquired from contact with sick or dead non-human primates in the more tropical countries to the north of South Africa. Rift Valley fever is a mosquito-borne virus infection of sheep and cattle which causes fatal disease in a small proportion of infected humans at irregular intervals of years when outbreaks of disease occur in livestock following heavy rains which favour the breeding of the vectors. Humans gain infection from contact with infected tissues of livestock or from mosquito bite. Arenaviruses cause chronic infection of rodents and humans gain infection from contact with virus excreted in rodent urine. The only African arenavirus to have been associated with haemorrhagic fever is Lassa fever virus of West Africa. Hantaviruses also cause chronic infection of rodents with excretion of virus in urine. They have been associated with human disease in Europe, Asia and the Americas, but have been poorly studied in Africa.

Yellow fever is a mosquito-borne virus which causes outbreaks of disease which may resemble the haemorrhagic fevers, but it occurs in West and East Africa and has never been diagnosed south of Angola. Dengue fever, caused by four serotypes of mosquito-borne virus, is generally associated with outbreaks of benign febrile illness, but fatal haemorrhagic infections can occur in a small proportion of patients. Dengue occurs in

Table 2.3. Detection of CCHF viral RNA by RT-PCR and Southern blot hybridization in 26 serum samples collected at various stages of illness from 19 CCHF patients and tested on arrival at the laboratory, shown in relation to isolation of virus and detection of antibody response.

Day of illness	PCR positive Virus positive	PCR positive Virus negative	PCR negative Virus positive	PCR negative Virus negative	Antibody positive	Total tested
3	1					1
4		1				1
5				1		1
6	4		1		2	5
7	3		1	1	5	5
8	6		1	2	8	9
9				2	2	2
11				1	1	1
12				1	1	1
	14	1	3	8	19	26

Table 2.2. Detection of viral RNA by RT-PCR and Southern blot hybridization in 80 serum samples which had been collected at various stages of illness from 45 confirmed CCHF patients and stored at -70 °C for up to 6 years, shown in relation to isolation of virus and detection of antibody response.

Day of illness	PCR positive Virus positive	PCR positive Virus negative	PCR negative Virus positive	PCR negative Virus negative	Antibody positive	Total tested
3	1	1		1		3
4	1	1			1	2
5	3			2	3	5
6	5	3	1	3	8	15
7	5	4			8	9
8	1	2		4	7	7
9		5		1	5	6
10		3			3	3
11	1	2	1	1	5	5
12		1		1	2	2
13	1	4		3	8	8
14		4	1	3	8	8
15		2		1	3	3
16		2		1	3	3
18				1	1	1
	18	34	6	22	65	80

Table 2.1. Detection of Crimean-Congo haemorrhagic fever virus in serum samples from experimentally infected mice using RT-PCR, isolation of virus and antigen detection in relation to day post-infection.

Day post-infection	No. of positive sera				No. of sera tested
	RT-PCR[1] 1	2	Virus isolation	Antigen detection	
1	1	3			5
2	1	2	2		5
3	5	5	5	1	5
4	4	4	5	4	5
5	5	5	5	4	5
6	4	5	5	5	5
7	5	5	4	5	5
8	3	3	3	5	5
	28	32	29	24	40

[1]RT-PCR products were detected by:
1. Ethidium bromide staining of agarose gels, and
2. Southern blot hybridization with radio-labelled probe.

2.3.2 Retrospective detection of CCHF viral RNA in stored human sera

A single DNA band corresponding to the predicted 536bp PCR product was detected in 25/80 (31.25%) stored serum samples from 18/45 (40%) confirmed CCHF patients in ethidium bromide stained agarose gels. Southern blotting of the gels and hybridization with a radio-labelled probe (Figure 2.1) confirmed the specificity of the assay and improved sensitivity markedly, resulting in detection of viral RNA in 52/80 (65%) sera from 32/45 (71.1%) patients. The findings in the RT-PCR assays are presented in Table

response. The methods included IF tests using fluorescein-labelled conjugates for the detection of IgG and IgM antibody, an IgM-capture ELISA, a sandwich ELISA for IgG antibody, and a competition ELISA (CELISA) which demonstrates total antibody activity.

3.2 Materials and methods

3.2.1 Antigens

Sucrose-acetone extracted antigen was prepared as described by Clarke and Casals (1958), from a stock of mouse brain from a previous experiment in which litters of 1 day old mice had been inoculated intracranially with 0.02 ml/mouse of CCHF virus isolate -/81 in the form of 10% mouse brain suspension. The brains of mice that died 8-9 days after inoculation were harvested, pooled and stored at -70 °C (Swanepoel *et al.*, 1983a). The organs were thawed and homogenised with 4 volumes of 8.5% sucrose solution. The homogenate was added drop-wise to 20 volumes of chilled acetone, with constant stirring after which the acetone was replaced with the same volume of fresh, chilled acetone and left for 1 h at 4 °C to dehydrate the precipitate. The acetone was removed and the deposit was ground in a small volume of acetone, centrifuged for 5 min at 500xg and the precipitate was left to dry for 1 h on a freeze drier before being rehydrated to the original volume of homogenate with 0.1M Tris buffer pH 9.0 and inactivated with 0.1% beta-propriolactone (BPL) (Sigma Chemical Company, St Louis, MO, USA) (Shope and Sather, 1979).

3.2.2 Antibodies

3. Serodiagnosis of Crimean-Congo haemorrhagic fever

3.1 Introduction

As discussed in sections 1.10 and 2.1 above, a clinical diagnosis of CCHF can be confirmed in the laboratory by isolation of virus, detection of viral antigen, demonstration of seroconversion or a ≥4-fold increase in antibody titre, or detection of specific IgM antibody activity. Chapter 2 reported investigation of the RT-PCR procedure for demonstration of viral RNA in serum, as an adjunct to isolation of virus and detection of antigen for rapid diagnosis of CCHF early in the course of the disease. The present chapter describes investigation of selected methods for making a rapid serodiagnosis of the disease.

Serological tests formerly used for the detection of antibody to the virus, such as complement fixation, immunodiffusion, haemagglutination-inhibition and reversed passive haemagglutination-inhibition lacked sensitivity and/or reproducibility (Casals and Tignor, 1974, Gaidamovich *et al.*, 1974; Swanepoel *et al.*, 1983a). The IF test, however, detects IgG and IgM antibody responses by day 7-9 of illness in all survivors of the infection, and occasionally even earlier (Shepherd *et al.*, 1989a). Specific IgG and IgM responses can also be detected by ELISA, but the tests have as yet been applied to very few patients (Saluzzo and Le Guenno, 1987, Shepherd *et al.*, 1989a).

In the present chapter, several serological methods were compared on sera from 101 confirmed CCHF patients, with particular reference to early detection of the antibody

blots and probes led to the detection of RNA in only 1 additional sample. No serum samples were available for testing from days 1 and 2 of illness, but viral RNA was detected in samples taken from day 3-16 of illness. Taking into account only the first serum sample received from each of 19 CCHF patients studied prospectively, the diagnosis could be confirmed in 12 instances by detection of viral RNA; in a further 6 instances the diagnosis was confirmed by detection of IgM antibody, and in the remaining patient the diagnosis was confirmed by demonstration of antibody seroconversion in a subsequent serum sample, before virus could be isolated from the first sample. No viral RNA was detected in 21 serum samples from 19 non-CCHF patients in whom alternative diagnoses were established.

In all instances where the RT-PCR failed to detect viral RNA in serum samples which yielded infectious virus in culture, it was subsequently confirmed that the PCR primers could amplify the nucleic acid of the isolates concerned in tests performed on infected cell cultures as part of a molecular epidemiology study (Chapter 5). Moreover, some of the isolates came from patients in whom a positive PCR result was obtained on an earlier serum sample.

2.4 Summary

The RT-PCR procedure detected CCHF viral nucleic acid in 32/40 serum samples collected during the first 8 days post-inoculation from experimentally infected infant mice; infectious virus was isolated from 29/40 samples, and viral antigen demonstrated in 24/40 samples. Viral RNA was first amplified from mouse serum taken on day 1 post-infection, 1 day earlier than infectious virus could be isolated, and 2 days before anti, became detectable. The presence of detectable viral RNA and infectious virus began to decline towards the end of the observation period, while antigen remained demonstrable in all sera on day 8. When the RT-PCR was applied retrospectively to serum samples which had been collected from confirmed CCHF patients and stored for up to 6 years at -70°C, and subjected to freezing thawing on several occasions, viral RNA was detected in only 15/80 samples on ethidium bromide stained gels, but the use of Southern blots with radio-labelled probes markedly increased the sensitivity of the assay and resulted in the detection of RNA in 52/80 samples. The increase in sensitivity was marginal when fresh serum samples were tested prospectively; positive RT-PCR results were detected on ethidium bromide gels in 14/26 sera from CCHF patients, while the use of Southern

solely on the basis of the detection of viral nucleic acid: in all instances positive RT-PCR findings were corroborated by isolation of virus and/or demonstration of an antibody response, involving either the same specimen or subsequent serum samples from the patient.

Taking into account only the first serum sample received from each of the 19 CCHF patients in order to compare the rapidity with which the diagnosis could be established by the various techniques, 12 were found to be positive by RT-PCR on ethidium bromide stained gels, and all of these 12 sera yielded virus in culture, while 8/12 also had IgM antibody activity indicative of current or recent infection. A further 6 sera were negative in RT-PCR tests and lacked infective virus, but had IgM antibody activity. The remaining serum sample was negative in RT-PCR tests and lacked demonstrable antibody, but subsequently yielded virus in culture. However, the diagnosis in the latter patient was established by demonstrating antibody seroconversion in a subsequent serum sample before virus could be isolated from the first sample.

No viral nucleic acid was detected in 21 serum samples received from 19/38 suspected viral haemorrhagic fever patients which were not found to be cases of CCHF, and alternative diagnoses including hepatitis A, rickettsial infection and bacterial septicaemias were established in these patients, either in the Special Pathogens Unit or in other laboratories.

2.3.4 Efficiency of the RT-PCR primers

2.2 in relation the day of illness on which the serum samples were taken, and the results obtained in virus isolation and antibody detection tests. Viraemia was demonstrable in a proportion of samples up to day 14 of illness, while viral RNA was detected up to day 16. Virus isolation and RT-PCR results were in agreement in 40/80 samples: 18 sera were positive by both techniques and 22 sera negative. However, the RT-PCR assay was positive on a further 34 samples in which no viraemia could be demonstrated, and virus was isolated from 6 sera in which no viral nucleic acid could be detected. Antibody response became detectable in a proportion of serum samples from day 4 of illness, and from day 8 onwards all samples were IgG and/or IgM antibody positive except for 1 taken on day 9 from a patient with fatal disease. No specimens were available from patients on days 1 or 2 after onset of illness.

2.3.3 Prospective application of RT-PCR as a diagnostic tool

Among the suspected viral haemorrhagic fever patients investigated prospectively, 19/38 proved to be cases of CCHF and the results obtained with the 26 serum samples received from these 19 patients are presented in Table 2.3. In contrast to the retrospective study, use of the Southern blot hybridization procedure on the fresh serum samples produced a positive result in only 1 more instance than did ethidium bromide staining of gels. Positive RT-PCR results were obtained in 15/26 (57.7%) sera and 12/19 (63.2%) patients. Nucleic acid was detected in 1 serum in which viraemia could not be demonstrated, and infective virus was isolated from 3 samples on which the RT-PCR assay was negative. Antibody was detected in a proportion of sera from day 6, and from day 9 onwards all samples were positive. None of the cases of CCHF was diagnosed

Figure 2.1 Demonstration, using Southern blot hybridization, of the 536 base pair (bp) PCR products amplified from the stored sera of 16 confirmed CCHF patients. Lane 1, CCHF virus positive control; lane 2, negative control; lanes 3-18, serum samples from CCHF patients.

read. Test specimens were considered CCHF antigen positive in wells where there was complete fixation of complement.

2.2.11 Indirect immunofluorescence tests

Human sera were tested for IgG and IgM antibody activity by IF at doubling dilutions from 1/8 upwards as described in section 3.2.5, using fluorescein-labelled anti-IgG or -IgM conjugate (Cappel, Organon Teknika nv, Turnhout, Belgium).

2.3 Results

2.3.1 Detection of CCHF viral RNA, infectious virus and antigen in mouse serum

The results of RT-PCR, virus isolation and antigen detection tests on mice sera are summarised in Table 2.1. A single DNA band corresponding to th predicted 536 bp PCR product was detected in 28/40 samples of mouse serum by ethidium bromide staining of agarose gels. Southern blotting of the gels and hybridization with the radio-labelled probe increased the sensitivity of the assay slightly, leading to detection of nucleic acid in 32/40 sera. There were 29/40 sera found to be virae n ic and 24/40 were CCHF antigen positive by ELISA. Viral nucleic acid was detected from day 1 post-infection onwards, infectious virus was isolated from day 2, and viral antigen was demonstrable by ELISA from day 3. The presence of detectable viral RNA and infectious virus began to decline in sera towards the end of the observation period, while antigen remained demonstrable in all sera on day 8.

Optimal dilutions of hemolytic serum and guinea pig complement were determined by chessboard titration, and the potency of the reference anti-CCHF hyperimmune mouse ascitic fluid (HMAF) was checked by chessboard titration against reference CCHF antigen. For preparation of sensitized cells, whole sheep blood collected at a dilution of 1/2 in Alsever's solution (2.05% dextrose, 0.42% sodium chloride, 0.8% trisodium citrate dihydrate, 10% citric acid) pH 6.1, was washed thrice with PBS and once with veronal buffered saline, pH 7.2 (VBS). The cells were resuspended in VBS at a final concentration of 4% and mixed 1:1 with rabbit anti-sheep erythrocyte haemolytic serum (Whittaker Bioproducts, Walkersville, Md, USA) pre-diluted to optimal concentration of 1/100.

For each mouse brain suspension to be tested for antigen, 2 rows of doubling dilutions of reference HMAF were prepared in microplates, starting at a dilution of 1/8. Brain suspensions at dilutions of 1/2 and 1/4 were added to the 2 rows respectively, together with equal volumes of complement diluted to contain three 100% lytic doses. Duplicate wells of brain suspension received VBS diluent in place of HMAF, and wells of HMAF at initial dilution received diluent in place of brain suspension, to act as controls for anti-complementary activity. The HMAF was also titrated in the presence of reference antigen at optimal dilution, and the potency of the complement was checked by testing doubling dilutions with 2 volumes of diluent in place of antigen and antibody. The tests were incubated at 4° C overnight, and next day the plates and sensitized sheep cells were incubated at 37° C for 30 min. 25 µl of cells were added to each well, the tests incubated at 37° C for 30 min, and the plates centrifuged at 200xg for 5 min before the results were

during the third week, while in the remaining 19 survivors there was a late rise in antibody level to maximum titre during the second to fifth month after onset of illness. Maximum IgG antibody titres recorded were nearly all in the range 256 to 4,096, but titres of 8,192 and 16,384 were determined in 2 patients, while in 1 patient there was a late increase in IgG antibody level to a titre of 32,768 at 16 months. Maximum IgG titres of 16 to 128 were recorded in a few survivors who were tested only during the first 2 weeks of illness. There was generally a 2-4-fold reduction in IgG antibody level within days of the maximum titre being attained, but thereafter levels declined gradually or stabilized, and 1 patient bled at 59 months still had a titre of 512. The only anomaly was that 1 patient lacked demonstrable IF IgG antibody on 1 occasion when tested a year after the onset of his illness.

The onset and early course of the IF IgM antibody response in all survivors of infection, plasma-treated and untreated, resembled the pattern of the IgG response in untreated survivors, with a slight tendency for IgM to become demonstrable a day or so earlier than IgG antibody in untreated patients, although the reverse also occurred in some individuals. As with IgG antibody, the attainment of maximum IgM antibody levels followed a bimodal pattern, with the highest titres in most patients being recorded towards the end of the second or into the third week of illness, but with a few peak titres being recorded 2–4 months after onset of illness. However, IgM antibody activity had declined to undetectable or minimal levels in most patients by 4 months, and thereafter IgM antibody was detected by IF on a single occasion in a patient who had an anomalous titre of 512 two years after the onset of illness (Figure 3.2). The maximum IF IgM

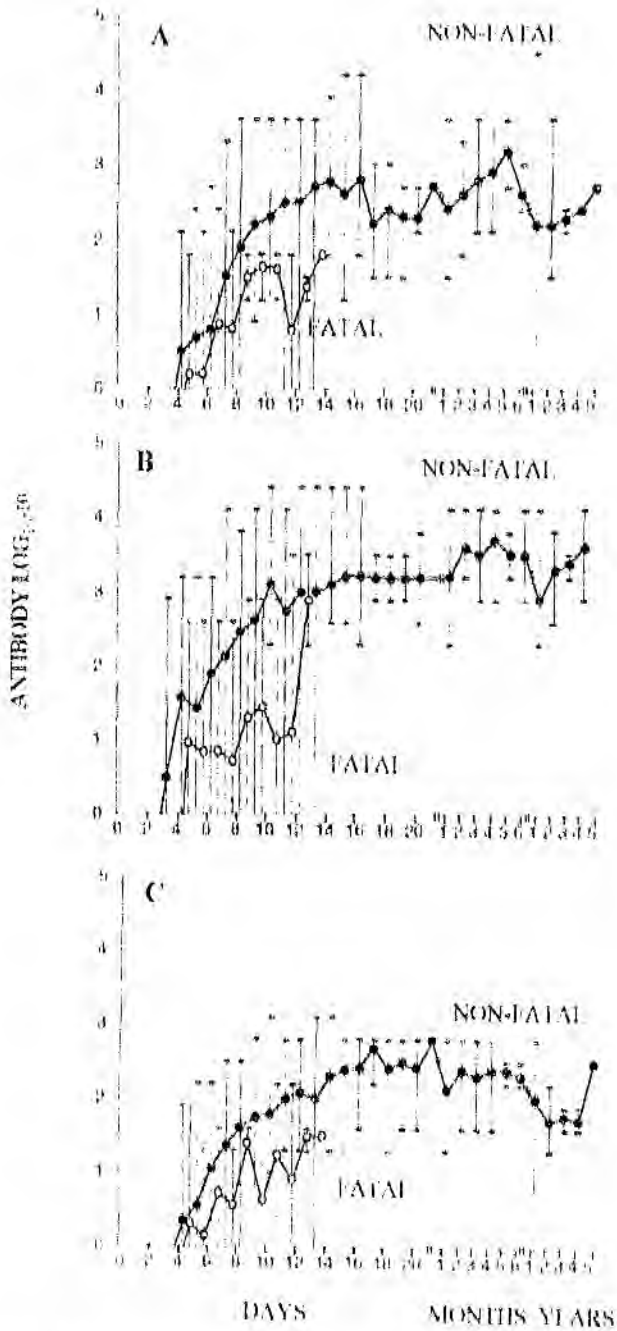


Figure 3.3. IgG antibody response in Crimean-Congo haemorrhagic fever patients detected by A. immunofluorescence; B. IgG sandwich ELISA and C. total antibody activity demonstrated by CFT ISA. Curves show geometric mean antibody titres and range at the indicated intervals after onset of illness.

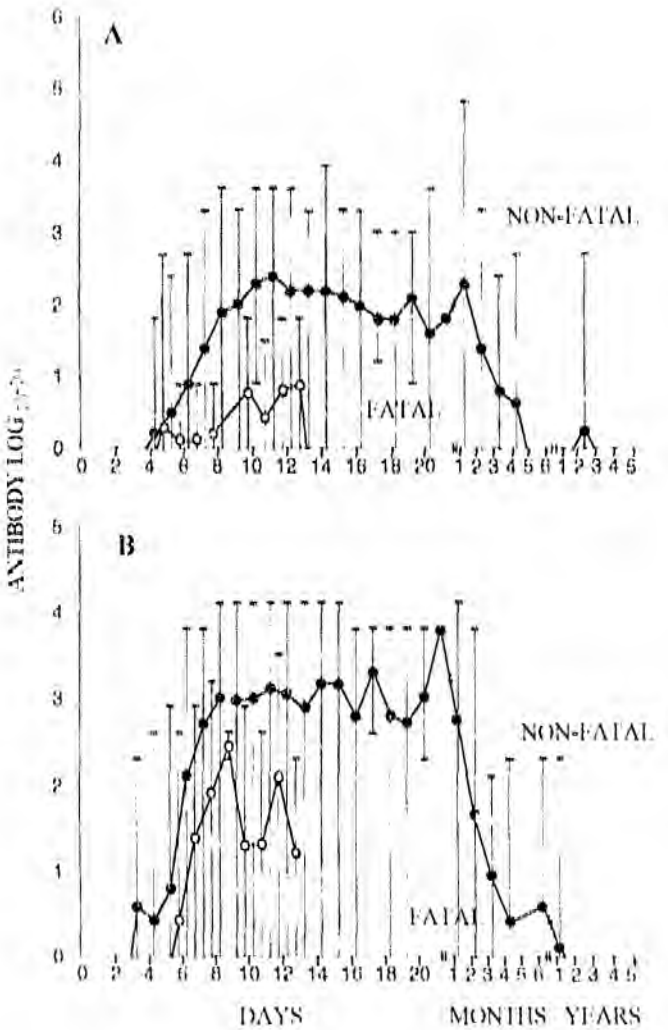


Figure 3.2. IgM antibody response in Crimean-Congo haemorrhagic fever patients detected by A. immunofluorescence and B. IgM-capture ELISA. Curves show mean antibody titres and range at the indicated intervals after onset of illness.

antibody activity or had minimal titres of 8, but had IF IgG titres of 256–4,096, and neutralizing (cell culture fluorescent-focus reduction) (Casals and Tignor, 1974; Shepherd *et al.*, 1989a) titres of 32–512. Recipients of immune plasma acquired demonstrable levels of serum IF IgG antibody activity within 2-4 hours of treatment. However, there was no corresponding immediate appearance of demonstrable IF IgM antibody activity in their sera, and treated patients seemed to acquire IgM antibody only from endogenous response at the same stage of illness as their untreated counterparts.

3.3.2 IF antibody response

No antibodies were demonstrable by IF in any sera taken during the first 3 days of illness (Figures 3.2A and 3.3A). Excluding those persons who received immune plasma (treated patients), only about 10% of survivors (range 1/12 to 2/13) tested on days 4 and 5 of illness had developed demonstrable IF IgM and/or IgG antibody activity. By day 6 11/17 untreated survivors tested had IgM antibody and 6 of them had IgG antibody demonstrable by IF. On day 7 IgG and/or IgM antibody was detected in 25/30 untreated survivors and this rose to 30/32 untreated survivors by day 8. All of 32 untreated survivors tested on day 9 had demonstrable IF IgM and IgG titres, but in a single patient IgG antibody continued to fluctuate between undetectable levels and a minimal titre of 8 during the first 3 weeks of illness, and in another patient IgM antibody behaved in the same way.

In 57 surviving patients (treated and untreated), the maximum IF IgG antibody titres were recorded towards the end of the second week of illness, particularly on day 11, or

In presenting the results of the serologic tests (Figures 3.2 and 3.3), distinction is made between fatal and non-fatal disease since the immune response differs markedly with the outcome of infection (Shepherd *et al.*, 1989a). A total of 25 patients died. The deaths of 23 patients on days 5-14 of illness were ascribed directly to the effects of CCHF infection, whereas 2 patients survived the acute illness and died later from other causes, and hence the results obtained on their sera are included with the findings on the remaining 76 survivors of the disease. One of these 2 patients was not initially recognized as a case of CCHF and was subjected to an operation for the drainage of cerebral haemorrhage; he removed a ventriculo-peritoneal bypass drainage tube himself and died on day 27 of illness from purulent meningitis associated with *Staphylococcus aureus* infection apparently gained through the surgical wound (Swanepoel *et al.*, 1985). Both IgM and IgG antibody responses were demonstrated in sera tested retrospectively, but no lesions or virological evidence of active CCHF infection were found at autopsy. The second patient had suffered for years from chronic bronchitis, coronary heart disease and hypertension for which he had frequently been treated in hospital; he appeared to recover from CCHF infection which had been confirmed by isolation of virus from serum and demonstration of an immune response, but died on day 24 of illness from pneumonia and a *Pseudomonas* septicaemia.

It must also be taken into account that 18 patients, 4 of whom died in the acute stage of illness plus 14 survivors, received intravenous treatment with 1 or more 250 ml units of immune plasma collected from recovered patients. Treatment with immune plasma was based on availability of the plasma. The plasma units lacked demonstrable IF IgM

doubling dilutions from 1/10 upwards, along with 50 µl of CCHF antigen diluted 1/100. The plates were incubated for 3 h at 37 °C with gentle mixing at 30 min intervals. After washing, anti-CCHF HRPO conjugate diluted 1/1,000 was added to the wells, left to react as described above and detected with ABTS substrate. The sera were considered positive if the optical density reading was ≥ 50% of that produced by human negative control serum (van der Groen *et al.*, 1989).

3.3 Results

3.3.1 Confirmation of the diagnosis in CCHF patients

Altogether, 2,998 specimens from 1,576 suspected cases of viral haemorrhagic fever were examined from January 1980-February 1992 inclusive. Among the 101 cases of CCHF encountered, the laboratory diagnosis was confirmed in 67 instances by isolation of virus from 1 or more serum samples taken during the first 13 days of illness, or from liver samples taken after death in fatal infections, together with the demonstration by IF of seroconversion or rising antibody titres in survivors of the disease. In a further 25 patients the laboratory diagnosis was based on the demonstration of seroconversion, or a ≥4-fold increase in IF antibody titres. In the remaining 9 patients the laboratory diagnosis depended on the demonstration of IF IgM antibody activity (at titres of 64-256) in single sera or repeat samples collected at close intervals, supported by a history of potential exposure to infection, the nature of the illness experienced by the patients, and other laboratory data (thrombocytopenia, haemostatic derangement and raised serum levels of transaminases and bilirubin).

wells. After further incubation and washing, anti-CCHF HRPO conjugate, diluted 1/1,000, was added to the wells and the plates were incubated. After further washing, the substrate, ABTS (Kirkgaard & Perry Laboratories) was added and the plates incubated at room temperature (22°C) for 30 min in the dark. The results were determined by reading the optical density at 402 nm. Specimens were considered to be IgM positive if the absorbance in the sample wells was at least twice the value of that for human negative control serum. Titres were recorded as reciprocals of the highest dilutions of test sera giving a positive result.

3.2.6.2 IgG sandwich ELISA

The IgG antibody response was measured by sandwich ELISA in which the plates were coated overnight at 4°C with monoclonal antibody 6F5 diluted 1/2,000 in carbonate buffer, pH 9.6. After post-coating, antigen diluted 1/200 was added to wells, the plates were incubated, washed and test sera were added in doubling dilutions from 1/100 upwards. The plates were incubated, washed and anti-human IgG HRPO conjugate (Zymed) was added at a dilution of 1/1,000. After further incubation and washing, substrate was added, allowed to react and the results recorded as above.

3.2.6.3 CELISA

Total antibody activity was determined in a CELISA in which test sera competed with coating antibody for binding of antigen, with immobilized antigen being detected by anti-CCHF HRPO conjugate. Plates were coated overnight at 4°C with monoclonal antibody 6F5 diluted 1/7,000. After post-coating, 50 µl of test serum was added to the wells in

Sera with IgM antibody activity were retested at starting dilutions of 1/16 after treatment for removal of rheumatoid factor. A 10 µl aliquot of serum was added to 150 µl serum precipitating reagent at room temperature for 30 min and then centrifuged at 800xg for 10 min (Serum Pretreatment Reagent) (Whittaker Bioproducts). In all of the serologic techniques, including IF tests, trials were done to establish the minimum incubation periods required to obtain reproducible results, and titres of sera were recorded as the reciprocals of the highest dilutions producing positive results.

3.2.6 Enzyme-linked immunoassays

The ELISA were performed in 96 well immunoassay plates (Nunc), and optimal working dilutions of the reagents were determined by chessboard titration. Throughout the assays, reagent volumes of 100 µl were used, the diluent for reagents was PBS, pH 7.4, containing 10% foetal calf serum, incubations were performed for 1 h at 37 °C, wells were post-coated with 200 µl PBS containing 2% bovine serum albumin (Calbiochem) and plates were washed thrice with PBS containing 0.1% Tween 20 unless specified.

3.2.6.1 IgM-capture ELISA using anti-CCHF HRPO conjugate

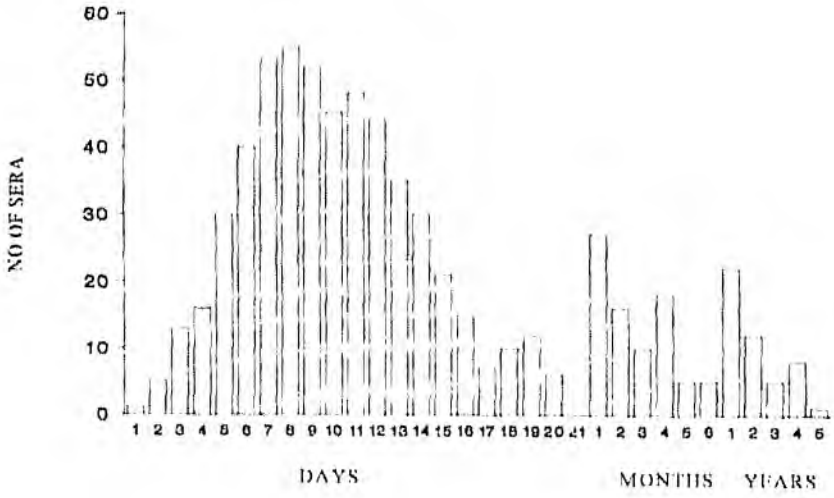
The presence of IgM antibody to CCHF virus was demonstrated by IgM-capture ELISA using rabbit anti-CCHF HRPO conjugate. Plates were coated overnight at 4 °C with µ-chain specific anti-human IgM (Zymed Laboratories Inc., San Francisco, CA, USA) diluted 1/1,000 in carbonate buffer, pH 9.6 (Ksaizek *et al.*, 1989). After the plates were washed, human sera were added to the wells in doubling dilutions from 1/200 upwards. The plates were incubated, washed, and CCHF antigen diluted 1/200, was added to the

3.2.5 Indirect immunofluorescence tests

Sera were tested by IF at doubling dilutions from 1/8 upwards for IgG and IgM antibody activity as described by Johnson *et al.*, (1981) using antigen slides prepared from CCHF virus infected cell cultures, and fluorescein-labelled anti-immunoglobulin conjugates (Cappel). Antigen slides were prepared from Vero 76 cells infected with CCHF virus strain SPU 4/81 isolated from a human patient. A confluent monolayer of cells grown in a 75 cm^2 tissue culture flask was infected with a high multiplicity of CCHF virus and the cells were incubated at 37 °C for 4 days to obtain at le. 1 80% virus infected cells. The media was discarded, the cells were washed with PBS, harvested and resuspended in saline containing 2% foetal calf serum to a concentration of 1×10^6 cells/ml. The cells were mixed at a ratio of 1:10 with an uninfected cell suspension prepared in the same way. Ten microliters of the cell suspension was applied to each well of 8-well multi-test slides (Flow Laboratories, Irvine, UK), dried for 20 min at 37°C and fixed in cold acetone for 20 min. The slides were stored until use at -70°C.

For IF tests, 10 µl of test sera dilutions were applied to each well of an antigen slide. For the IgG tests the slides were incubated in a moist chamber at 37 °C for 20 min. For the IgM tests the slides were incubated overnight at 37°C. The slides were washed for 3 min in PBS and 1 min in water and dried. Ten microliters of fluorescein-labelled anti-IgG or -IgM conjugate diluted 1:100 with Evans blue as a counterstain were applied to each well and the slides were incubated at 37 °C for 20 min. The slides were washed and dried and mounted with glycerol mounting fluid and coverslipped. The slides were read using a Nikon ultraviolet light microscope.

Figure 3.1. Histogram showing the numbers of Crimean-Congo haemorrhagic fever patients from whom serum samples were received at the indicated intervals after onset of illness.



(Sigma) (Wilson and Nakane, 1978; Smith and Tedder, 1981). Briefly 200 µl of freshly prepared 0.1M sodium periodate was added to a solution of 4 mg/ml HRPO and incubated for 30 min at room temperature. The HRPO-aldehyde solution was dialysed overnight in 1mM sodium acetate pH 4.4. The pH was adjusted to 9.5 by addition of 0.01M carbonate buffer pH 9.6 and immediately 1 ml of 8 mg/ml of purified IgG fraction was added and gently mixed at room temperature for 2 h. The conjugate was dialysed against PBS and freeze dried.

3.2.4 Serum specimens

Six hundred and sixty seven serum samples were collected at various intervals from the day of onset of illness up to 59 months later from 101 cases of CCHF diagnosed from February 1981-February 1992 (Figure 3.1). Sera taken during the acute illness were routinely tested on receipt at our diagnostic laboratory by IF for IgG and IgM antibody activity to agents associated with viral haemorrhagic fevers in Africa, inoculated into mice and cell cultures for the isolation of virus, and subjected to diagnostic tests for a range of other aetiological agents as deemed appropriate, after which residual samples were stored at -70°C for further tests. Post-convalescent sera were taken as the opportunity arose to monitor the persistence of antibody activity, or to assess the potency of immune plasma collected for therapeutic use. Adequate samples of 546 sera from 96 patients remained available for testing by ELISA for IgM antibody and by CELISA for total antibody activity to CCHF virus, whilst 425 of the sera from 86 patients were available for testing by sandwich ELISA for IgG antibody.

A monoclonal antibody, 6H5, directed against CCHF viral nucleocapsid was used as a coating antibody in the ELISA (Blackburn *et al.*, 1987).

Hyperimmune mouse ascitic fluid (HMAF), stored freeze-dried at -70°C, had been prepared from adult mice inoculated intraperitoneally (ip) with 0.2 ml of a 1/10 dilution of mouse brain CCHF virus suspension (isolate 4/81) in Freund's complete adjuvant, once weekly for 4 weeks. On the fifth week the mice were inoculated ip with 0.2 ml of a suspension of sarcoma 180 cells. One week later the ascitic fluid was harvested by euthanasing the mice, cutting an incision in the abdominal cavity and removing the ascitic fluid with a pasteur pipette (Sartorelli *et al.*, 1966). The cells were sedimented by centrifugation at 800xg for 5 min and the supernatant was stored at -70°C. The potency of the HMAF was determined by antibody tests (refer to Section 2.2.5).

3.2.3 Anti-CCHF horseradish peroxidase conjugate

Immune rabbit serum stored at -70°C from a previous experiment was used to prepare an anti-CCHF horseradish peroxidase (HRPO) conjugate. The New Zealand giant rabbits had been inoculated subcutaneously with $10^{4.5}$-$10^{7.0}$ tissue culture infective doses ($TCID_{50}$) of CCHF virus 4/81, given a booster 1 month later, and exsanguinated after a further 2 weeks, and the serum stored at -70°C. The IgG fraction was isolated from pooled immune rabbit serum by ammonium sulphate precipitation (31%, pH 7.4), followed by affinity chromatography on a Protein A-Sepharose column according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The IgG fraction was dialysed against 0.01M carbonate buffer pH 9.6 and conjugated to HRPO type VI

4.2.6 *In situ* hybridization

For ISH assays the tissue sections were deparaffinized and digested as described in Section 4.2.4. The digested tissue was post-fixed in 4% paraformaldehyde and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine. The tissue sections were denatured in 2x SSC containing 70% diethyl formamide for 10 min at 70°C, plunged into cold 70% ethanol for 10 min and dehydrated through a series of graded alcohol solutions. The slides were air dried and prehybridized for 30 min at room temperature in hybridization buffer containing 50% diethylformamide, 4x SSC, 1x Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA, 0.25 mg/ml yeast tRNA and 10% dextran sulphate. The RNA probes were optimally diluted in hybridization buffer, denatured for 5 min at 95°C and applied to the sections. The tissue sections were coverslipped with parafilm and hybridization was carried out overnight at 42°C. After washing the slides a blocking step was performed using 20% NSS/TST. Anti-DIG antibody (Boehringer-Mannheim) diluted 1/400 was applied to the sections and detected using the labelled streptavidin-biotin IHC technique as described above for the IHC assays.

The specificity of the CCHF probes was confirmed using a DIG-labelled RNA probe specific for parvovirus as well as hybridizing the CCHF RNA probes to non-CCHF autopsy tissue sections.

4.2.7 Serological tests

Sera collected from the patients were tested on submission by the diagnostic laboratory for IgG and IgM antibody to CCHF virus by IF tests as described in Section 3.2.5.

phosphatase conjugate diluted 1/5,000 in blocking buffer for 1 h at room temperature with shaking. The membrane was washed twice in TST containing Triton-X-100 for 10 min and twice in 0.1M Tris-HCl, 0.1M sodium chloride, 0.05M magnesium chloride pH 9.5. The blot was incubated overnight in a sealed bag containing 45 µl nitroblue tetrazolium salt, NBT, (75 mg/ml in dimethyl formamide) and 35 µl BCIP (50 mg/ml in 100% dimethyl formamide) in 9.92 ml buffer (0.1M Tris-HCl, 0.1M sodium chloride, 0.05M magnesium chloride, pH 9.5). The amount of labelled RNA generated was estimated by comparison with the labelled control RNA. Formalin-fixed paraffin-embedded CCHF infected cells were used to optimise the hybridization conditions and 2 RNA probes, 450 and 571 bases in length, were selected for use in the ISH assays on human tissues.

Table 4.2. Oligonucleotide primer designations and their corresponding nucleotide positions and primer sequences.

Primer designation	Nucleotide positions[1] 5'	3'	Primer sequence
17sF2	135	153	5'GGTACTAATACGACTCACTATAGGGAGATGGACCTTCACAAACTC 3'
12sF3	290	309	5'GGTACTAATACGACTCACTATAGGGAGAGAATGTGCATGGTTAGCTC 3'
SP6-R2	540	560	5'GGTACTATTTAGGTGACACTATAGAATAGGACATCACAATTTCACCAGG 3'
SP6-R3	670	688	5'GGTACTATTTAGGTGACACTATAGAATAGGACAAATTCCTGCACCA 3'

[1]Nucleotide positions relative to the positive sense strand of CCHF virus strain IbAr 10200.

[2]The underlined sequences contain 23 base T7 or SP6 promotor sequences 3' to a 6 base clamping sequence

positive sense strand of CCHF virus strain IbAr 10200 are shown in Table 4.2. The forward primers, designated T7-F2 and T7-F3, were complementary to viral RNA and contained the T7 promotor sequence at the 5' end. The reverse primers, designated SP6-R2 and SP6-R3, were complementary to the message sense RNA and had the SP6 promotor sequence at the 5' end. Four DIG-labelled RNA probes complementary to viral RNA were generated by *in vitro* transcription of the PCR products with T7 RNA polymerase using a DIG RNA labelling kit (SP6/T7) (Boehringer-Mannheim). Four DIG-labelled probes complementary to message sense RNA were synthesised from PCR products using SP6 RNA polymerase. For the transcription, the following reagents were added to 1 µg of PCR product: 2.0 µl NTP mix, 2.0 µl 10x buffer, 1.0 µl RNAse inhibitor, 2.0 µl RNA polymerase, either T7 or SP6, in a total volume of 20 µl and the mixture was incubated for 2 h at 37 °C. One microliter of DNAse I was added and incubated for 15 min at 37 °C. The reaction was stopped by addition of 2.0 µl 0.2M EDTA and 2.5 µl 4M lithium chloride. The RNA was precipitated in cold absolute ethanol at -70 °C for 1 h, and retrieved by centrifugation at 10,000g for 20 min. The pellet was washed in 70% ethanol, dried and resuspended in 40 µl water containing 1 unit/µl of RNAsin and stored at -20 °C. Adequate labelling of the RNA probes was confirmed by dot-blot analysis (Boehringer-Mannheim). Serial 1/5 dilutions of the probes and control RNA (supplied in the kit) were prepared and 1 µl of each dilution was applied to Nitroplus 2000 membranes. The dots were dried and the RNA cross-linked by exposure to ultraviolet light. A blocking step was performed by immersing the membrane in blocking buffer (TBT containing 0.3% triton-X-100 and 2% normal sheep serum) for 30 min at room temperature. The membrane was incubated in anti-DIG alkaline

applying a series of titrations to control slides. The primary antibody was applied to the tissue section and incubated for 90 min. Sections were washed and biotinylated swine anti-mouse and anti-rabbit immunoglobulins (LSAB kit, Dako Corporation, Carpinteria, CA, USA) were applied to the sections and incubated for 15 min.

The slides were washed and streptavidin alkaline phosphatase conjugate (LSAB kit, Dako Corporation) was applied to the sections and incubated for 15 min. After a further washing step the alkaline phosphatase activity was detected by applying napthol/fast red substrate (Dako Corporation) to the sections and incubating the slides for 20 min. The slides were washed in distilled water and the sections were counterstained in Mayer's haematoxylin (Fisher Scientific) for 2-3 min and mounted with aqueous mounting medium (Signet Laboratories, Dedham, MA, USA).

The specificity of the CCHF histochemical staining was confirmed using non-CCHF hyperimmune mouse sera or non-CCHF immune rabbit sera to replace the primary antibody as well as applying the primary CCHF antibody to non-CCHF autopsy tissue sections, and Dengue infected cells.

4.2.5 RNA probes

The RNA probes used in this study were generated from PCR products amplified from the S segment of CCHF virus using primers with CCHF specific and polymerase promotor sequences (Collis *et al.*, 1991; Smith *et al.*, 1991; Young *et al.*, 1991; Loget *et al.*,1993).The nucleotide sequences of the primers and their positions relative to the

Table 4.1. Antibodies evaluated for use in immunohistochemistry assays

Antibody	Antigen	Working dilution
Polyclonal		
HMAF*	CCHF	1/500
Rabbit no.1	CCHF	
Rabbit no.2*	CCHF	1/500
Monoclonal		
VH-1A5-1-1	G1	
VH-2C9-1-1	G2	
VH-6H3-1-1	G2	
VH-8H12-1-2	G1	
VH-11B10-1-1*	G1	1/500
H-23E11-1-1	NP	
H-5E4-1-1	NP	
H-21B2-2-3	NP	
H-17F9-1-1	NP	
H-29D3-1-1	NP	
H-17F9-5-2	NP	
H-7F8-1-2	NP	
H-12G10-1-2	NP	
H-2G10-1-1	NP	
H-17B6-2-2	NP	
H-5G2-1-1*	NP	1/250
H-2B11-1-1	NP	
H-11C5-1-1	NP	
H-1F5-1-1	NP	
H-11F10-1-1	NP	
6-F-5	NP	
5-H-2	NP	
H-9D6-1-1	NP	
H-12F8-1-1	NP	
H-12G10-2-2	NP	

*Antibodies selected for use in IHC assays

The antibodies listed in Table 4.1 were evaluated for CCHF antigen detection in formalin-fixed paraffin-embedded control positive and negative tissues using IHC assays. CCHF-HMAF and rabbit CCHF-immune sera (supplied by the Special Pathogens Branch, Centers for Disease Control, Atlanta, Georgia), prepared against CCHF virus strain IbAr 10200, and 2 monoclonal antibodies, VII-11B10-1-1 directed against the viral glycoprotein G1, and II-5G2-1-1 directed against the viral nucleoprotein (NP) (supplied by the Virology Division of USAMRIID) were selected for use in IHC assays on human tissues.

4.2.4 Immunohistochemistry

The IHC assays were performed using a labelled streptavidin-biotin method as described by Zaki *et al.* (1995) for the detection of Sin Nombre antigen in paraffin-embedded tissues. For IHC and ISH assays the sections were washed thrice for 5 min in Tris-saline-triton (TST) (0.1M Tris pH7.5, 0.1M sodium chloride, 0.005M magnesium chloride, 0.25% Triton-X-100) and incubations were performed at room temperature unless otherwise stated. Briefly, 4μ sections of paraffin-embedded tissues placed on Fisher Plus slides (Fisher Scientific, Pittsburgh, PA, USA) were deparaffinized through 2 changes of xylene substitute. The sections were rehydrated in graded alcohol solutions, absolute alcohol, 95% and 70% and a final incubation in water. The tissue sections were digested for 20 min in 0.1 mg/ml Proteinase K (Boehringer Mannheim) in 0.6M Tris (pH 7.5) containing 0.1% calcium chloride. A blocking step was performed by incubating the sections in 20% normal swine serum in TST (NSS/TST) for 30 min. The optimal working dilutions for the primary antibodies, as shown in Table 4.1, were determined by

In the course of the study, the opportunity arose to apply IHC prospectively to liver tissue from a patient who died during a common source outbreak of CCHF at an ostrich abattoir in South Africa.

4.2 Materials and methods

4.2.1 Patient tissues

Archival paraffin-embedded tissues from 12 confirmed CCHF patients were included in the study. In all cases routine haematoxylin-eosin sections were reviewed, and clinical and laboratory reports were reviewed where available.

Additionally, formalin-fixed liver tissue taken with a biopsy needle after death was submitted from a patient during an outbreak of CCHF among workers in an ostrich abattoir in South Africa in November, 1996, and this was examined prospectively using IHC. No other specimens were available from the patient for serological or virological examination.

4.2.2 Control cells and tissues

Assays were developed using formalin-fixed and paraffin-embedded pellets of minced normal human tissue mixed with Vero E6 (ATCC CRL No. 1586) cells infected with CCHF virus strain IbAr 10200, and uninfected Vero E6 cells and cells infected with Dengue virus serotype 1 as negative controls.

4.2.3 Antibodies

4. Detection of CCHF virus in human tissues by immunohistochemistry and *in situ* hybridization of viral nucleic acid

4.1 Introduction

The cumulative fatality rate for CCHF infections in South Africa is 19.9% (28/141), with specific deaths occurring on days 5-14 of illness, although 2 patients died later from complications to the primary disease. Antibody response is frequently not detected in fatal cases, and although the diagnosis can often be confirmed by demonstration of viral antigen or nucleic acid in serum, or isolation of infectious virus, it sometimes occurs that only tissue samples of internal organs are submitted to the laboratory; most commonly liver samples taken with a biopsy needle after death (unpublished laboratory records). Virus can often be isolated from fresh liver samples, and antigen can sometimes be detected by IF in impression smears, or by ELISA in liver homogenates (Shepherd *et al.*, 1988). In some instances, however, only formalin-fixed tissue samples are submitted, and the histopathologic lesions seen in tissue sections may be suggestive of CCHF, but are not pathognomonic. There have been relatively few histopathologic studies of the disease, involving small numbers of specimens, and little is known of the cellular targets and distribution of the virus (Swanepoel *et al.*, 1987; Joubert *et al.*, 1985; Baskerville *et al.*, 1981). Hence, it was undertaken to explore the use of immunohistochemistry (IHC) and *in situ* hybridization (ISH) techniques for diagnosing CCHF infection on autopsy tissue samples, and for studying the cellular distribution of the virus. Preserved tissue samples from a series of fatal cases of CCHF in South Africa were used.

capture and IgG sandwich ELISAs demonstrated higher antibody titres than did IF, and each detected a response in 4 fatal infections in which no response could be found by IF. Among survivors of the disease, the IgM and IgG ELISAs detected an immune response at an earlier stage of infection than did IF tests in 10 and 15 patients respectively, but the reverse was true in similar numbers of patients (11 and 14 respectively). The failure of the ELISAs to produce positive results on occasion with sera which had low IF titres, was possibly related to the higher starting dilutions used in these tests. The CELISA, in which sera were tested at a starting dilution of 1/10, produced lower titres than did the IgM and IgG ELISAs, but yielded results which were in close agreement with the findings in IF tests.

ELISAs were found to be most consistent if plates were coated freshly before use, rather than stored at -70°C. The sensitivities of the IgM-capture and IgG sandwich ELISAs could be improved slightly by using a 1/50 starting dilution for test sera, but this advantage was outweighed by the tendency to obtain occasional false-positive results in non-infected persons at the lower dilution.

3.4 Summary

Several methods for demonstrating antibody to Crimean-Congo haemorrhagic fever virus were compared on serum samples taken from 101 patients during the acute stage of illness and at intervals for up to 59 months thereafter, with emphasis on early detection of the immune response. Twenty-three patients died on days 5-14 of illness, and their deaths were ascribed directly to the effects of the disease, whereas a further 2 patients died later from other causes. Very few of the patients who died from the acute illness mounted an antibody response which was detectable by any of the methods tested. Four of the patients who died and 18 who recovered were treated with immune plasma collected from recovered patients. Treated patients acquired IgG antibody from the plasma, but it was possible to discern the onset of an endogenous IgM response in those individuals who survived the disease by all of the methods tested.

It was found that routine IF tests detected IgM and/or IgG antibodies at the earliest on day 4 of illness in about 10% of patients who survived the disease, and by day 9 all survivors had antibodies demonstrable by IF. Antibody was demonstrable by IF in only 4/19 patients who died from the disease and had not received immune plasma. The IgM-

little or no difference to the interpretation of IgM IF tests, but it was discovered that patients with severe or fulminant hepatitis A (and suspected to be suffering from viral haemorrhagic fever) frequently had non-specific IgM activity in IF tests, with titres of up to 32,768 despite pre-treatment of sera. Sera from patients with malaria, or from malaria-affected areas, often exhibited non-specific fluorescence at low titre in both IgG and IgM tests, but it was not established whether this was possibly associated with the use of anti-malarial drugs, or with raised serum immunoglobulin levels.

3.3.3 ELISA antibody response

Antibody levels recorded in IgM-capture and IgG sandwich ELISAs (Figures 3.2B and 3.3B) were much higher than in the corresponding IF tests, with maximum titres recorded in most survivors of infection falling in the range 200-25,600. Moreover, IgM antibody was detected in 4 fatal infections in which it had not been demonstrable by IF, and in 15/76 non-fatal infections IgM antibody was demonstrable 1 to 3 days earlier than by IF, being recorded as early as day 3 of illness in 2 patients. However, in a further 14 non-fatal infections IgM antibody was detected earlier by IF than by ELISA. Similarly, IgG antibody was detected by ELISA in 3 fatal infections in which it had not been demonstrable by IF, and in 10 non-fatal infections IgG antibody was detected earlier by ELISA, as early as day 3 in 1 instance, but in a further 11 survivors the antibody response was detected earliest by IF. The C-ELISA produced lower antibody titres than either the IgM-capture or IgG sandwich ELISAs, but the results obtained (Figure 3.3C) were generally consistent with those obtained in IF IgG tests. Maximum titres recorded in individual survivors of infection fell into the range 80-640. Results obtained in

antibody titres recorded in patients were generally 2-4-fold lower than the peak IgG titres in the same individuals. Maximum titres of 32 were recorded in 2 patients who were tested only during the first 2 weeks of illness; otherwise peak titres fell into the range 64-4,096, except for titres of 8,192 recorded in 2 patients.

Among the 23 patients who succumbed during the acute illness, the 4 who received immune plasma therapy all acquired IgG antibody activity demonstrable by IF at maximum titres of 16-256, and 3 of these had weak IgM antibody activity, fluctuating between minimal titres of 8 and undetectable levels. Among the 19 untreated patients antibody response was detected in only 4 individuals who were admitted to hospital at an advanced stage of illness and died within 48 hours. Three had peak IgG titres of 16-64 and IgM titres of 64-512, while the fourth had only IgM antibody activity at a titre of 64.

It was possible to detect IgG and/or IgM IF antibody responses at an earlier stage in some patients by lowering the starting dilution of serum to 1/4, but in many instances non-specific fluorescence interfered with the interpretation of results at this dilution, possibly because patients had been treated with high doses of fluorescing drugs such as tetracycline. Hence the starting dilution of 1/8 was adopted for routine use. Consistent results could be obtained in IgG IF tests with minimum incubation periods of 20 min for sera and conjugates on antigen slides at 37° C. Although positive results could be obtained in IgM IF tests after 1.5 or 3 hours incubation of sera on slides, results were most consistent and titres highest after overnight incubation. No overt problems were encountered with rheumatoid factor in CCHF patients, and pre-treatment of sera made

The ISH demonstrated viral nucleic acid in 5/12 CCHF patients. Examination of liver sections showed evidence of viral replication occurring predominantly within endothelial cells (Figure 4.7) and to a lesser extent in hepatocytes.

Endothelial staining by both IHC and ISH was observed in spleen, lung, heart and intestinal tissues. Figure 4.8 shows immunolocalization of CCHF antigen in splenic tissue.

4.3.6 Prospective diagnosis of a fatal case of CCHF

Subsequent to examining the series of CCHF cases, a common source outbreak of CCHF virus occurred among workers in an ostrich abattoir in Oudsthoorn in the Western Cape Province of South Africa, in November 1996. A total of 16 non-fatal infections were confirmed serologically and virologically. A seventeenth case involved fatal infection and the diagnosis was confirmed by detection of CCHF antigen in liver sections using IHC. Formalin fixed liver was the only specimen received from the patient. Isolated antigen positive hepatocytes were observed scattered throughout the parenchyma of the liver section.

4.4 Summary

The utility of IHC as a diagnostic procedure was investigated by examining archival tissues from confirmed CCHF patients. It was found that in the absence of pathognomonic lesions, histopathologic findings could be rendered diagnostic through

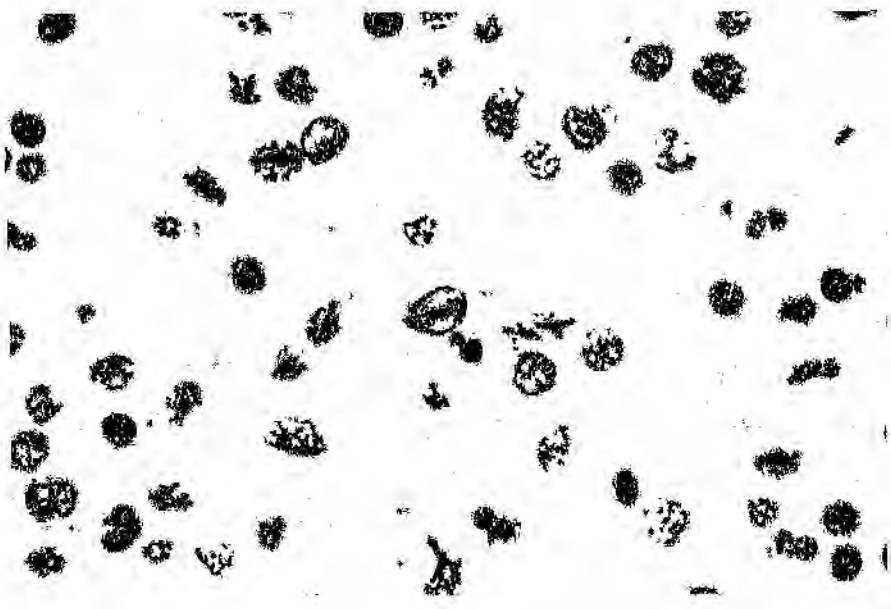


Figure 4.8. Immunolocalization of CCHF antigen in splenic tissue of human CCHF patient using CCHF-HMAF. (Original magnification x100). Naphthol/fast red substrate with haematoxylin counterstain.

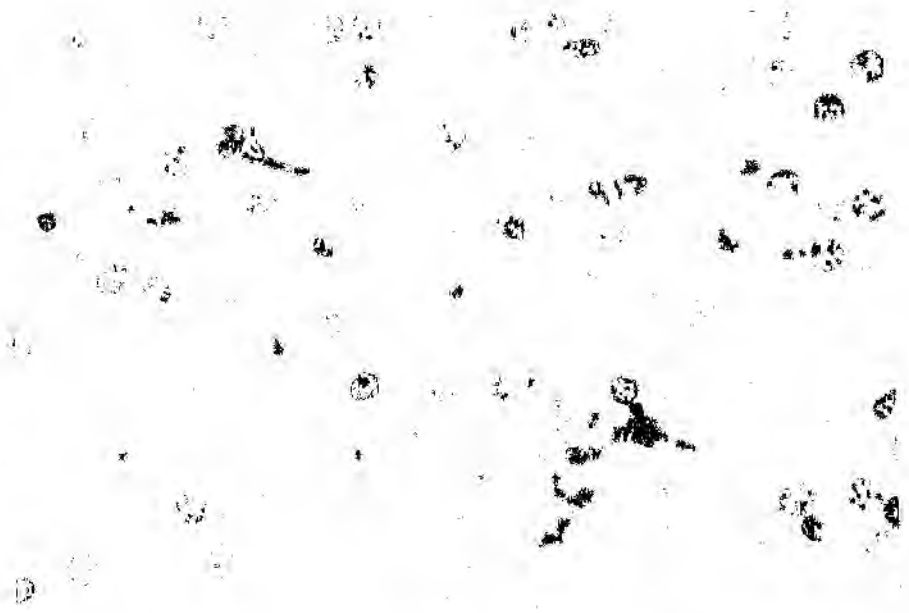


Figure 4.6. Immunolocalization of CCHF antigen in cytoplasm of sinusoidal lining cells including endothelial cells and Kupffer cells of human CCHF patient using CCHF-HMAF. (Original magnification x63). Naphthol/fast red substrate with haematoxylin counterstain.



Figure 4.7. Demonstration of CCHF viral nucleic acid in the cytoplasm of sinusoidal lining cells by ISH using CCHF RNA probe. Note endothelial distribution. (Original magnification x100). Naphthol/fast red substrate with haematoxylin counterstain.



Figure 4.4. Immunolocalization of CCHF antigen in hepatocytes of human CCHF patient using CCHF-HMAF. (Original magnification x100). Naphthol/fast red substrate with haematoxylin counterstain.



Figure 4.5. Immunolocalization of CCHF antigen in hepatocytes of human CCHF patient associated with hepatic necrosis. (Original magnification x100). Naphthol/fast red substrate with haematoxylin counterstain.

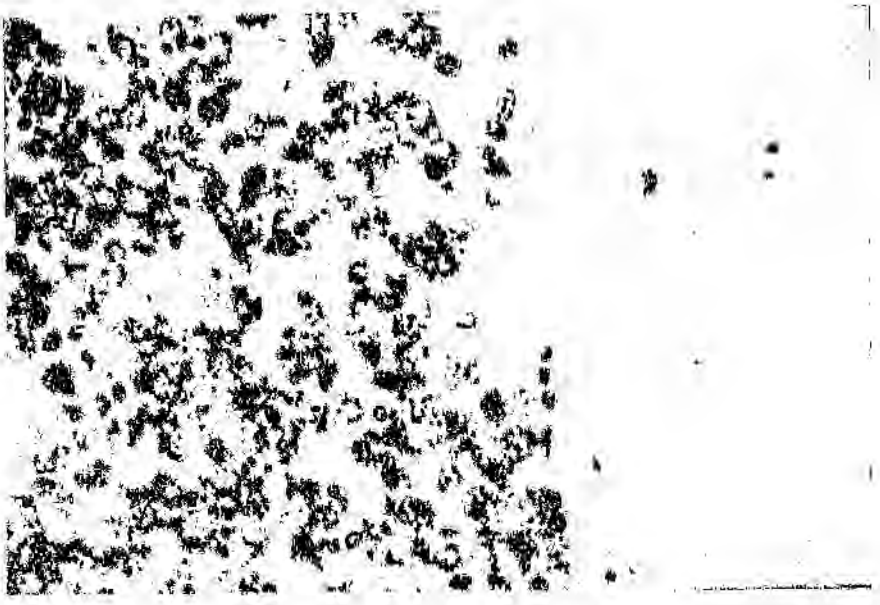


Figure 4.2. Immunohistochemical detection of CCHF antigen in infected cell lines using CCHF-HMAb. Note absence of staining of adjacent non-infected tissue.(Original magnification x40). Naphthol/fast red substrate with haematoxylin counterstain.

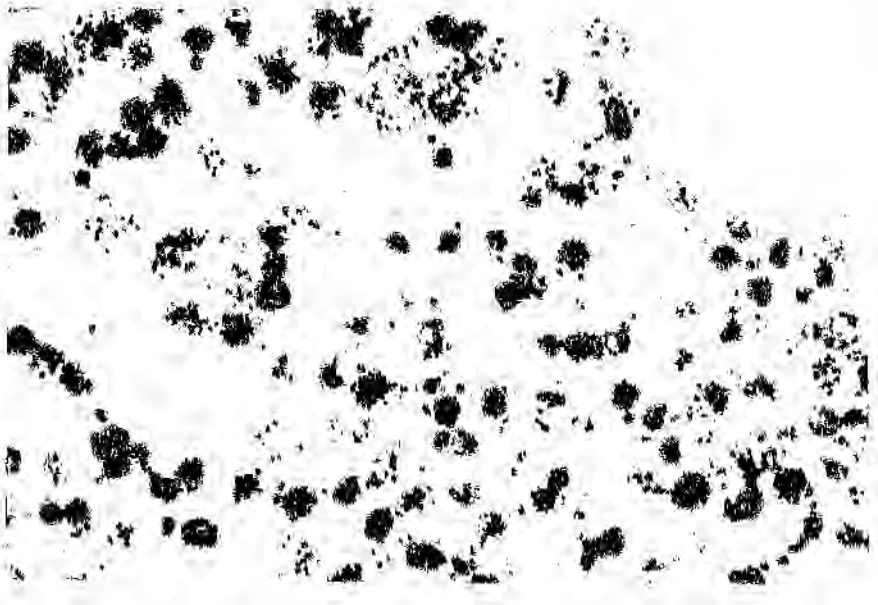


Figure 4.3. Detection of CCHF viral nucleic acid by ISH in infected cell lines using CCHF RNA probe. Note absence of staining of non-infected tissue.(Original magnification x63). Naphthol/fast red substrate with haematoxylin counterstain.

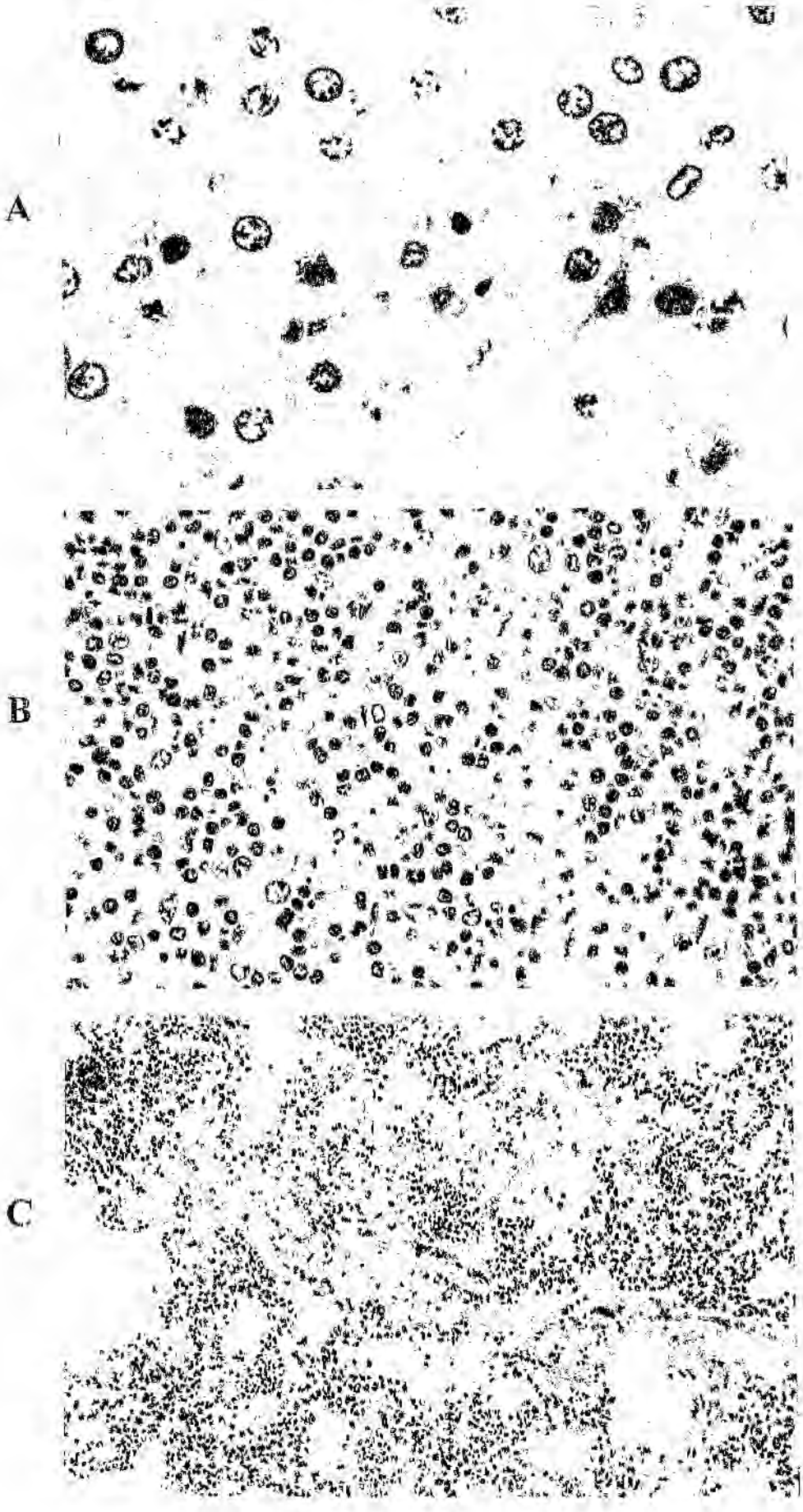

A
B
C

Figure 4.1. Photomicrograph of haematoxylin and eosin stained sections showing some histopathological features of CCHF. A. Liver section showing eosinophilic changes in hepatocytes; B. Splenic tissue showing local necrosis and lymphoid depletion; C. Lung section showing intra-alveolar haemorrhage and hyaline membrane formation. (Original magnifications: A. x100; B. x63; C x20).

probes, generated using T7 polymerase and the primer pairs T7-F2 and SP6-R2, and T7-F2 and SP6-R3, showed more intense staining with the CCHF infected cells and hence were selected for use in the ISH assays. The human tissues were examined for CCHF viral nucleic acid by ISH assays using the 2 RNA probes optimally diluted at 1/200 and viral RNA was demonstrated in a total of 6/12 cases. Infrequent CCHF viral RNA positive cells were detected in the tissue sections. The frequency of positive cells was unaffected by applying the probes to the tissue sections at lower dilutions (eg. 1/50 and 1/100).

4.2.4 Concordance of IHC, ISH, and serology

Of the 12 patients in whom CCHF was diagnosed by virus isolation, 10 cases were positive by IHC, 5 cases by ISH, and 5 cases by serology. Consequently, IHC appears to be a more sensitive diagnostic assay than ISH and serology.

4.3.5 Cellular targets and CCHF virus distribution

The IHC results on human tissues are summarised in Table 4.3. Twelve patients were included in the study of which abundant antigen staining in liver sections was detected in 7 cases, infrequent staining was observed in 3 cases and in 2 cases no staining could be detected. The CCHF antigen was detected in hepatocytes with focal areas of antigen positive cells scattered throughout the parenchyma (Figure 4.4), generally associated with areas of necrosis (Figure 4.5). Antigen was also observed in sinusoidal lining cells, including endothelial and Kupffer cells (Figure 4.6).

4.3.2 CCHF IHC assays

Initially several antibodies were evaluated for use in IHC assays, of which 2 polyclonal antibodies, 1 rabbit serum and the other an HMAF, plus 2 monoclonal antibodies were selected for their reactivity with CCHF antigen in formalin-fixed tissues. The 2 monoclones were specific for viral glycoprotein G1 and nucleocapsid protein, NP (Table 4.1). A second rabbit antibody did not react with formalin-fixed tissues and the remaining monoclonal antibodies that were tested either did not react or could not be used because of non-specific epithelial staining. All patients were tested with polyclonal HMAF and a total of 10/12 were CCHF-antigen positive. Six of the cases were diagnosed positive after a single IHC examination, and a result was obtained on the remaining 4 cases after a second experiment. Selected cases were tested with the rabbit antiserum and the 2 monoclonal antibodies which had reacted with control antigens.

The staining patterns were characteristic for different antibodies. The HMAF reacted with the CCHF antigen to give a diffuse staining pattern shown in Figure 4.2, and the staining observed with the rabbit serum appeared more granular. The anti-G1 monoclonal antibody showed a staining pattern similar to the HMAF and the anti-NP monoclonal antibody showed a more punctate pattern similar to that observed with the rabbit serum.

4.3.3 CCHF ISH assays

All 8 RNA probes which had been generated were evaluated for use in ISH assays using formalin-fixed paraffin embedded CCHF infected cells. Four probes reacted with CCHF RNA to give a granular staining pattern as shown in Figure 4.3. However, 2 of the

were generally below reference values. Abnormal clotting profiles were reported from onset of illness with prolonged activated partial thromboplastin times (PTTs>35s) and thrombin times. Levels of serum fibrin degradation products were elevated and fibrinogen levels were depressed in 4 patients and elevated in 2 patients.

Examination of haematoxylin and eosin stained liver sections showed evidence of hepatocellular necrosis in all cases. The severity of damage varied from mild to moderate and severe. Five cases exhibiting mild damage showed eosinophilic changes in isolated hepatocytes and rare Councilman bodies. Seven cases exhibiting moderate to severe damage showed necrotic areas ranging from small foci to extensive geographic necrosis. Cell loss, eosinophilic change of hepatocytes and Councilman bodies were observed in necrotic areas (Figure 4.1A). Haemorrhage was evident and generally associated with necrosis. Fatty change, Kupffer cell hyperplasia and mononuclear portal inflammatory infiltrates were observed. Sinusoidal dilatation and bile stasis were noted in 10 cases. Other noteworthy hepatic histopathologic features include prominent hyperplastic and hypertrophic Kupffer cells containing phagocytosed cellular debris in all patients. Necrosis of sinusoidal lining cells including these swollen Kupffer cells was not prominent, although it was difficult to discern by light microscopy. Splenic tissue from 2 patients showed lymphoid depletion, focal necrosis and scattered lymphoblasts in periarterial sheath (Figure 4.1B). Diffuse alveolar damage, intra-alveolar haemorrhage, hyaline membrane formation, and a mononuclear interstitial pneumonitis were present in the lung (Figure 4.1C). Congestion and slight interstitial edema were present in the heart.

Table 4.3. CCHF virus in human tissues by immunohistochemistry and *in situ* hybridization.

Case	Age	Gender	Day of death	Serology	Virus isolation	Tissue	IHC	ISH
1	36	M	5	-	+	liver	rare[1]	-
2	48	M	7	-	+	liver	rare	-
3	47	F	13	+	+	liver	-	-
4	59	M	9	-	-	liver	-	rare
5	40	M	7	-	+	liver	+	rare
6	30	F	12	+	+	liver	+	-
7	37	F	8	-	+	liver	+	+
8	68	M	7	-	-	liver	+	-
9	63	M	24	+	+	liver	rare	+
10	36	M	6	-	+	liver	+	+
11	48	M	6	-	+	liver	+	+
						spleen	+	+
12	15	M	7	-	+	liver	+	+
					-	spleen	+	rare
					-	lung	+	rare
					-	intestine	rare	rare
					-	heart	rare	rare

IHC: immunohistochemistry
ISH: *in situ* hybridization
rare[1]: rare positive

The laboratory data showed that thrombocytopenia with platelet counts of <18 000 was present from an early stage of illness in all CCHF patients. Total white cell counts recorded at onset varied for different patients and ranged from low to normal or elevated levels but generally dropped during the first week of illness to leukopenic levels subsequently became elevated before death. There was a tendency for haemoglobin levels to fall below normal values (female< 12.0 g/dl; male<13.0g/dl) during the first 7-8 days of illness. Aspartate aminotransaminase, alanine amino transaminase and creatine kinase levels were markedly raised in all the patients early after onset of illness and remained elevated. Creatinine and urea levels increased with progression of the illness. Direct and total bilirubin levels were elevated. Total serum protein and albumin levels

4.2.8 Virus isolations

Sera and liver biopsy specimens were tested on submission by the diagnostic laboratory for the presence of CCHF virus by intracerebral inoculation of day-old mice, and inoculation of monolayer cultures of Vero 76 cells in 8 chamber culture slides (as described in 2.2.9). Brain tissue harvested from mice succumbing to infection was tested by IF for CCHF antigen to confirm virus isolation and duplicate cell cultures were examined for isolation of virus on days 3 and 6 after inoculation by performing IF tests with reference antiserum (Swanepoel *et al.*, 1983a).

4.3 Results

4.3.1. Clinical and pathological findings

Serology and virus isolation data on the 12 patients included in the study are summarized in Table 4.3.

The source of infection for 2 of the patients was unknown, 4 had a history of tickbite, 1 patient had a nosocomial infection and 5 patients had exposure to livestock and ticks and could have become infected from either source. The common symptoms reported on admission included severe headache, fever, myalgia, neck stiffness, rigors, vomiting, diarrhoea, epistaxis and gingival bleeding. Petechiae were noted in 4 cases and all patients generally had episodes of haematemesis, melaena and hematuria. Active haemorrhaging from multiple sites was evident in 5/12 patients. Death generally occurred on days 5-14 after onset of illness, with 1 exception where a patient died on day 24

RFLP group	Isolate (SPU no)	Patient no	Outcome	Restriction endonuclease							
				DdeI	DraII	RSAI	NheI	AvaII	HinfI	TaqI	HaeIII
[illegible]	378/90	14	Non-fatal	1	1	3	1	1	1	1	1
[illegible]	380/90	14	Non-fatal	1	1	3	1	1	1	1	1
[illegible]	381/90	14	Non-fatal	1	1	3	1	1	1	1	1
[illegible]	382/90	14	Non-fatal	1	1	3	1	1	1	1	1
[illegible]	388/90	14	Non-fatal	1	1	3	1	1	1	1	1
[illegible]	15/92	15	Non-fatal	1	1	3	1	1	1	1	1
[illegible]	16/92	15	Non-fatal	1	1	3	1	1	1	1	1
4	50/93	16	Non-fatal	1	1	3	1	1	1	1	1
5	381/85	17	Non-fatal	4	1	3	1	1	1	1	1
5	361/86	18	Non-fatal	4	1	3	1	1	1	1	1
[illegible]	422/86	19	Non-fatal	4	1	3	1	1	1	1	1
[illegible]	509/86	20	Non-fatal	4	1	3	1	1	1	1	1
[illegible]	560/86	21	Non-fatal	4	1	3	1	1	1	1	1

Table 5.4. Grouping of CCHF isolates by the RFLP patterns.

RFLP group	Isolate (SPU no)	Patient no	Outcome	Restriction endonuclease							
				DdeI	DraII	RSAI	NheI	AvaII	HintI	TaqI	HaeIII
1	259/84	1	Fatal	1[1]	1	1	1	1	1	1	1
2	94/85	2	Fatal	2	2	2	1	1	1	1	2
3	187/86	3	Non-fatal	4	1	3	1	1	2	1	1
4	12/86	4	Non-fatal	1	1	3	1	1	1	1	1
4	23/86	5	Fatal	1	1	3	1	1	1	1	1
4	196/86	6	Fatal	1	1	3	1	1	1	1	1
4	177/87	7	Non-fatal	1	1	3	1	1	1	1	1
4	383/87	8	Non-fatal	1	1	3	1	1	1	1	1
4	509/87	9	Non-fatal	1	1	3	1	1	1	1	1
4	486/87	10	Non-fatal	1	1	3	1	1	1	1	1
4[2]	18/88	11	Fatal	1	1	3	1	1	1	1	1
4[2]	19/88	11	Fatal	1	1	3	1	1	1	1	1
4[7]	21/88	tick		1	1	3	1	1	1	1	1
4	497/88	12	Fatal	1	1	3	1	1	1	1	1
4	130/89	13	Non-fatal	1	1	3	1	1	1	1	1

Table 5.3. RFLP groups as defined by cDNA fragment patterns obtained after digestion of the PCR products with restriction endonucleases.

RE	Groups									
	1	2	3	4	5	6	7	8	9	10
DdeI	280[1]	280	265	280	265	265	NC[2]	280	280	280
	250	150	175	250	175	175		150	150	150
		110	100		100	100		110	110	110
DraII	NC	350	NC	NC	NC	NC	NC	350	NC	NC
		200						200		
RSAI	240	NC	410	410	410	410	410	NC	410	NC
	150		140	140	140	140	140		140	
	140									
NheI	NC	NC	NC	NC	NC	300	NC	NC	NC	NC
						250				
AvaII	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
HinfI	420	420	NC	420	420	420	420	420	420	420
	110	110		110	110	110	110	110	110	110
TaqI	320	320	320	320	320	320	320	320	320	320
	210	210	210	210	210	210	210	210	210	210
HaeIII	440	300[3]	440	440	440	440	440	440	440	440
	70	130	25	70	70	70	70	70	70	70
	40	70	40	40	40	40	40	40	40	40
No of isolates	1	1	1	20	18	5	7	1	1	2

RE:restriction endonuelease
[1]Fragment size in bp
[2]NC:no cut
[3]One band - 40 bp not resolved

Table 5.2 GenBank accession numbers, year and locality of isolation (where available) for CCHF, Hazara and Dugbe isolates included in partial nucleotide sequence analysis of geographically distinct isolates. (Karabatsos, 1985; Zeller *et al*, 1994, Ward *et al*., 1990a)

Isolate	Year	Origin	Locality	GenBank no.
AP 92	1982	Tick	Greece	U01958
IBD 49199	1 6 88	Human	Mauritania	U15023
ArMg 951	28 3 85	*Boophilus microplus*	Madagascar	U15024
ArD 8194	5 12 73	Caprine	Bandia, Senegal	U15020
ArD 15786	4 3 69	*Hyalomma truncatum*	Dakar, Senegal	U15021
ArD 39554	1 3 84	*H. marginatum rufipes*	Mauritania	U15089
ArD 97264	22 4 93	*H. marginatum rufipes*	Barkedji	U15090
ArD 97268	22 4 93	*H. truncatum*	Barkedji	U15091
ArB 601	1 9 73	*H. rufipes*	Central African Republic	U15092
IBD 38562	1983	Human	Burkino Faso	U15093
Pak IBD 206*	1965	Tick	Pakistan	NS
HY-13*	1968	Tick	China	NS
Drazdov*	1967	Human	Russia	NS
UG3010*	1956	Human	Democratic Republic of Congo	NS
247/85	1985	Human	South Africa	U84636
582/86	1986	Human	Namibia	U84635
91/85	1985	Human	South Africa	NS
566/86	1986	Human	South Africa	NS
215/87	1987	Human	South Africa	U84639
60/89	1989	Human	South Africa	NS
422/86	1986	Human	South Africa	U84638
187/86	1986	Human	South Africa	NS
259/84	1984	Human	South Africa	NS
45/88	1988	Human	South Africa	U84637
115/85	1985	Human	South Africa	NS
536/8.	1986	Human	Namibia	NS
378/90	1990	Human	South Africa	NS
18/88	1988	Human	South Africa	NS
281/89	1989	Human	South Africa	NS
Hazara Ib 280	1964	*Ixodes redikorzevi*	Pakistan	M86624
Dugbe ArD 14313	1985	*Amblyomma variegatum*	Senegal	M28130

*Unpublished data supplied by the Virology Division, USAMRIID
NS: no submitted
NA: not available

Phylogenetic relationships among the southern African CCHF isolates and 14 isolates from other geographic regions, plus 2 other nairoviruses, Dugbe and Hazara, were examined by including sequence data obtained from GenBank and the Virology Division of USAMRIID. The locality, origin and GenBank accession numbers of the isolates where available are shown in Table 5.2.

5.3 Results

5.3.1 Restriction endonuclease analysis

Ten RFLP groups were defined from the fragment lengths obtained from digestion of the PCR products with 8 restriction endonucleases (Table 5.3), and each isolate was assigned to a group (Table 5.4). Seventeen isolates represented repeat isolations on different days from 5 patients, and in all instances isolates obtained from the same patient had identical RFLP patterns. Furthermore, an isolate from a tick which had been removed from 1 patient, had the same RFLP pattern as 2 isolates from the patient's serum. Two isolates from separate patients in a common source outbreak also had identical RFLP patterns. No correlation could be made between the RFLP grouping and source of infection, year of infection or pathogenicity of the virus, but 3 isolates, 247/85 and 281/89 (RFLP group 10) and 259/84 (RFLP group 1) were from areas where the tick *H. truncatum* is prevalent and *H. m. rufipes* and *H. m. turanicum* are absent.

5.3.2 Nucleotide sequence analysis

The CCHF isolates selected for cDNA sequencing included 5 which each had unique

7.5, 100mM magnesium chloride, 250mM sodium chloride), 1 µl 0.1M dithiothreitol, 2 µl 1/4 dilution of labelling mixture (7.5µM 7-deaza-dGTP, 7.5µM dCTP and 7.5µM dTTP), 0.5 µl [α-^{35}S]-dATP (specific activity 1000 Ci/mmol) (Amersham), and 2 µl Sequenase DNA polymerase. The labelling reaction was allowed to proceed at room temperature for 5 min and 1 µl manganese buffer (0.15M sodium isocitrate, 0.1M manganese chloride) was subsequently added to each tube. The reaction was terminated by transferring 3.5 µl of labelling reaction mixture to 2.5 µl of each termination mix, ddG, ddA, ddT and ddC, and incubating the samples at 37 °C for 10 min. The reaction was stopped with 4 µl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF). The samples were heated to 80 °C for 2 min and the products were resolved on 8% polyacrylamide-8M urea gels in 1x glycerol tolerant buffer (1.8M Tris, 0.6M Taurine, 0.01M EDTA). The gels were fixed in 5% acetic acid, 20% methanol for 15 min, vacuum dried and the products visualised by exposing the gels to β-Max hyperfilm (Amersham) overnight at room temperature.

5.2.6 Sequence data analysis

Alignment of sequence data and similarity among the southern African isolates was performed using HIBIO DNASIS (Version 7.08). Phylogenetic relationships among the isolates were examined using PHYLIP (Phylogenetic Inference Package, Version 3.51c). SEQBOOT was used to make 100 bootstrap replications of the nucleotide data. A maximum likelihood tree was generated with DNAML using the bootstrapped data and the occurrence of each node determined using CONSENSE.

min in a total volume of 25µl. Digested products were separated by electrophoresis on 4% agarose gels (3% Nusieve, 1% UE agarose) (FMC Bioproducts) in Tris-borate-EDTA (TBE) buffer containing 1 µg/ml ethidium bromide, and the DNA fragments visualised on a UV-transilluminator. Fragment lengths were estimated from the migration distances of the fragments relative to the migration distances of the molecular weight marker fragments of known size, using marker V (pBR322 DNA cleaved with *Hae* III) (Boehringer Mannheim). Mean values were calculated from plotting fragment size (in bp) against migration distance on semi-logarithmic graph paper.

5.2.5 Sequencing of PCR products

Fifteen CCHF isolates representing the range of RFLP groups identified, were chosen for sequencing. The selected isolates were amplified by RT-PCR using the primers F2 and R3 ,and a 450 base pair region of the amplified product, from nucleotide positions 186 to 635 relative to the complete S segment of Nigerian tick isolate IbAR 10200, was sequenced directly using the Sequenase kit, Version 2.0 (United States Biochemical Corp., Cleveland, OH, USA). In a pre-treatment step, 0.5 pmol of the PCR products were incubated for 15 min at 37 °C with 1.0 unit exonuclease I and 2.0 units shrimp alkaline phosphatase in a total volume of 7 µl. The enzymes were inactivated by incubating the samples at 80 °C for 15 min. For each isolate 4 sequencing reactions were performed using 1 of the 4 primers shown in Table 5.1 for each reaction. Five microliter aliquots of the pre-treated PCR products were annealed to 10 pmol primer at 95 °C for 3 min followed by 5 min on ice. The labelling reaction mixture was added to 5 µl of annealed DNA so that each reaction mixture contained 2 µl reaction buffer (200mM Tris-HCl, pH

5.2.3 RNA extraction and RT-PCR

Confluent monolayers of Vero 76 cells grown in 150 cm^2 tissue culture flasks were infected with a 10^{-1} dilution of suckling mouse brain suspension and maintained in EMEM containing 2% foetal calf serum at 37 C for 4 days to achieve 80-100% infection. The cells were washed once with PBS pH 7.4, harvested and total RNA was extracted from the infected cells using the acid guanidium thiocyanate-phenol-chloroform method as described in Section 2.2.4. The RNA was quantitated spectrophotometrically assuming that an optical density reading of 1 at a wavelength of 260 nm corresponds to approximately 40μg/ml of single-stranded RNA (Maniatis *et al.*, 1982), and aliquoted and stored in 2.5M ammonium acetate and 2.5 volumes of ethanol. Before use, RNA was centrifuged for 20 mins at 10,000xg, washed once with chilled 70% ethanol and dried. The pellet was resuspended in RNase free water at a concentration of 1 µg/µl and 5 µg RNA was subjected to RT-PCR using the protocol described in Section 2.2.5, and a 536 bp fragment of the S segment of the viral genome was amplified. Amplification was confirmed by electrophoresis of 10 µl aliquots of the PCR products on 1.2% Seakem agarose gels in TAE buffer containing 1 µg/ml ethidium bromide, and the DNA bands were visualised on a UV-transilluminator.

5.2.4 Restriction endonuclease digestion of PCR products

PCR products were analysed by restriction fragment length polymorphism (RFLP) using 8 restriction endonucleases: *Hae* III, *Hinf* I, *RSA* I, *Nhe* I, *Dde* I, *Dra* II, *Taq* I and *Ava* II (Boehringer Mannheim). Twenty microliter aliquots of the PCR products were digested using 10 units of enzyme diluted in the optimal buffer system at 37 C for 60

blood of sheep or cattle, and 11 lived in or visited a rural environment where contact with livestock and ticks was possible, but were unaware of specific incidents which would have constituted exposure to infection. There were 15/46 deaths among the patients. The isolates had been stored at -70 °C in the form of freeze-dried 10% suckling mouse brain suspensions, all at the level of second or third mouse brain pass.

5.2.2 Oligonucleotide primers

RT-PCR reactions were performed using the primer pair designated F2 and R3, and the methods described in Chapter 2. The PCR products were sequenced using the PCR primers, F2 and R3, plus an additional pair of primers designated F3 and R2. The nucleotide sequences and primer positions relative to the positive sense strand of the S segment of CCHF virus isolate IbAr 10200 are shown in Table 5.1. The nucleotide sequences for the primers were supplied by the Virology Division, USAMRIID.

Table 5.1. Nucleotide sequences of primers used in RT-PCR and DNA sequencing reactions, and their positions relative to the S segment RNA of reference virus IbAr 10200.

Primer	Position 5'	Position 3'	Nucleotide sequence
F2	135	153	5' TGGACACCTTCACAAACT 3'
F3	290	309	5' GAATGTGCATGGGTTAGCTC 3'
R2	549	530	5' GACATCACAATTTCACCAGG 3'
R3	670	653	5' GACAAATTCCCTGCACCA 3'

representative of the genus (Swanepoel *et al.*, 1987).

The severity and outcome of illness vary for reasons that are incompletely understood, but age, underlying health, secondary infection, and the timeliness and adequacy of supportive therapy are probably important factors. Initially it was believed that African strains of CCHF virus were less pathogenic for humans than Asian strains (Hoogstraal, 1979), and although this is no longer thought to be correct (Swanepoel *et al.*, 1987), it was considered necessary to determine whether genetic diversity of the virus in South Africa correlates with pathogenicity for humans, year and source of infection, or geographic origin. Restriction fragment length polymorphism (RFLP) analysis was used to screen CCHF isolates for genetic diversity, and representative isolates from each of the RFLP groups identified were selected for nucleotide sequencing of a region of the S segment of the viral genome. Phylogenetic relationships were examined among southern African and other isolates by including sequence data obtained from GenBank and the Virology Division of USAMRIID.

5.2 Methods and materials

5.2.1 CCHF virus isolates

The 57 southern African CCHF isolates included in the study were cultured in the Special Pathogens Unit between 1984-1993, from 46 patients and a tick removed from 1 of the patients. The isolates came from widely separated locations in southern Africa, and 26/46 patients became infected from known tick bite. 1 patient contracted nosocomial infection from exposure to human blood. 8 patients had contact with the

5. Investigation of genetic heterogeneity of CCHF virus in southern Africa

5.1 Introduction

One hundred and eight outbreaks of CCHF, involving 141 patients, have been recorded from widely scattered locations in southern Africa since the first case was recognised in 1981. The largest group of cases, 63/141 (44.7%), arose from known or potential contact with fresh blood or other tissues of livestock and/or ticks. 55/141 (39%) arose from known tick bite. 7/141 (5%) nosocomial infections arose from contact with blood or fomites of known CCHF patients while in 16/141 (11.3%) cases there was no direct evidence of contact with livestock or ticks, but the patients lived in or visited a rural environment where such contact was possible. The case fatality rate fluctuated around 30% for the first few years after CCHF was initially recognized in southern Africa but it has declined to 19.9% (28/141), probably because greater awareness of the disease leads to earlier recognition and better management of patients in most instances.

Antibody surveys on the sera of cattle and wild vertebrates have shown that the distribution of CCHF virus in South Africa broadly corresponds with that of ticks belonging to the genus *Hyalomma*, the main vectors of the virus (Swanepoel *et al.*, 1987). The ticks are xerophilic and are widely distributed in the drier interior of the country, being absent only from the higher rainfall areas of the eastern seaboard (Howell *et al.*, 1978). There are 3 species of *Hyalomma* in the country, and higher prevalences of antibody to the virus have been demonstrated in sera collected from areas where *H. m. rufipes* and *H. m. turanicum* are present, than from areas where *H. truncatum* is the sole

demonstrating the presence of viral antigen by IHC. Viral antigen was detected in 10/12 patients in which a diagnosis had been confirmed by isolation of virus. Viral RNA was detected by ISH in 5/12 patients, and antibody response in 5/12.

In a prospective investigation, viral antigen was detected by IHC in a formalin-fixed liver specimen submitted from a patient with fatal infection during an outbreak of CCHF at an ostrich slaughterhouse in South Africa in 1996. No other specimens were available from the patient.

From the IHC and ISH examination of tissues available it appears that the reticuloendothelial system and hepatocytes are the main targets of infection. The widespread infection of endothelium probably plays a major role in the pathogenesis of the bleeding tendency and disseminated intravascular coagulopathy, which commonly characterize the disease. The association of necrotic areas in liver with detection of viral antigen suggests that hepatocyte damage may be mediated by a direct cytopathic effect.

```
          121                                                                 180
10200     GCTCCACTGG CATTGTAAAA AAGGGACTTG AATGGTTCGA GAAAAATGCA GGAACCATTA
247/85    .......C.. ...A..C..G .....C.... ........ .....A..G. ......T..C.
582/86    .......C.. T..A..C..G .....C.... .G...... .....A..G. ......T..C.
94/85     .......C.. ...A..C..G .....C.... .......... .....A..G. ......T....
566/86    .......C.. ......G..G .......... .......... .....A.... ..C.......
245/87    .......... ......G..G .......... .......... .....A.... ..C.......
60/89     .......A.. ...A..G... .G...C.... .G.....T.. A.....CA.. .........C
422/86    .......... ......G..G .G........ .......... .....A..... ..C.......
187/86    .......... ......G..G .G........ .......... .....A.....G ..C.....C.
259/84    .......... ......G..G .GA..T.... .......... .....A.....G ..T.......
45/88     .......... ......G..G .G........ .......... .....A.....G ..C.......
415/85    .......... ......G.... .G...C.... .......... .....A..G... .....T..C.
536/86    .......... ......G.... .G........ .......... .....A.....G ..C.......
378/90    .......... ......G..G .G........ .......... .....A.....G ..C.......
18/88     .......... ......G..G .G........ .......... .....A.....G ..C.......
281/89    .......C.. ...A..C..G .....C.... .......... .....A..G... .........C.

          181                                                                 240
10200     AGTGCTGGGA TGAAAGTTAT ACTGAGCTAA AGGTCGACGT CGCGAAAATA GAGCAGCTTA
247/85    ....T..... ...G..C... .........T ..A..T.GT. ...C...... ..........C
582/86    ....T..... ...G..C... .........T ..A..T.GT. ...C...... ..........G
94/85     ....T..... ...G..C... .........T ..A..T.GT. ...C...... ..........G
566/86    .......... .........C .....\.... ..A..... .......... ..A..A...G
245/87    .......... .........C .....\.... ..A..T.... .......... ..A..A...G
60/89     ....T..... ...G..C..C .....A..G. ..A..G..T. G..C.....T ..A......G
422/86    ....T..... .......... .......... ..A..T.... ...A...... ..A......G
187/86    ....T..... .......... .......... ..A..T.... .......... ..A..A...G
259/84    ....T..... .........C .......... ..A..T.... .......... ..A..A...G
45/88     ....T..... .......... .......... ..A..T.... .......... ..A......G
415/85    ....T..... ...G..C... .........T ..A..T.GT. ...C...... ..........G
536/86    ....TG.... .......... ..G....... ..A..... ...A...... ..A.....AG
378/90    ....T..... .......... .......... ..A..T.... .......... ..A..A...G
18/88     ....T..... .......... .......... ..A..T.... ...A...... ..A..A...G
281/89    ....T..... ...G..C... .........T ..A..T.GT. ...C...... ..........G
```

10200. Alignment of the predicted amino acid sequences is shown in Figure 5.4. There were 16 amino acid substitutions among the southern African isolates of which 10 were conservative changes and 6 were changes from hydrophobic or hydrophilic amino acids to neutral and polar amino acids. No change resulted in replacement of a hydrophobic amino acid with a hydrophilic amino acid, or vice versa. However, there were 2 substitutions that involved a proline residue (isolate 247/85, residue 80; isolate 45/88, residue 145).

A graphic representation of the phylogenetic relationships among 15 southern African isolates as determined by using a maximum likelihood method (DNAML) to estimate the phylogeny and 100 bootstrap replicates of the sequence data, is presented in Figure 5.5. The number of times each node occurred in the consensus from 100 trees is shown beneath the branches. There appear to be 2 distinct clades with isolates 245/87 and 566/86 constituting clade a) and isolates 60/89, 281/89, 217/85, 94/85 and 582/86 constituting clade b). The remaining isolates branch off singly.

The phylogenetic relationships as determined by DNAML between 15 southern African CCHF isolates, and 14 isolates from other geographic regions, plus 2 non-CCHF nairoviruses, Dugbe, and Hazara, is presented graphically in Figure 5.6. Hazara virus was used to outgroup the tree. Southern African isolates clustered into 3 groups with isolates from other countries on the continent, while isolates from Asia and Madagascar grouped together, and a Greek isolate branched off on its own.

	121		150
10200	SVKEMLSDMI	RRRNLILNRG	GDENPRGPV
247/85	. . . . .	. . . . .	. . . . .
582/86	. . . . .	. . . . .	. . . . .
94/85	. . . . .	. . . . .	. . . . .
566/86	. . . . .	. . . . Q. . . .	. . . . .
245/87	. . . . .	. . . . Q. . . .	. . . . .
60/89	. . . . .	. . . . .	. . . . .
422/86	. . . . .	. . . . Q. . . .	. . . . .
187/86	. . . . .	. . . . Q. . . .	. . . . .
259/84	. . . . .	. . . . Q. . . .	. . . . .
46/88	. . . . .	. . . . Q. . . .	. . . . G. . . .
415/85	. . . . .	. . . . Q. . . .	. . . . .
536/86	. . . . .	. . . . Q. . . .	. . . . .
378/90	. . . . .	. . . . Q. . . .	. . . . .
18/88	. . . . .	. . . . Q. . . .	. . . . .
281/89	. . . . .	. . . . .	. . . . .

Figure 5.4. Alignment of predicted amino acid sequences for southern African CCHF virus isolates. The region is compared with a reference isolate, IbAr 10200, from Nigeria. Dots indicate sequence identity.

```
          1                                                                  60
10200     RFVFQMASAT DDAQKDSIYA SALVEATKFC APIYECAWVS STGIVKKGLE WFEKNAGTIK
247/85    .. ........ .......... .......... .......... .......... ..........
582/86    .......... .......... .......... .......... .......... ..........
94/85     .......... .......... .......... .......... .......... ..........
566/86    .......... .......... .......... .......... .......... ..........
245/87    .......G.. .......... .......... .......... .......... ..........
60/89     .......... .... ..... .........Y .......... ......R... .....T....
422/86    K......... .......... .......... .......... ......R... ..........
187/86    K......... .......... .......... .......... ......R... ..........
259/84    K......... .......... .......... .......... ......R... ..........
46/88     K......... .......... .......... .......... ......R... ..........
415/85    K......... .......... .......... ..V....... ......R... ..........
536/86    K......... .......... .......... ..V....... ......R... ..........
378/90    K......... .......... .......... .......... ......R... ..........
18/88     K......... .......... .......... .....Y.... ......R... ..........
281/89    K......... ...... .. .......... .......... .......... ..........

          61                                                                 120
10200     SWDESYTELK VDVPKIEQLT GYQQAALKWR KDIGFRVNAN TAALSNKVLA FYKVPGEIVM
247/85    .......... .G.......P N......... .......... .......... ..........
582/86    .......... .G.......A N......... .......... .......... ..........
94/85     .......... .G.......A N......... .......... .......... ..........
566/86    .......... .........A N......... .......... .......... ..........
245/87    .......... .........A N......... .......... .......... ..........
60/89     .......... .........A N......... .......... .........S ..........
422/86    .......... .........A N......... .......... .......... ..........
187/86    .......... .... ....A N......... .......... .......... ..........
259/84    .......... .........A N.........E .......... .......... .....D.L.
46/88     .......... .........A N......... .......... .......... ..........
415/85    ........ .. .G.......A N..... .... .......... .......... ..........
536/86    .G........ .........A N......... .......... .......... ..........
378/90    .......... .........A N..... . .. .......... .......... ..........
18/88     ........ .. .........A N......... .......... .......... ..........
281/89    .......... .G.......A N......... .......... .......... ..........
```

Figure 5.3. Pairwise comparison of percentage similarity of predicted amino acid sequences between southern African Crimean-Congo haemorrhagic fever isolates.

Isolate No	247/85	582/86	94/85	566/86	245/87	60/89	422/86	187/85	259/84	45/88	415/85	536/86	378/90	18/88	281/89
247/85	100														
582/86	99	100													
94/85	94	100	100												
566/86	98	99	99	100											
245/87	97	98	98	99	100										
60/89	96	97	97	97	96	100									
422/86	97	97	97	99	98	97	100								
187/85	97	97	97	99	98	97	100	100							
259/84	95	96	96	99	96	95	98	98	100						
45/88	96	97	97	98	97	96	99	99	97	100					
415/85	97	97	97	97	97	96	99	99	97	98	100				
536/86	95	96	96	97	96	95	98	98	96	97	98	100			
378/90	97	97	97	99	98	97	100	100	98	99	99	98	100		
18/88	96	97	97	98	98	96	99	99	98	99	98	97	99	100	
281/89	99	99	99	98	97	97	98	98	96	97	98	96	98	97	100

Figure 5.2. Pair-wise comparison of percentage nucleotide similarity between southern African Crimean-Congo haemorrhagic fever virus isolates.

Isolate No	247/85	582/86	94/85	566/86	245/87	60/89	422/86	187/85	259/84	45/88	415/85	536/86	378/90	18/88	281/89
247/85	100														
582/86	98	100													
94/85	98	98	100												
566/86	84	84	84	100											
245/87	84	83	84	97	100										
60/89	84	84	84	83	82	100									
422/86	85	85	85	92	93	84	100								
187/85	84	84	85	91	91	84	96	100							
259/84	82	82	83	93	93	82	94	94	100						
45/88	84	83	84	93	93	83	97	97	91	100					
415/85	88	88	88	89	89	85	92	93	91	92	100				
536/86	82	82	83	92	93	82	97	96	94	97	93	100			
378/90	82	82	83	93	91	82	96	97	97	96	92	95	100		
18/88	83	83	83	93	93	82	96	95	96	95	91	95	98	100	
281/89	98	98	98	84	84	84	86	85	83	85	89	84	85	84	100

```
          361                                                                 420
10200     TGTCTGTCAA AGAGATGCTG  CAGACATGA  TTAGGAGAAG  GAAGCTGATT  CTAAACAGGG
247/85    ..........  ...A...T.A  .........  ....A.....  ....T....C  ..C.....A.
582/86    ..........  ...A...T.A  .........  ....A.....  ....T....C  ..C.....A.
94/85     ..........  ...A...T.A  .........  .C..A.....  ....T....C  ..C.....A.
566/86    ....C.....  ..........  ..T......  ..........  A.....AA..  ..........
245/87    ....C.....  ...A......  ..T......  ..........  .....AA..A  ..........
60/89     ..........  .........T  .........  .C..A..G..  A........C  ..T.....A.
422/86    ..........  ...A......  .........  ..........  .....A....  .........A.
187/86    ..........  ..........  .........  ..........  .....A....  ..........
259/84    ..........  ..........  ..T......  ..C.......  .....AA...  ..........
45/88     ..........  ..........  .........  ..........  .....A....  ..........
415/85    ..........  ..........  .........  ..........  .....A....  ..........
536/86    ..........  ..........  .........  ..........  .....A....  ..........
378/90    ..........  ..........  .........  ..C.......  .....AA...  ..........
18/88     ..........  ..........  .........  ..C.......  .....AA...  .........A
281/89    ..........  ...A...T.A  .........  ....A.....  ....T....C  ..C.....A.

          421                                                   460
10200     GTGGTGATGA  GAACCCACGT  GGCC..ATGA
247/85    ....C.....  .........C  ..T......
582/86    ....C.....  .........C  ..T......
94/85     ....C.....  .........A  ..T.....A.
566/86    ..........  ...T......  .....T....
245/87    ..........  ...T......  ..........
60/89     .G........  ........A.A  .........A.
422/86    ....C.....  ...T......  .....T....
187/86    ..........  ...T......  .....T....
259/84    ..........  ...T......  .....T....
45/88     ..........  ...TGG....  .....T....
415/85    ..........  ...T......  .....T....
536/86    ..........  ...T......  ....T.....
378/90    ..........  ...T......  .....T....
18/88     ..........  ...T......  .....T....
281/89    ....C.....  .........C  ..T..T....
```

```
          241                                                                 300
10200     CGGTTACCA ACAAGCTGCC TTGAAGTGGA GAAAAGACAT AGGTTTCCGT GTCAATGCCA
247/85    .AAAC..... ...G..... TC.A..... .G..G..T.. .......... .....C....
582/86    .AAAC..... ...G..... TC.A..... .G..G..T.. .......... .....C....
94/85     .AAAC..... ...G..... TC.A..... .G..G..T.. .......... .....C....
566/86    ..AA..... .......... .......... .G........ .......... .......A.
245/87    ..AA..... .......... .......... .......... .......... .......A.
60/89     ..AAC..... G..G....T C.C..... .G..G..... ...C.....C .......A.
422/86    ..AA..... ...G..... .....A.... .G........ .......... .......A.
187/86    .TAA..... ...G..... .....A.... .G........ .......... .......A.
259/84    ..AA..... .......... .....A.... ....G..A.. .......... .......A.
45/88     .TAA..... .......... .....A.... .G........ .......... .......A.
415/85    .AAAC..... ...G....T C.A..... .G..G..T.. .......... .......A.
536/86    ..AA..... ...G..... .....A.... .G........ .......... .......A.
378/90    ..AA..... .......... .....A.... ....G..... .......... .......A.
18/88     ..AA..... .......... .....A.... ....G..... .......... .......A.
281/89    .AAAC..... ...G....T C.A..... .G..G..T.. .......... .....C....

          301                                                                 360
10200     ACACAGCAGC TCTGAGCAAC AAAGTCCTCG CAGAATACAA AGTCCCTGCT GAGATTGTGA
247/85    .T..G..... CT.A..... .......T.. .......... .......... ..A.......
582/86    .T..G..... CT..... .......T.. .......... .......... ..A.......
94/85     .T..G..... CT..... .......T.. .......... .......... ..A.......
566/86    .......G.. .T..... .......T.. ....G..... ...T.....C ..........
245/87    .......G.. .T..... .......T.. ....G..... ...T.....C ..........
60/89     .......G.. C..A..... .......T ....G..... G..T..... ..........
422/86    .......G.. .......... .......... ....G..... ...T.....C ..........
187/86    .......G.. .......... .......... ....G..... ...T.....C ..........
259/84    .......G.. .T..... .......... ....G..... ...T.....C ..T...C...
45/88     .......G.. .......... .......... ....G..... ...T.....C ..........
415/85    .......G.. .....T.... .......... ....G..... ...T.....A ..........
536/86    .......G.. .....T.... .......... ....G..... ...T.....C ..........
378/90    .......G.. .T..... .......... ....G..... ...T.....C ..........
18/88     .......G.. .T..... .......... ....G..... ...A.....C ..........
281/89    .T..G..... CT..... .......T.. .......... .......... ..A.......
```

Figure 5.1. Nucleotide sequence comparison of southern African Crimean-Congo haemorrhagic fever isolate . The region is compared with a reference isolate, IbAr 10200, from Nigeria. Dots indicate sequence identity

```
          1                                                          60
10200     ACAGGTTTGT GTTTCAGATG GCCAGTGCCA CCGATGATGC ACAGAAGGAC TCCATCTACG
247/85    .T........ A..C...... .........A .......... ...A.....T .........T
582/86    .T........ A..C...... .........A .......... ...A..... .........T
94/85     .T........ A..C...... .........A .......... ...A.....T .........T
566/86    ....A..... ...C..A... .......... .T........ ...A.....T .....  .T
245/87    ...A..... ...C..A... ...G...... .T........ ...A. ...T .........T
60/89     .......... A..C...... ..T....A. .......... ...A..A..T .........T
422/86    ...A..... ...C..A... .......... .......... ...A.....T ..........
187/86    ...A..... ...C..A... ......C... .T........ ...A.....T .........T
259/84    . .A..... ...C..A... .......... .T........ ...A.....T ..T.......
45/88     ...A..... ...C..A... .......... .......... ...A.....T ..........
415/85    ...A..... ...C..A... .......... .T........ ...A.....T ..........
536/86    ...A..... ...C..A... .......... .......... ...A.....T ..........
378/90    ...A..... ...C..A... .......... .T........ ...A.....T ..T.......
18/88     ...A..... ...C..A... .......... .T........ ...A.....T ..T. ....
281/89    ...A..... A..C...... .........A .......... ...A. ...T .........T

          61                                                         120
10200     CATCTGCTCT GGTGGAGGCA ACAAAGTTTT GTGCACCTAT ATATGAGTGC GCATGGGTTA
247/85    .......... A.......... ..T.....C. .......... ...C.....T .........C
582/86    .......... A.......... ..T.....C. .......... ...C.....T .........C
94/85     .......... A.......... ..T.....C. .......... ...C.....T ..... ..C
566/86    .......... .......... .......... .......... ...C.....T ..........
245/87    .......... .......... .....A.... .......... ...C.....T ..........
60/89     .......CT. ...T.....T ..C....A.. ....T..C.. ......A..T ..C.......
422/86    .G........ ...A...... .....A.... .......... .........T ..........
187/86    .G........ .......... .....A.... .......... .......  ..T ..... ....
259/84    .G........ .......... ...A..... .......... .........T ..G.......
45/88     .G........ ......A... ....A..... .......... .........T ..........
415/85    .G........ .......... .....A.... .......G.. .........T ..........
536/86    .G........ .......... ...  A.... .......G.. .........T ..........
378/90    .G........ .......... .......... .......... .........T ..G.......
18/88     .G........ .......... .....A.... .......... ....  ..AT ..G.......
281/89    .......... A.......... ..T.....C. .......... ...C. ...T .........C
```

RFLP patterns (isolates 259/84, 94/85, 187/86, 582 86 and 60/89, from RFLP groups 1, 2, 3, 8 and 9 respectively) and 2 isolates from each of the remaining 5 groups(isolates 378/90, 18/88, 422/86, 45/88, 536/86, 415/85, 566/86, 245/87, 247/85 and 281/89, from RFLP groups 4, 5, 6, 7 and 10) (Table 5.4).

The nucleotide sequences of the partial S segment of the southern African isolates were aligned with the sequence generated for the reference Nigerian tick isolate IbAr 10200 (sequence data supplied by USAMRIID) (Figure 5.1). The sequence similarity between southern African isolates and IbAr 10200 ranged from 84-92% (data not shown). Base changes were observed throughout the portion of the genome that was analysed, with the longest conserved regions among the southern African isolates ranging from 11-17 bases. Conserved regions were observed in positions 33-46, 279-295 (with the exception of isolate 60/89), 318-328 and 358-373 (with the exception of isolates 566/86 and 245/87). A pairwise comparison of sequence homology among southern African isolates generated with DNASIS, is shown in Figure 5.2. Similarity between the isolates ranged from 82 to 98%.

To determine the significance of the nucleotide changes a pairwise comparison of the predicted amino acid sequences of southern African isolates was generated using DNASIS (Figure 5.3). The protein sequence similarity ranged between 94-100%, and hence the majority of the nucleotide changes did not result in amino acid changes. The predicted amino acid sequences of the partial S segment of the southern African isolates were aligned with the sequence generated for the reference Nigerian tick isolate IbAr

RFLP group	Isolate (SPU no)	Patient no	Outcome	Restriction endonuclease							
				DdeI	DraII	RSAI	NheI	AvaII	HinfI	TaqI	HaeIII
6	536/86	35	Fatal	4	1	3	2	1	1	1	1
7	97/85	36	Fatal	3	1	3	1	1	1	1	1
7	566/86	37	Non-fatal	3	1	3	1	1	1	1	1
7	103/87	38	Non-fatal	3	1	3	1	1	1	1	1
7[8]	244/87	39	Fatal	3	1	3	1	1	1	1	1
7[8]	245/87	40	Non-fatal	3	1	3	1	1	1	1	1
7	556/87	41	Fatal	3	1	3	1	1	1	1	1
7	273/88	42	Non-fatal	3	1	3	1	1	1	1	1
8	582/86	43	Non-fatal	2	2	2	1	1	1	1	1
9	60/89	44	Non-fatal	2	1	3	1	1	1	1	1
10	281/89	46	Non-fatal	2	1	2	1	1	1	1	1

[1] Numbers refer to RFLP patterns .
[2, 3, 4, 5, 6] CCHF isolates from the same patient on different days of illness
[7] Tick isolate
[8] Two patients from 1 outbreak

RFLP group	Isolate (SPU no)	Patient no	Outcome	Restriction endonuclease							
				DdeI	DraII	RSAI	NheI	AvaII	HinfI	TaqI	HaeIII
5	105/87	22	Fatal	4	1	3	1	1	1	1	1
5[a]	45/88	23	Fatal	4	1	3	1	1	1	1	1
5[a]	49/88	23	Fatal	4	1	3	1	1	1	1	1
5	203/88	24	Fatal	4	1	3	1	1	1	1	1
5	465/88	25	Non-fatal	4	1	3	1	1	1	1	1
5	498/88	26	Non-fatal	4	1	3	1	1	1	1	1
5	337/89	27	Non-fatal	4	1	3	1	1	1	1	1
5	372/89	28	Non-fatal	4	1	3	1	1	1	1	1
5	407/89	29	Non-fatal	4	1	3	1	1	1	1	1
5	48/90	30	Non-fatal	4	1	3	1	1	1	1	1
5	187/90	31	Fatal	4	1	3	1	1	1	1	1
5	215/90	32	Non-fatal	4	1	3	1	1	1	1	1
5	30/93	33	Non-fatal	4	1	3	1	1	1	1	1
6[a]	415/85	34	Fatal	4	1	3	2	1	1	1	1
6[a]	428/85	34	Fatal	4	1	3	2	1	1	1	1
6[a]	430/85	34	Fatal	4	1	3	2	1	1	1	1
6[a]	431/85	34	Fatal	4	1	3	2	1	1	1	1

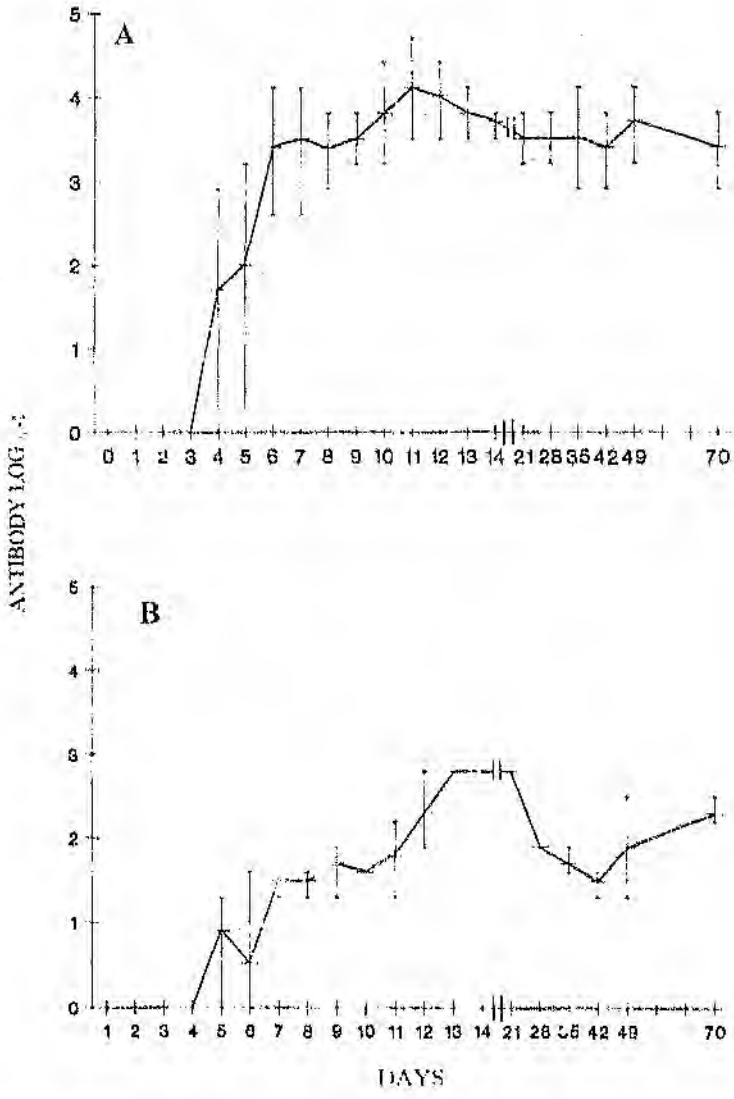


Figure 6.2. Antibody response to CCHF virus in sheep sera detected by A. sandwich ELISA for IgG antibody activity using anti-sheep HRPO conjugateand B. CELISA for total antibody activity. Curves show mean antibody titres plus range.

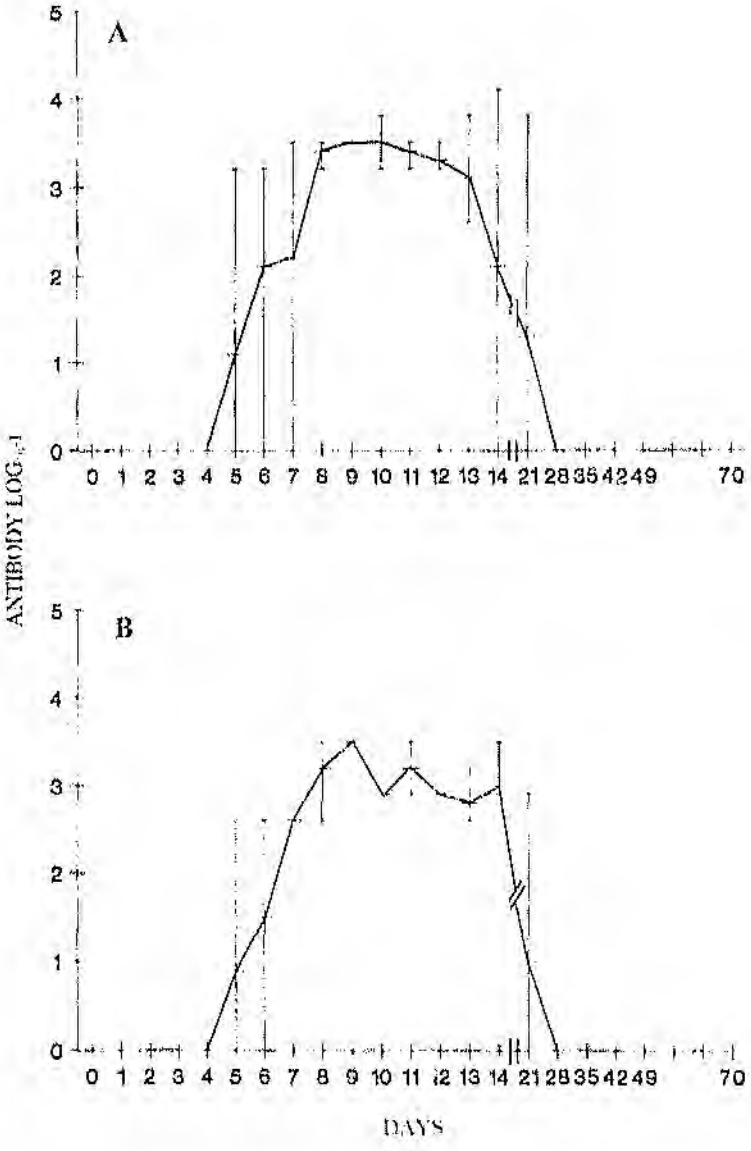


Figure 6.1. IgM antibody levels to CCHF virus in sheep sera detected by IgM capture EIISAs using two different detection methods: A. anti-species HRPO conjugate and B. anti-CCHF HRPO conjugate. Curves show mean antibody titres plus range.

4 post-infection at the earliest (Figure 6.2a), while the CELISA, which does not discriminate between IgG and IgM antibody, demonstrated antibody by day 5 at the earliest (Figure 6.2b) (Results presented in Appendices C and D). Antibody was still demonstrable by both methods on day 70 in all 3 sheep, but the titres determined by the sandwich ELISA were slightly higher than those measured by the CELISA.

The results obtained in the capture ELISA for IgM and CELISA for total antibody activity on the sera of eleven cattle are shown in Figure 6.3 (results presented in Appendices E and F). IgM antibody was detectable from day 7, but 5 of the cattle failed to develop demonstrable IgM response (Figure 6.3b). Total antibody response was detected from day 6 by CELISA (Figure 6.3a) and was still detectable on day 56, whereas IgM antibody titres declined after day 28 and were no longer demonstrable by day 49. Three cattle failed to develop any demonstrable antibody response.

Mouse brain and cell culture lysate antigens produced similar results in all ELISA and CELISA tests.

6.3.2 Small mammals

Antibody titres detected by CELISA in the sera of 34 experimentally infected small mammals are summarised in Table 6.1 and presented in full in Appendix G. All 9 guinea pigs tested had demonstrable antibodies by day 21 post-infection. On day 21 post-inoculation, the 2 bushveld gerbils had antibody to CCHF which had declined in titre by day 35. A similar trend was observed with the white tailed rats and scrub hares in that

6.2.4.4 CELISA

The CELISA (van der Groen *et al.*, 1989) was performed as described in Section 3.2.6.3. The sera were considered positive if the optical density reading was ≥ 50% that for control serum taken from the animals prior to infection. An attempt was made to perform the test as a blocking rather than as a competition assay by adding antigen and test sera sequentially instead of simultaneously.

6.2.4.5 IF tests

IF tests for the detection of IgG antibody to CCHF virus had been performed on the sera of small mammals as described in Section 3.2.5 using CCHF antigen slides and commercially available anti-mouse, -rabbit and -guinea pig immunoglobulin FITC conjugates (Cappel).

6.3 Results

6.3.1 Sheep and cattle

The results obtained in IgM-capture ELISAs on sheep sera are shown in Figure 6.1 and presented in Appendices A and B. IgM antibody to CCHF virus was detectable from day 5 to day 21 after infection. The method using the anti-CCHF conjugate (Figure 6.1b) demonstrated slightly lower titres than that using the HMAF detection system (Figure 6.1a), but detected antibody more frequently in sera taken early and late in the course of the response than the HMAF system

The sandwich ELISA detected IgG antibody activity to CCHF virus in sheep sera by day

substrate. ABTS was added and the plates incubated at room temperature (22° C) for 30 min in the dark. The results were determined by reading the optical density at 402nm on a Multiscan spectrophotometer. Specimens were recorded as IgM antibody positive if the absorbance in the sample wells was at least twice the value of that for control serum taken from the animals prior to infection. Titres were recorded as reciprocals of the highest dilutions of test sera giving a positive result.

6.2.4.2 IgM-capture sandwich ELISA

Plates were coated overnight with μ-chain specific anti-sheep IgM antibody, post-coated and reacted with sheep sera as above. After washing, 1/200 CCHF antigen in diluent containing 2% normal sheep serum was added and the plates incubated. The plates were washed and anti-CCHF HMAF diluted 1/1,000 was added, left to react, and detected with 1/1,000 anti-mouse IgG HRPO conjugate (Zymed) and ABTS substrate. The results were recorded as described above. Cattle sera were not tested by this method.

6.2.4.3 IgG sandwich ELISA

Plates were coated overnight at 4°C with anti-CCHF monoclonal antibody 6H5 diluted 1/2,000. After post-coating, CCHF antigen at a dilution of 1/200 was added, the plates were incubated, washed and sheep sera were added in doubling dilutions from 1/100 upwards. The plates were incubated, washed and anti-sheep IgG HRPO (Zymed) was added at a dilution of 1/1,000. The peroxidase was detected with ABTS substrate as above. Cattle sera were not tested by this method.

Sera used in evaluating the ELISA and C'ELISA techniques included 63 serial samples from 3 sheep, 145 samples from 11 cattle, and 69 samples from 34 small mammals, that had been collected at the intervals indicated in the results from animals infected subcutaneously with $10^{7.5}$-$10^{8.5}$ $TCID_{50}$ of CCHF isolate 4/81, and stored at -70 °C (Shepherd *et al.*, 1989c; Shepherd *et al.*, 1991). Pre-inoculation serum from each animal served as a negative control. The C'ELISA was applied to 960 serum samples from 29 species of wild vertebrate, which had been collected in the Kruger National Park, South Africa, from 1974-1992 for unrelated purposes and stored at -70 °C.

6.2.4 Serological tests

The plates, buffers, test volumes, incubation times and washing procedures for the ELISA and C'ELISA were essentially as described in Section 3.2.6, and optimal working dilutions of reagents were determined by chessboard titration.

6.2.4.1 IgM-capture ELISA using anti-CCHF HRPO conjugate

Plates were coated overnight at 4 °C with μ-chain specific anti-sheep IgM (Zymed) diluted 1/1,000 in carbonate buffer, pH 9.6. It had previously been established that major cross-reactions occurring in the IgM of bovids render this capture antibody suitable for testing cattle as well as sheep sera (Ksiazek *et al.*, 1989). After the plates were washed, sheep or cattle sera were added to the wells in doubling dilutions from 1/200 upwards. The plates were incubated, washed, and CCHF antigen diluted 1/200, was added to the wells. After further incubation and washing, anti-CCHF HRPO conjugate, diluted 1/1,000, was added to the wells and the plates were incubated. After further washing, the

6.2 Materials and methods

6.2.1 Antigens

Sucrose-acetone extracted antigen was prepared from suckling mouse brain infected with South African CCHF isolate 4/81 (Swanepoel *et al.*, 1983a) and inactivated with 0.1% beta-propriolactone as described in Section 3.2.1 (Clarke and Casals, 1958).

For preparation of cell lysate antigen, confluent monolayers of Vero 76 cells grown in 150cm^2 flasks were infected with CCHF isolate 4/81 at a multiplicity of 1 tissue culture infective dose ($TCID_{50}$) per cell and maintained in EMEM supplemented with 2% foetal calf serum. The cells were incubated for 5 days at 37 °C, harvested, washed in borate buffered saline (BBS), pH 9.0, and the pellet was resuspended in BBS containing 0.5 ml of 1% triton X-100 and 0.1% SDS, and subjected to ultrasonic disruption using a Branson 220 sonic disruptor (Branson Cleaning Equipment Company, Shelton, CT, USA) for 15 min. After centrifugation for 10 min at 10,000xg and 4 °C, the supernatant fluid was inactivated with 0.1% BPL and stored at -70 °C for use as antigen.

6.2.2 Antibodies

Monoclonal antibody 6D5, specific for CCHF viral nucleocapsid protein (Blackburn *et al.*, 1987), was used as a coating antibody for ELISA. Anti-CCHF IMAF, and anti-CCHF horseradish peroxidase (HRPO) conjugate prepared from purified immune rabbit immunoglobulin (Section 3.2.3), were used as detector antibodies.

6.2.3 Test sera

Prior to developing ELISA to test for antibodies to other tick-borne viruses in human and livestock sera (Chapter 7), it was considered logical to perform a feasibility study by developing CCHF sandwich IgG antibody and IgM-capture antibody ELISA for sheep and cattle sera, using commercially available anti-species immunoglobulin peroxidase conjugates, and the sera of experimentally infected sheep and cattle. Following this, it was decided to determine whether the tests could be adapted for use on the sera of wild vertebrates.

Anti-species immunoglobulin conjugates are commercially available for performing IF and ELISA tests on the sera of various domestic animals, and some of these can be used on the sera of wild vertebrates, e.g. anti-dog and anti-cat conjugates can be used on the sera of wild canids and felids. Nevertheless, it was found that the CCHF ELISA techniques developed for sheep and cattle sera could be applied to the sera of some but not all African species of antelope: anti-sheep and anti-cattle immunoglobulins bound poorly or not at all to the immunoglobulins of some antelope in ELISA or immunodiffusion tests (unpublished laboratory records). Hence, the competition ELISA (CELISA) which had been developed using anti-CCHF horseradish peroxidase conjugate prepared from immune rabbit serum (see Section 3.2.3 above), was evaluated for its wider applicability, using the sera of experimentally infected small mammals, sheep and cattle. The sera of the small mammals had been tested by IF. The CELISA was then applied to the sera of 960 wild vertebrates collected in a nature reserve.

6. Detection of antibody to CCHF virus in the sera of livestock and wild vertebrates using enzyme-linked immunosorbent assays

6.1 Introduction

Certain diagnostic problems encountered in the Special Pathogens Unit suggested that there was a need to investigate the possibility that tick-borne viruses other than CCHF were causing human disease in South Africa, and it was decided to conduct surveys on cattle sera for antibodies to selected viruses in order to seek evidence that the viruses were present in the country (see Chapter 7). Serological techniques such as immunodiffusion, complement-fixation, IF, haemagglutination-inhibition, passive haemagglutination-inhibition and virus neutralization had been used in the past to investigate CCHF (Woodall *et al.*, 1967; Casals, 1969; Casals, 1978; Zavodova *et al.*, 1971; Casals and Tignor, 1974; Buckley, 1974; Chaidamovich *et al.*, 1974; Saidi *et al.*, 1975; Tignor *et al.*, 1980; Donets *et al.*, 1982). Despite the fact that reversed passive haemagglutination inhibition was used successfully to conduct CCHF studies on the sera of cattle and wild animals in southern Africa (Swanepoel *et al.*, 1983b; 1987; Shepherd *et al.*, 1987a) epidemiologic studies have always been hampered by the lack of sensitivity, reproducibility and amenity to automation of the available serological techniques. The problems were potentially overcome by the development of a solid-phase radioimmunoassay and an indirect or sandwich ELISA for CCHF (Donets *et al.*, 1982). Although the ELISA was used for the detection of antibody in human sera (Shepherd *et al.*, 1989a), the technique was not applied to studies on the sera of domestic or wild animals.

5.4 Summary

Fifty seven southern African isolates were assigned to 10 groups according to the RFLP patterns obtained using 8 restriction endonucleases on a 536 bp PCR product amplified from the S segment of CCHF virus genome between positions 186 and 635 relative to the positive sense strand. The majority of isolates, 20/57 and 18/57, belonged to RFLP groups 4 and 5. Five isolates had unique RFLP patterns. No correlation could be made between the RFLP grouping and source of infection, year of infection or pathogenicity, where pathogenicity is defined as severity of illness and fatality. Three isolates, 247/85 and 281/89 (RFLP group 10) and 259/84 (RFLP group 1) were from areas where the tick *H. truncatum* is prevalent and *H. m. rufipes* and *H. m. turanicum* are absent.

Analysis of the nucleotide sequence data for a 450bp region of the PCR product of the S segment of the CCHF virus genome between positions 135 and 670, of 15 southern African isolates, showed that the majority of nucleotide changes were conserved with 94-100% similarity of predicted amino acid sequences among the isolates. No correlation could be made between the phylogenetic relationship of the 15 southern African isolates and source of infection, year of infection or pathogenicity of the virus, but 7 of the isolates fell into 2 clades, with the remaining isolates branching off singly.

In a phylogenetic comparison with 14 CCHF isolates from other geographic regions, 15 southern African isolates clustered into 3 groups with isolates from other countries on the continent, while isolates from Asia and Madagascar grouped together, and a Greek isolate branched off on its own.

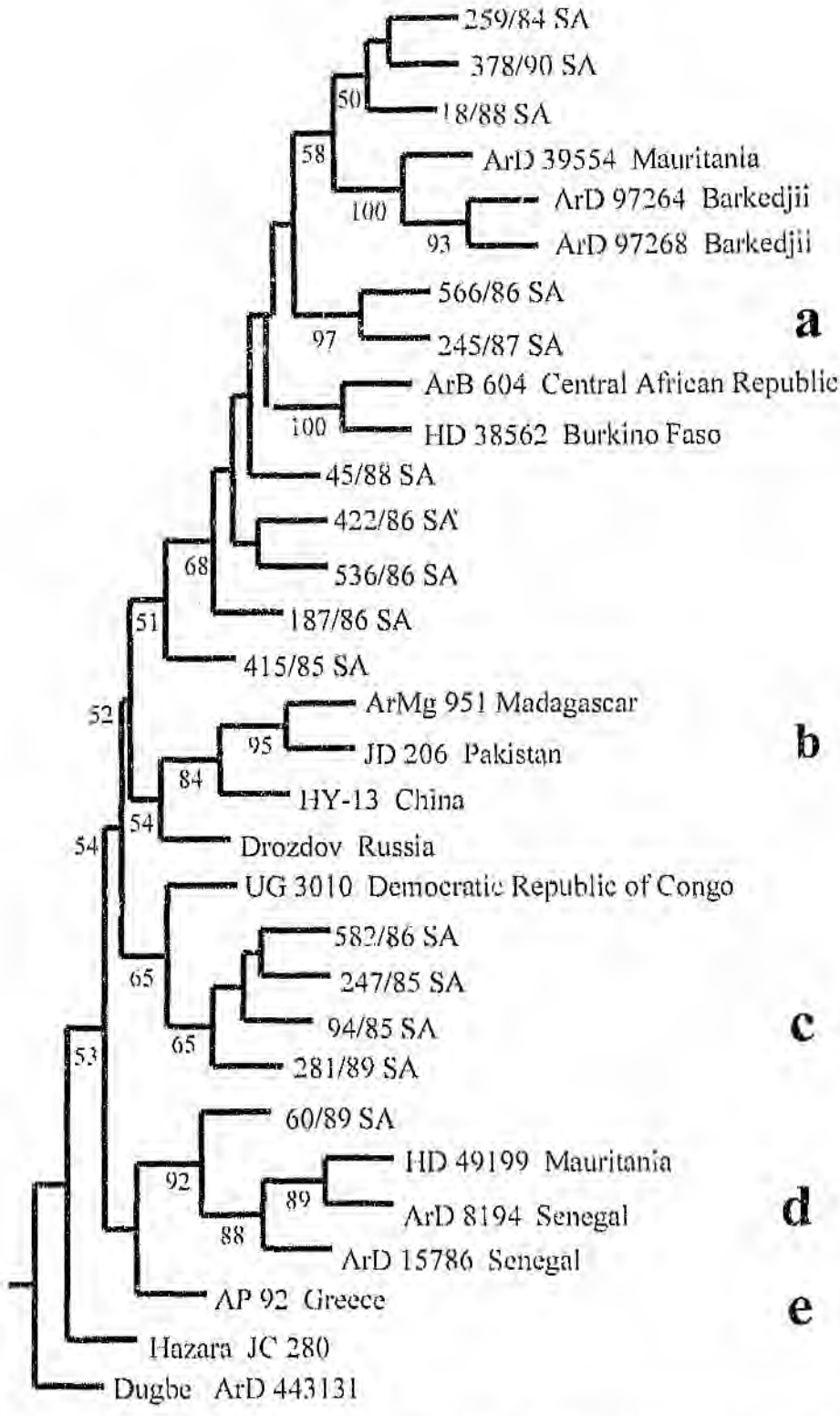


Figure 5.6. Phylogenetic relationship among geographically distinct CCHF isolates determined using DNAML and 100 bootstrap replications of the data. The number of times each node occurred in the consensus tree is shown beneath the branches (values less than 50 have been omitted). a, b, c, d and e represent groups of CCHF virus isolates.

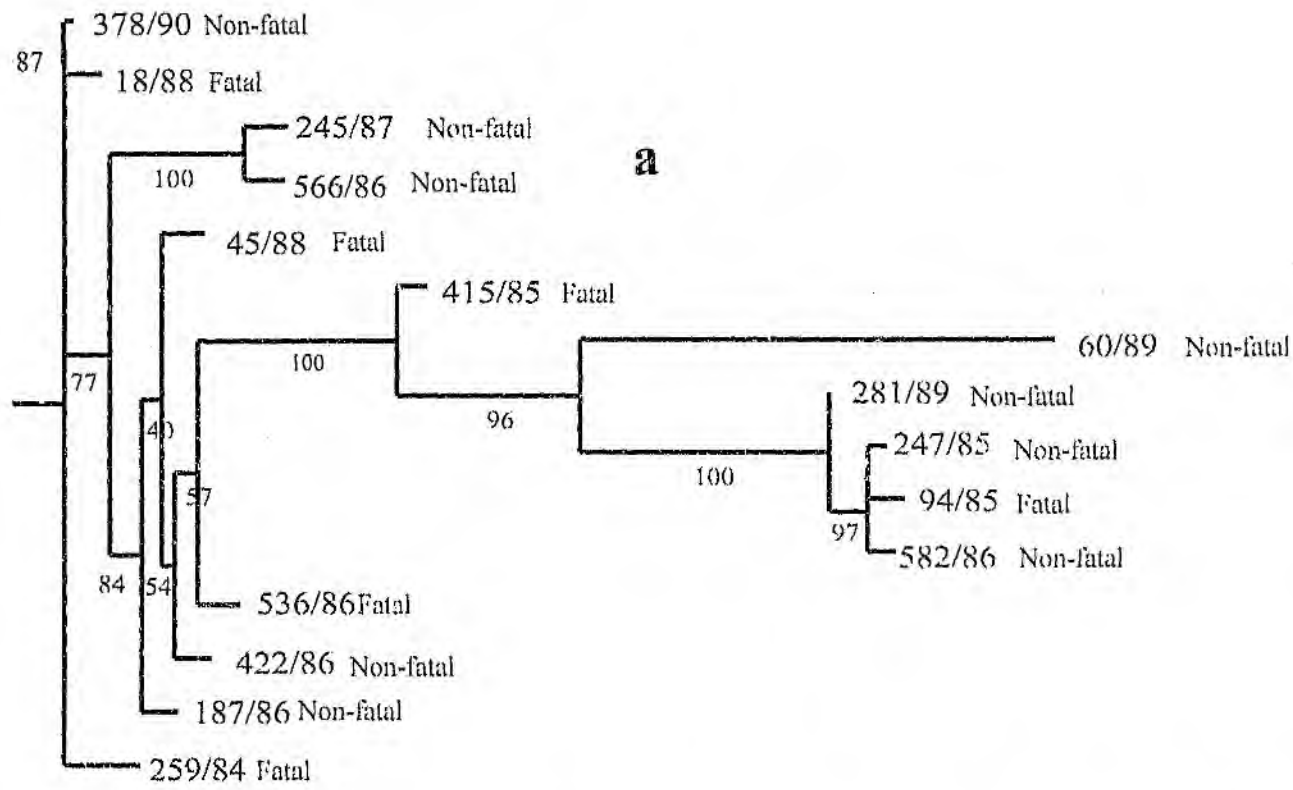


Figure 5.5. Phylogenetic relationship among southern African isolates determined using DNAML and 100 bootstrap replications of the data. The number of times each node occurred in the consensus from 100 trees is shown beneath the branches, and a) and b) refer to two groups of isolates. The outcome of the illness is shown for each isolate.

were prepared in Liebowitz medium containing 2% foetal calf serum as diluent. Equal volumes of virus containing a calculated 50-100 fluorescent focus units (FFU) per 0.05 ml were added to the tubes of serum, and the mixtures incubated for 45 min at 37° C. The medium was removed from cell monolayers in 8-well slide culture chambers, 0.1 ml of each serum-virus mixture was added to duplicate wells and the slides incubated at 37° C for 1 hour. To each well was then added 0.3 ml of Liebowitz medium containing 2% foetal calf serum and the slides were incubated for 48 hours at 37°C. Slides were fixed in cold acetone for 10 min and IF test performed with reference HMAF as described previously (Swanepoel *et al.*, 1983a).

A control back titration of the virus used in the test consisted of working strength virus plus 2 serial 10-fold dilutions incubated with equal volumes of 1/4 dilution of foetal calf serum. Each control serum-virus mixture was inoculated onto monolayers which were incubated and stained by IF along with the test cultures. The average number of FFU was determined in the lowest control virus dilution which could be counted and the number of FFU used in the test calculated. Serum titres were recorded as the reciprocals of the highest dilutions which produced a ≥90% reduction in the number of FFU.

7.3 Results

7.3.1 Antibody survey on cattle sera

The results of ELISA for antibodies to the tick-associated viruses in cattle sera are summarised in Table 7.1 and presented in full in Appendix II. Findings for CCHF virus were similar to those reported previously for the RPHI technique (Swanepoel *et al.*,

Vero 76 cell cultures as described previously (Swanepoel *et al.*, 1983a; Swanepoel *et al.*, 1987). Cerebrospinal fluid was also tested for virus on 1 occasion. In addition, attempts were made to demonstrate the presence of Dugbe virus S segment RNA in serum samples by means of RT-PCR, using primer sequences described by Ward *et al.* (1990b) and the protocol described in Section 2.2.5. Vero 76 cell cultures infected with reference virus and non-infected cells were used as positive and negative controls, and PCR products were analysed on ethidium bromide stained agarose electrophoresis gels. Tests for neutralizing antibody to Dugbe virus were performed as described below (section 7.2.7). Serum samples were also tested (in another laboratory) for the presence of DNA of parvovirus B19 by PCR (Koch and Adler, 1990), and IgG and IgM antibodies to parvovirus B19 by means of commercially available ELISA kits (Genoclin GmBH Laboratories, Hamburg, Germany). Monitoring of clinical pathology values and tests for the diagnosis of a further range of disease conditions as indicated in the results, including tests for the presence of anti-platelet antibody in serum, were performed at other laboratories.

7.2.7 Neutralization tests

Fluorescent focus neutralization tests for antibody to Dugbe virus were performed by a constant virus-varying serum dilution method based on fluorescent focus reduction in Vero 76 cell cultures as described previously for CCHF virus (Casals and Tignor, 1974; Tignor *et al.*, 1980; Shepherd *et al.*, 1989a). Sera were extracted by the acetone-ether method for removal of non-specific inhibitors of virus infectivity (Casals and Tignor, 1974). Reconstituted sera were inactivated at 59 °C for 30 min and serial 4-fold dilutions

Walker, 1991), as illustrated for Dugbe virus in the results.

Sera from human patients had been submitted to the laboratory from 1984-1993 for the diagnosis of suspected viral haemorrhagic fever, and had been stored at -70°C after the initial investigations had been performed. Specimens which had been collected from patients during convalescence 1-2 months after the onset of illness, were selected for inclusion in the study where possible, although a few sera taken earlier were also tested. The sera included samples from 72 patients in whom a diagnosis of CCHF had been confirmed by isolation of virus and/or demonstration of seroconversion, or ≥4-fold increase in antibody titres, or presence of IgM antibody activity by IF. A further 172 sera came from 162 patients with a history of tick bite or possible exposure to ticks, in whom no evidence of CCHF infection was found. Serological evidence of *R. conorii* infection had been found in 28 of these patients, and other diagnoses had been established in a further 19 instances, but no definitive diagnosis had been obtained in the remaining 115 cases.

7.2.6 Investigation of patient with suspected Dugbe virus infection

In the course of the study rising titres of antibody to Dugbe virus were detected in serum samples from a patient who had developed febrile illness with prolonged thrombocytopenia after exposure to tick bite in 1990, and further observations were made on the patient at intervals over a period of 34 months following the onset of illness. As with all other patients, initial tests performed on his serum samples in the Special Pathogens Unit included attempts to isolate virus by inoculation of suckling mice and

7.2.4 IgM capture ELISA for antibodies to tick-associated viruses

An ELISA for the detection of IgM antibodies to the test viruses in human sera was performed using μ-chain specific anti-human IgM (Zymed) as capture antibody and ABTS substrate. Antigens immobilized by captured IgM were detected with reference HMAFs and anti-mouse IgG HRPO conjugate (Zymed). ABTS was used as the substrate. Optimal dilutions of the coating antibody (1/5,000) and anti-mouse IgG HRPO conjugate (1/2,000) were determined by chessboard titration with known CCHF positive serum. Antigens and HMAF of the tick-associated viruses were used at the optimal dilutions determined above. Human sera were tested in doubling dilutions from 1/200 upwards, and results recorded as described above.

7.2.5 Test sera

A total of 2,116 cattle sera from 46 herds distributed throughout South Africa, which had been tested by reversed passive haemagglutination inhibition (RPHI) for antibody to CCHF virus in a survey in 1983-1984 (Swanepoel *et al.*, 1987) and stored at -70° C, were screened at initial dilution for antibody to CCHF virus by ELISA in order to evaluate the latter technique by comparing the 2 sets of results. Fifty sera were tested per herd where possible, and in smaller herds all available cattle were tested. A subset of 1,358 sera from 28 herds was screened by ELISA for antibodies to Dugbe, louping ill, NSD and Thogoto viruses, while batches of 400 to 785 sera were screened for antibodies to the remaining 7 study viruses, as indicated in the results. Herds to be screened for antibody to each virus were selected to include areas where known vector species or at least ticks of the same genus are present, as well as areas where the ticks are absent (Howell *et al.*, 1978;

Sucrose-acetone extracted antigens were prepared from infected mouse brain (as described in section 3.2.1.) and HMAF were prepared in adult mice for all of the viruses included in the study, as described previously (see section 3.2.2.) (Shope and Sather, 1979).

Positive control sera were prepared by inoculating 2 guinea pigs per virus intraperitoneally with 0.2 ml of a 1/10 dilution of infected mouse brain suspension. Boosters were administered 1 and 3 weeks later, and the guinea pigs were exsanguinated 2 months after receiving the initial dose of virus. Sera from non-infected guinea pigs were used as negative controls.

7.2.3 IgG sandwich ELISA for antibodies to tick-associated viruses

A sandwich ELISA for the detection of IgG antibodies to the test viruses in cattle and human sera was performed as described previously for CCHF virus (Section 3.2.6.2) using the appropriate anti-species immunoglobulin horseradish peroxidase (HRPO) conjugate (Zymed), and ABTS substrate. Optimal working dilutions of HMAF coating antibodies (1/1,000-1/5,000) and antigens (1/50-1/200) for the tick-associated viruses were determined by chessboard titration with control guinea pig sera and anti-guinea pig IgG HRPO conjugate. Optimal dilutions of anti-cattle and anti-human IgG HRPO conjugates (1/500-1/2,000) were determined in chessboard titrations with known CCHF positive cattle and human sera. Specimens were tested in doubling dilutions from 1/50 upwards, and recorded as IgG antibody positive if the absorbance at 402 nm was at least twice that of negative control serum. Titres were recorded as the reciprocal of the highest serum dilution producing a positive result.

cross-reactive with antigenically related viruses, and to check the possibility that dual infections may have occurred in some instances. Next, tests for antibodies to the selected viruses were applied to convalescent phase sera from patients who lacked evidence of CCHF infection, but either had a history of being bitten by ticks, or lived in or visited a rural location where exposure to ticks was possible. Alternative causes of illness had been identified in some of these patients, but no definitive diagnosis had been established in most instances. Antibody activity to 1 or more members of the genus *Nairovirus* of the family *Bunyaviridae*, viruses related to CCHF, was detected in serum samples of 4 patients. Three of these patients were no longer accessible for further investigation ; rising titres of antibody to Dugbe virus were detected in the fourth who developed febrile illness with a petechial rash, bruising tendency, transient epididymo-orchitis and encephalitis, plus prolonged thrombocytopenia, after being bitten by larval ticks.

7.2 Materials and methods

7.2.1 Virus strains

Viruses used in the study included strains of CCHF, Chenuda and West Nile isolated in South Africa (Swanepoel *et al.*, 1983a; McIntosh, 1980; Kokernot and McIntosh, 1959), and Hazara, Dugbe, Bahig, Bhanja, louping ill, Kadam, Thogoto and Dhori obtained from Yale Arbovirus Research Unit, New Haven, Ct, USA. Nairobi sheep disease (NSD) virus was in stock in the Arbovirus Unit of the Institute and is believed to have been obtained from a veterinary laboratory in Kenya.

7.2.2 Antigens and antisera

7. Tick-borne viruses in the differential diagnosis of CCHF in South Africa

7.1 Introduction

From January 1980-December 1993 inclusive the Special Pathogens Unit examined 3,219 specimens from 1,717 patients in southern Africa suspected to be suffering from viral haemorrhagic fever, and diagnosed 109 cases of Crimean-Congo haemorrhagic fever (CCHF) (Swanepoel *et al.*, 1983a; Swanepoel *et al.*, 1987; Swanepoel *et al.* 1989; unpublished laboratory records). Among those patients for whom no cause of illness could be found there were some with a history of having been bitten by ticks. Preliminary screening had eliminated tick-borne typhus (*Rickettsia conorii* infection), Q fever (*Coxiella burnetti* infection), ehrlichiosis (*Ehrlichia* spp. infection), Lyme disease (*Borrelia burgdorferi* infection), and CCHF virus infection in these patients, and it was decided to investigate the possibility that tick-borne viruses other than CCHF were involved. Twelve viruses, including CCHF, were selected for the investigation. Not all were known to occur in Africa or to cause human disease, or even to be transmitted principally by ticks.

As a preliminary, an ELISA was developed to test for antibodies to the selected viruses. Most of the ticks which bite humans parasitize farm animals by preference, and hence it was convenient to conduct an antibody survey on cattle sera to determine whether there was evidence that the selected viruses occur in South Africa. Sera taken during convalescence from patients with confirmed CCHF infection were tested to determine whether the causative agent of this disease induces production of antibody which is

6.4 Summary

It was shown that IgM antibody to CCHF virus can be demonstrated in the serum of experimentally infected sheep by IgM-capture ELISA with either of 2 detection methods, using rabbit anti-CCHF conjugate, or a sandwich technique with anti-CCHF HMAF and a commercial anti-mouse immunoglobulin conjugate. The titres determined by use of the anti-CCHF conjugate were slightly but not significantly lower than those detected by the sandwich method. Total and IgG antibody activities can be detected by the CELISA and a sandwich method using commercial anti-species conjugate. The CELISA can also be used to detect antibody in the sera of cattle and small and large wild vertebrates. In tests on 960 sera of 29 species of wild vertebrate from the Kruger National Park, the highest prevalences of antibody to CCHF virus were found in the sera of the largest animals.

Table 6.2. Prevalence of antibody to CCHF virus in wild vertebrate sera collected in the Kruger National Park.

Common name	Scientific name	No.positive/no. tested(%)
Lion	*Panthera leo*	0/116(0)
Cheetah	*Acinonyx jubatus*	0/14(0)
Leopard	*Panthera pardus*	0/6(0)
Genet	*Genetta genetta*	0/1(0)
Wild dog	*Lycaon pictus*	3/62(5)
Elephant	*Loxodonta africana*	0/23(0)
Black rhino	*Diceros bicornis*	2/5(40)
White rhino	*Ceratotherium simum*	21/31(68)
Zebra	*Equus burchelli*	2/28(7)
Warthog	*Phacochoerus aethiopicus*	0/21(0)
Hippopotamus	*Hippopotamus amphibius*	0/15(0)
Giraffe	*Giraffa camelopardelis*	10/44(23)
Impala	*Aepyceros melampus*	5/47(11)
Blue wildebeest	*Connochaetes taurinus*	0/31(0)
Tsessebe	*Damaliscus lunatus*	0/2(0)
Buffalo	*Syncerus caffer*	31/312(10)
Nyala	*Tragelaphus angasii*	0/1(0)
Bushbuck	*Tragelaphus scriptus*	0/1(0)
Kudu	*Tragelaphus strepsiceros*	2/4(50)
Duiker	*Sylvicapra grimmia*	0/1(0)
Roan	*Hippotragus equinus*	0/8(0)
Sable	*Hippotragus niger*	3/49(6)
Hartebeest	*Sigmoceros lichtensteinii*	0/1(0)
Suni	*Neotragus moschatus*	0/4(0)
Baboon	*Papio ursinus*	0/21(0)
Vervet monkey	*Cercopithecus pygerythrus*	0/1(0)
Porcupine	*Hystrix africaeaustralis*	0/2(0)
Hare	*Lepus saxatilis*	0/63(0)
Guinea fowl	*Numida meleagris*	2/37(5)
Ostrich	*Struthio camelus*	0/9(0)

Table 6.1. Detection of antibody to CCHF virus in experimentally infected small mammals by IF and CELISA.

Species	n	Day after inoculation	Antibody titre (range)	
			ELISA	IF
Cavia porcellus (Guinea pig)	9	21	160-320	5120
Tatera brantsii (Bushveld gerbil)	2	1	0	0
	2	21	160	640
	2	35	20-40	0
Tatera leucogaster (Highveld gerbil)	4	1	0	0
	3	28	0-320	80-640
Mystromys albicaudatus (White-tailed rat)	3	1	0	0
	3	14	0-80	0-640
	3	21	20-160	640
	3	28	20-40	160-640
Aethomys chrysophilus (Red veld rat)	2	1	0	0
	2	14	80	160-640
Xerus inauris (Cape ground squirrel)	2	43	160	640
Lepus saxatilis (Scrub hare)	4	1	0	0
	2	2-6	0	0
	2	7	10-80	40
	3	14	20-320	640
	2	21	320	320-640
	2	27	160	320-640
	2	35	80	160-320
	3	45	20-40	0
Oryctolagus cuniculi (New Zealand giant rabbit)	8	28	0-160	0-640

the antibody titres declined after day 21, with 1 hare having a consistently low antibody titre (20-40) in specimens tested from day 21-45. One highveld gerbil and 1 rabbit did not have demonstrable antibody on day 28, and no earlier or later sera were available from these animals.

These sera had previously been tested by IF tests and the results are shown in Table 6.1. Although the antibody titres were lower by CELISA they bore a consistent relationship to those detected by IF (correlation coefficient 0.8). The only exceptions were 2 bushveld gerbils and 3 hares which had low antibody titres by ELISA on days 35 and 45 post-infection respectively but were negative by IF, and 1 highveld gerbil which was negative by ELISA but had a low IF antibody titre in day 28.

6.3.3 Wild vertebrates

The numbers of wild vertebrate sera from the Kruger National Park that were found to have antibody to CCHF virus demonstrable by CELISA are shown in Table 6.2 The highest prevalences of antibody were found in some of the larger mammals such as buffalo, white rhinoceroses and giraffe. A low prevalence of antibody was found in guinea fowl, some of the antelope species, black rhino and wild dog. The wild felids, elephant, baboons, warthogs, some antelope species and ostrich were all CCHF antibody negative by CELISA. Contrary to previous findings (Swanepoel *et al.*, 1987), no antibody was found in any of the 63 hares that were screened.

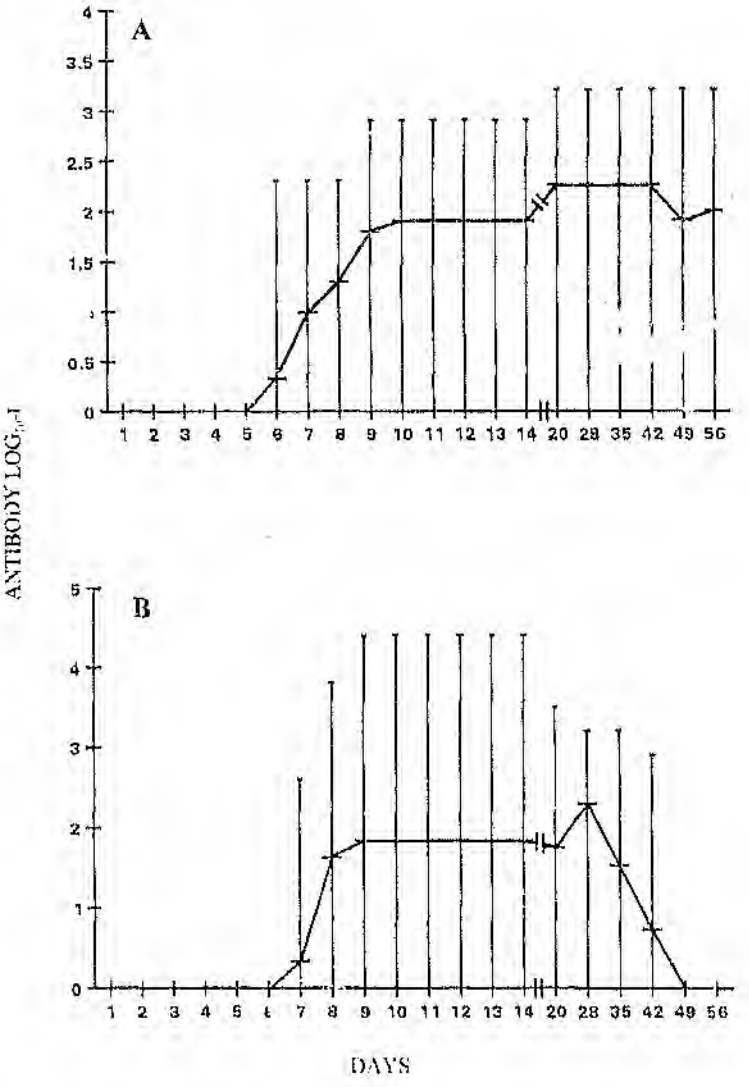


Figure 6.3. Antibody response to CCHF virus in cattle sera detected by A. CELISA for total antibody activity and B. IgM capture antibody ELISA. Curves show mean antibody titres plus range.

and as a preliminary cattle sera were tested by enzyme-linked immunoassay to seek information on the occurrence of the study viruses in the country. The prevalence of antibody activity in cattle sera was found to be 905/2116 (42.8%) for CCHF virus antigen, 70/1,358 (5.2%) for Dugbe, 21/1,358 (1.5%) for louping ill, 6/450 (1.3%) for West Nile, 7/1,358 (0.5%) for NSD, 3/625 (0.5%) for Kadam and 2/450 (0.4%) for Chenuda, while no reactions were recorded with Hazara, Bahig, Bhanja, Thogoto and Dhori antigens. The findings on CCHF confirmed previous observations that the virus is widely prevalent within the distribution range of ticks of the genus *Hyalomma*. Antibody activity to Dugbe antigen was detected only in locations within the distribution range of the tick *Amblyomma hebraeum*. Cross-reactivity for the antigens of the nairoviruses, Hazara, Nairobi sheep disease and Dugbe, was detected in serum samples from 3/72 human patients with confirmed CCHF infection, while serum from 1/162 other patients reacted monospecifically with Dugbe antigen. The latter patient suffered from febrile illness with transient encephalitis, petechial rash, bleeding tendency and orchitis, plus prolonged thrombocytopenia after being bitten by larval ticks, and observations were made on his haematological and antibody status at intervals over a period of 34 months following the onset of illness.

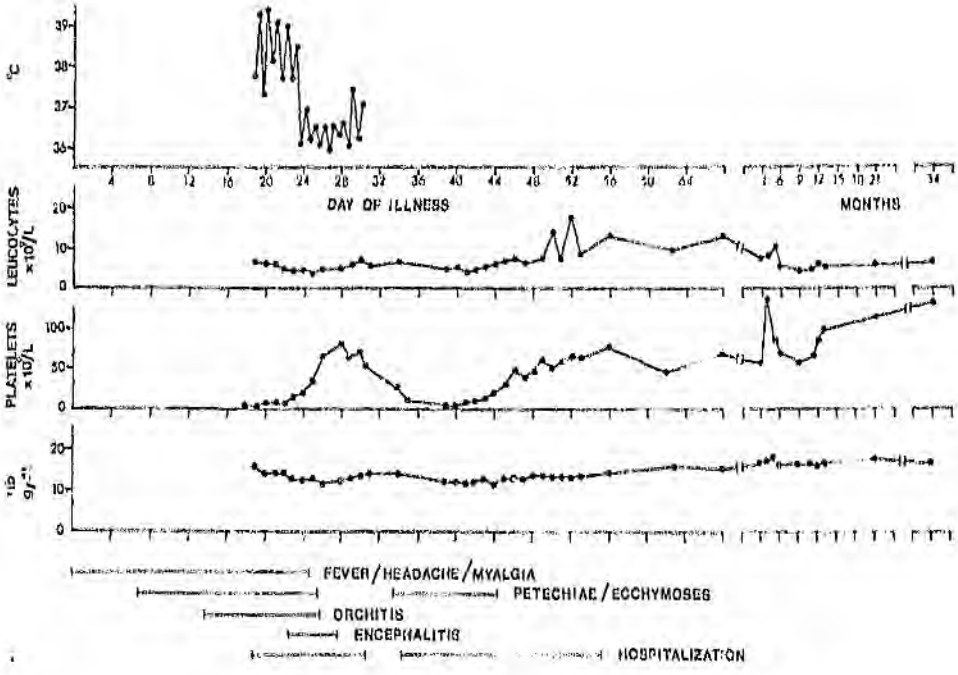


Figure 7.2. Curves showing temperature, blood leukocyte, platelet and haemoglobin levels monitored over a period of 34 months in a patient with suspected Dugbe virus infection, in relation to the main signs and symptoms of illness.

were unremarkable throughout the periods of hospitalization. Serum alkaline phosphatase levels were slightly elevated during the initial period in hospital, and a maximum value of 148 U/l, was recorded in March 1997, 3 months after the initial onset of illness (reference value 5-90 U/L). Negative results were reported from other laboratories for blood cultures for bacteraemia, bloodsmears for malaria, neutralization tests for antibodies to Coxsackieviruses B1-6, and ELISAs for antibodies to mumps, Epstein-Barr and human immunodeficiency viruses. Initial screening for antibodies to haemorrhagic fever viruses ruled out CCHF, Marburg, Ebola, Rift Valley fever, arenavirus and hantavirus infections, and IF tests also eliminated the non-viral tick-borne agents mentioned in the introduction. Haemagglutination-inhibition tests for antibodies to chikungunya, Sindbis, West Nile, Wesselsbron and Rift Valley fever arthropod-borne viruses were reported to be negative. No evidence of parvovirus B19 infection was found by PCR and ELISA. No virus was isolated from serum samples and RT-PCR for Dugbe virus nucleic acid was negative on samples collected during hospitalization. Rising titres of IgG and IgM antibody to Dugbe virus antigen, with post-convalescent decline, were demonstrated by ELISA, but neutralizing antibody was detected on a single occasion only 65 days after the onset of illness, at the lowest dilution of serum tested, 1/10. Negative results were obtained in ELISA for antibodies to all of the other tick-associated viruses included in the study.

7.4 Summary

In the course of investigating suspected cases of viral haemorrhagic fever in South Africa patients were encountered who had a history of being bitten by ticks, but who lacked evidence of infection with CCHF virus or non-viral tick-borne agents. Hence, the possibility that tick-associated viruses other than CCHF were involved was investigated.

partial thromboplastin times or fibrin degradation products, to indicate the occurrence of intravascular coagulopathy. Bone marrow samples taken on 9 and 19 December were described as being of normal active appearance. Fever subsided on 13 December and the patient remained afebrile thereafter. Other signs and symptoms of illness abated over the next 3 days, while the platelet count rose steadily to 80×10^9/L on 17 December (Figure 7.2). The patient was discharged on 20 December, but re-admitted on the 24th with renewed skin and palatal petechiae. Platelet counts were again < 20×10^9/L. Although tests had failed to reveal the presence of anti-platelet antibody in serum, immune thrombocytopenic purpura was considered as a possible diagnosis. Large doses of pooled immunoglobulin (28 g/day) were administered intravenously for 4 days, and the patient was placed on prednisolone therapy which was discontinued after 4 weeks. Tests for anti-nuclear and rheumatoid factors were also negative during the period of hospitalization. Platelet counts rose to 66×10^9/L by 10 January, while total leucocyte counts rose transiently, to a maximum of 18.0×10^9/L (Figure 7.2). The patient was finally discharged from hospital on 14 January, and was still well when last seen 34 months after the onset of illness, despite the fact that his platelet counts had remained < 100×10^9/L for most of the time. It can nevertheless be discerned that there was an upward trend in platelet counts towards the end of the period of monitoring (Figure 7.2), and a count of 158×10^9/L was recorded on the last occasion. No blood counts had been performed on the patient prior to the illness reported here for comparison of the results with the present findings.

Results of tests for liver and renal function (alanine and aspartate transaminases, serum proteins, bilirubin, urea, creatinine and blood electrolyte levels, and urine examination)

he felt "light-headed" at work and that evening, when he had developed fever, headache and malaise, consulted his doctor and was placed on oral tetracycline therapy (this was discontinued and replaced by a course of oral ampicillin on 28 November). Over the next 2 weeks he experienced bouts of fever, headache, weakness, fatigue and severe myalgia (backache and thigh pains), which were exacerbated on days that he returned to work. On 26 November he became aware of "flecks" (petechiae) on his arms and legs, and on 3 December developed swelling and tenderness of his right testicle, and noticed that he bruised easily. His rash worsened on 7 December, and because blood taken that evening was found to have a platelet count of 3×10^9/L, he was admitted to hospital on the following morning.

On admission the patient was febrile, in marked discomfort with pain in the lower back and thighs readily elicited by minor movement, had numerous lesions suggestive of healing tick bites, bruises, petechiae and ecchymoses on the lower trunk and limbs, subconjunctival haemorrhages, palatal petechiae, generalized lymphadenopathy, swollen right testicle and epididymis, a blood pressure of 130/80 mm Hg, a pulse rate of 86 per minute and respiratory rate of 28 per minute. On 12 December the patient developed visual hallucinations, nightmares and hyperaesthesia of the chest and trunk. Leucocyte, differential and erythrocyte counts fluctuated within reference value ranges, and haemoglobin values declined from 15.9 g/dL on 8 December to 12.0 g/dL at the lowest (Figure 7.2). Thrombocyte counts initially remained $<20\times10^9$/L (Figure 7.2) despite the administration of 750 ml of platelet concentrate over the first 5 days in hospital. Prolonged prothrombin times (INR 1.3–1.4) were recorded only on 8 and 13 December, and at no stage were there abnormal findings in other coagulation studies, e.g. activated

in serum samples from 4 patients; 3/72 in whom a diagnosis of CCHF infection had been made, and 1/162 of the remaining patients included in the study (Table 7.3). All 4 patients were ostensibly exposed to infection in locations which fell within the distribution range of the tick *A. hebraeum*, where antibody activity to Dugbe virus antigen was found in cattle sera (Figure 7.1). All 4 survived their illnesses, but the 3 patients in whom a diagnosis of CCHF had been made came from remote rural locations and could not be traced for further investigation. The diagnosis of CCHF infection in the first patient (number 1 in Table 7.3) was based on isolation of virus and demonstration of seroconversion, while rising titres of antibody were detected by IF in paired sera from patients 2 and 3 (Table 7.3). In addition, CCHF virus was isolated from and seroconversion demonstrated in the daughter of patient 2, who became ill 2 days after her father (Table 7.3). However, serum samples from the daughter reacted monospecifically with CCHF antigen. Patient 4 (Table 7.3), in whom rising titres of antibody to Dugbe virus antigen were demonstrated, was an urban resident of the Transvaal, and extended observations were made on his haematological and antibody status after his recovery from acute illness, as described below. His serum samples reacted monospecifically with Dugbe antigen throughout the period of monitoring. None of the patients in the study had antibody to viruses tested other than nairoviruses.

7.3.3 Findings on patient with suspected Dugbe virus infection

The patient (number 4 in Table 7.3), a previously healthy 51 year old male who lived and worked as an artisan in Johannesburg, visited a farm in the north-western Transvaal (Figure 7.1) from 16-18 November 1990, and on the first evening discovered that he had acquired about 300 larval tick bites, mainly in the groin and armpits. On 20 November

Table 7.3. Antibody reactions to nairovirus antigens detected by ELISA in serum samples of four suspected haemorrhagic fever patients.

		ELISA titres							
		Serogroup CCHF				Serogroup Nairobi sheep disease			
		CCHF		Hazara		Nairobi sheep disease		Dughe	
Patient	Day of illness	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
1	12	3,200	3,200	200	-	100	-	3,200	-
2	14	12,800	3,200	400	-	800	1,600	12,800	1,600
3	13	800	-	100	-	-	-	3,200	-
4	29	-	-	-	-	-	-	12,800	51,200

Patients diagnosed as cases of CCHF infection on the basis of virus isolation and or IF antibody response.

Table 7.2. Cross-reactivity for nairovirus antigens in 1385 cattle sera detected by ELISA.

	Numbers of sera reacting with indicated antigens							
Total sera tested (herds)	CCHF	CCHF Dugbe	CCHF Dugbe NSD	CCHF NSD	Dugbe	Dugbe NSD	NSD	Hazara
785 (16)	365	24	3	1	24	1	2	0
600 (12)[c]	167	11	0	0	7	0	0	NT

Tested with CCHF, Dugbe, NSD and Hazara antigens
Tested with CCHF, Dugbe and NSD antigens
NT not tested

overlaps that of *A. hebraeum* but extends to the southern Transvaal and the Orange Free State (data not shown) (Howell *et al.*, 1978). This tick is the principal vector of NSD virus in East Africa (Terpstra, 1994). No reactions were recorded in cattle sera with the remaining nairovirus, Hazara. The cross-reactivity of individual cattle sera for nairovirus antigens is analysed in Table 7.2, in which distinction is made between 785 sera tested with all 4 nairovirus antigens included in the study and 600 sera which were not tested with Hazara antigen. It can be seen that 532/571 (93.2%) sera which reacted with CCHF antigen were monospecific, while 31/70 (44.3%) Dugbe reactors and 2/7 NSD reactors were monospecific.

Very few sera reacted with West Nile and Kadam antigens, but antibody activity to louping ill antigen was detected in 11 widely scattered locations, with a maximum prevalence of 7/37 (18.9%) in a herd in the Cape Province. The locations where antibody activity to louping ill antigen was detected appear to coincide with the patchy areas of distribution of the 2 ticks of the genus *Ixodes* which parasitize livestock in South Africa, *I. pilosus* and *I. rubicundus* (data not shown) (Howell *et al.*, 1978). No single serum sample reacted with more than 1 of the flavivirus antigens, louping ill, West Nile or Kadam, but reactions to all 3 antigens occurred in 3 herds. Only 2 cattle sera reacted with Chenuda antigen, and no antibodies were detected to the remaining 4 viruses in the study, Bahig, Bhanja, Thogoto and Dhori (Table 7.1).

7.3.2 Antibody tests on sera of suspected haemorrhagic fever patients

Antibody activity to 1 or more nairoviruses, besides CCHF, was detected by ELISA

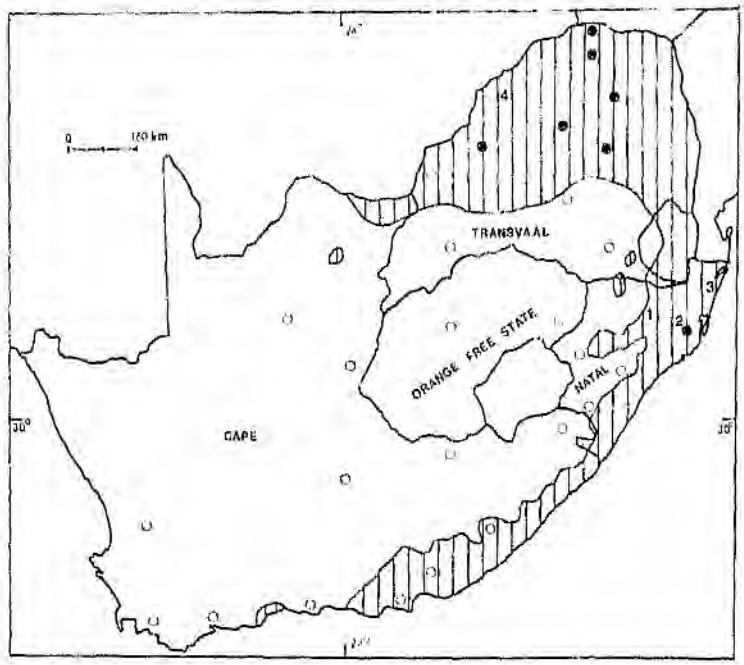


Figure 7.1. Map showing locations where antibody to Dugbe virus antigen was detected by ELISA in cattle and human sera, in relation to the known distribution of the tick *Amblyomma hebraeum* (vertical hatching): closed circles (●), herds in which antibody was detected; open circles (o), herds in which antibody was not detected; arabic numerals, locations where the corresponding patients in Table 13 were exposed to infection.

Table 7.1. Results of ELISA for antibodies to tick-associated viruses in cattle sera from South Africa.

Virus	Sera positive/tested (%)	Herds positive/tested
Family *Bunyaviridae*, Genus *Nairovirus*, Serogroup CCHF		
CCHF	905/2,116 (42.8)	41/46
Hazara	0/785 (0)	0/1.
Serogroup Nairobi sheep disease		
Nairobi sheep disease	7/1,358 (0.5)	3/28
Dugbe	70/1,358 (5.2)	7/28
Genus *Bunyavirus*, Serogroup Tete		
Bahig	0/400 (0)	0/9
Genus unassigned, Serogroup Bhanja		
Bhanja	0/435 (0)	0/9
Family *Flaviviridae*, Genus *Flavivirus*, Antigenic complex Tick-borne encephalitis		
Louping ill	21/1,358 (1.5)	11/28
Antigenic complex St Louis encephalitis		
West Nile	6/450 (1.3)	4/9
Antigenic complex unassigned		
Kadam	3/625 (0.5)	3/13
Family *Reoviridae*, Genus *Orbivirus*, Serogroup Kemerovo		
Chenuda	2/450 (0.4)	2/9
Family *Orthomyxoviridae*, Genus unassigned		
Thogoto	0/1,358 (0)	0/28
Dhori	0/78	0/16

1987): antibody to the virus was found to be widely distributed in South Africa in accordance with the known distributions of the 3 *Hyalomma* tick species which occur in the country, with a tendency for herds to be seronegative or have a low prevalence of antibody along the southern coast where *H. truncatum* is the sole representative of the genus. Results were concordant (either positive or negative) for 1,681/2,010 (83.6%) sera tested by both RPHI and ELISA. However, ELISA appeared to be the more sensitive technique and produced positive results for 231/2,010 (11.5%) sera for which RPHI was negative, while the reverse occurred in only 98/2,010 (4.9%) instances. There was a tendency for discrepancies between the 2 techniques to manifest as differences in prevalence of antibody within seropositive herds, and 5 herds lacked antibody by both methods.

Dugbe was the only other virus for which moderately high prevalences of antibody were detected in cattle sera (Table 7.1), and this occurred only in 7 herds which fell within the known distribution range of the tick *Amblyomma hebraeum* in South Africa (Howell *et al.*, 1978) (Figure 7.1). Antibody was present in 70/350 (20%) sera from the 7 seropositive herds, and the prevalence in the individual herds ranged from 2-39% (=20% in 5 instances). However, 200 sera from 4 other herds within the distribution range of the tick lacked antibody to Dugbe virus (Figure 7.1). NSD virus is the closest known antigenic relative of Dugbe, and 6/7 reactions recorded with this virus occurred in herds which contained reactors to Dugbe antigen, while the seventh seropositive animal was a lone reactor in a herd in the Orange Free State Province. The 7 NSD reactions were recorded within the distribution range of the tick *Rhipicephalus appendiculatus* which

isolates from each of the RFLP groups identified were selected for partial RNA sequencing. Phylogenetic relationships were examined among southern African and other isolates by including existing sequence data.

A total of 57 southern African isolates were assigned to 10 groups according to the RFLP patterns obtained using 8 restriction endonucleases. No correlation could be made between the RFLP grouping and source of infection, year of infection or pathogenicity of the virus. However, 2 RFLP groups came from areas where the tick *H. truncatum* is prevalent and *H. m. rufipes* and *H. m. turanicum* are absent. Analysis of the nucleotide sequence data for a 450 bp region of cDNA of the S segment of the CCHF virus genome of 15 southern African isolates, revealed up to 18% genetic differences, but the majority of nucleotide changes were conserved with 94-100% similarity existing between predicted amino acid sequences among the isolates. It is well known that RNA viruses are capable of showing greater genetic diversity than DNA viruses (Holland *et al.*, 1992). No correlation could be made between the phylogenetic relationship of the 15 isolates and source of infection, year of infection or pathogenicity of the virus. In phylogenetic comparison with 14 CCHF isolates from other geographic regions, however, the 15 southern African isolates clustered into 3 groups with isolates from other countries on the continent, while isolates from Asia and Madagascar grouped together, and a Greek isolate branched off on its own.

The only inference which can be drawn from the RFLP and sequence data is that the broad geographic distribution of strains of CCHF virus may be related to the distribution and dispersal of the vectors of the virus. CCHF virus has been isolated from 30 species

Involvement of the mononuclear phagocyte system is a common feature of many of the viral haemorrhagic fever viruses (Jaax *et al.*, 1996; Peters *et al.*, 1996; Zaki *et al.*, 1996; Shieh *et al.*, 1996; Murphy and Nathanson, 1997; Shieh *et al.*, 1997; Zaki and Peters, in press). There is little information on the role of cytokines and other inflammatory mediators in the pathogenesis of viral haemorrhagic fevers. Although the pathogenesis of haemorrhage in CCHF patients remains unclear, it can be postulated that hepatocellular necrosis may lead to release of tumour necrosis factor and other procoagulants into the circulation, and ultimately to impairment of the synthesis of coagulation factors to replace those which are consumed. Widespread infection of endothelium, with degenerative changes dominating rather than necrosis, could signify that there is capillary dysfunction which contributes to the occurrence of a haemorrhagic diathesis and the generation of a petechial rash. Immune reaction to endothelial infection could lead to activation of complement in the capillary bed, with consequent triggering of the intrinsic coagulation cascade contributing to the occurrence of DIC.

Factors affecting the pathogenesis of CCHF are incompletely understood, but host factors such as age, underlying health, secondary infection, and the timeliness and adequacy of supportive therapy are probably important determinants of the severity and outcome of illness. Initially it was believed that African strains of CCHF virus were less pathogenic for humans than Asian strains (Hoogstraal, 1979), and although this is no longer thought to be correct (Swanepoel *et al.*, 1987), it was considered necessary to determine whether genetic diversity of the virus in South Africa correlates with pathogenicity for humans, year and source of infection, or geographic origin. RFLP analysis was used to screen CCHF isolates for genetic diversity, and representative

IHC are sometimes difficult to obtain, while RNA probes such as those used here are easily synthesized; they were generated by incorporation of T7 and SP6 promotor sequences into PCR products, with subsequent *in vitro* transcription of amplified DNA. The approach is quicker and simpler than conventional techniques which require cloning of DNA into vectors containing RNA polymerase promotors.

After completion of the study, a formalin-fixed liver sample was received from a suspected fatal case of CCHF in an outbreak of the disease at an ostrich abattoir. No other specimens were available from the patient, and it proved possible to confirm the diagnosis through the use of IHC.

From detailed observations made on cellular targets of the virus, it emerged that in the liver the cells lining hepatic sinusoids, including Kupffer and endothelial cells, and hepatocytes, are the main targets of infection. Involvement of the mononuclear phagocyte system may represent a mechanism for viral clearance, or the virus may replicate in these cells and be released back into the circulation. Infection of hepatocytes is probably due to either passive transfer of the virus, or release of replicating virus, from the Kupffer cells. There were minimal inflammatory infiltrates associated with necrotic areas in the liver suggesting that the cellular damage is mediated by a direct viral cytopathic effect. Necrosis and lymphoid depletion were observed in the spleen. Infection of mononuclear phagocytes and depletion of lymphoid cells may protect the virus from phagocytosis and immune inactivation, and enhance the systemic spread of infection.

performed and only tissue samples taken with a biopsy needle after death, most commonly liver samples, are submitted to the laboratory (Shepherd *et al.*, 1988; unpublished laboratory records). In some instances only formalin-fixed tissue samples are submitted, and the histopathologic lesions seen in sections may be suggestive of CCHF, but are not pathognomonic. There had been relatively few histopathologic studies of the disease prior to the present investigation, involving small numbers of specimens, and little was known of the cellular targets and distribution of the virus (Swanepoel *et al.*, 1987; Joubert *et al.*, 1985; Baskerville *et al.*, 1981). Thus, it was undertaken to explore the use of the IHC and ISH techniques for diagnosing CCHF infection on autopsy tissue samples, and for studying the cellular distribution of the virus. Preserved tissue samples from 12 fatal cases of CCHF in South Africa, mainly liver samples taken after death with biopsy needles, were available for study.

Common histopathologic findings in liver of all cases that were examined included eosinophilic hepatocyte necrosis, Councilman bodies and Kupffer cell hyperplasia, while common findings in spleen were lymphoid necrosis and depletion. The findings were similar to those observed in other viral haemorrhagic fevers. Virus had been isolated from 12/12 of the patients, and low antibody titres demonstrated in 5/12. In 4 instances the presence of antibody activity may have resulted from the administration of immune plasma. Virus antigen was demonstrated by IHC in 10/12 patients, and viral nucleic acid demonstrated by ISH in 5/12. It can be concluded that IHC can be used to render histopathologic findings diagnostic in a high proportion of cases of fatal CCHF. Although ISH was less sensitive than IHC, there was 1 case in which viral nucleic acid was detected while antigen was not. Furthermore, specific antibodies suitable for use in

antibody on day 9 of illness in patients who were not moribund at that stage, rendered a diagnosis of CCHF extremely unlikely. This information proved to be clinically useful since it facilitated the establishment of alternative diagnoses.

Nairoviruses generally induce weaker neutralizing antibody responses than do members of the other genera of the family *Bunyaviridae* (Peters and Le Duc, 1991), and it was previously shown that this was true for CCHF infection (Shepherd *et al.*, 1987). It can be added from the present observations that the appearance of circulating antibody did not correlate directly with the clearance of viraemia, and virus could be isolated with facility from patients' sera up to day 13 of illness despite the presence of antibody at high titre as demonstrated by any of the methods used here, and despite the fact that survivors felt better and their clinical pathology values such as platelet counts and serum transaminase levels improved from about day 10 of illness onwards. Nor did the administration of immune plasma appear to eliminate demonstrable viraemia or improve the chances of surviving the disease. On the other hand, it must be noted that individual patients received from 1–9 units of plasma which were not uniform with respect to neutralizing antibody potency, and were treated at various stages of illness up to and including terminal coma, so that no firm conclusions can be drawn on the efficacy of the treatment.

A diagnosis of CCHF can often be confirmed in fatal infections by demonstration of viral antigen or nucleic acid in serum, or by isolation of infectious virus, but frequently there is no detectable antibody response. Virus can be isolated from, or antigen demonstrated in, suspensions prepared from certain visceral organs, but usually no autopsy is

Early recognition of CCHF usually occurs only when patients or clinicians are alert to incidents constituting specific exposure, as in nosocomial infections or following the occurrence of a bite by a *Hyalomma* tick vector of the virus, so that comparatively few specimens are submitted for the diagnosis of the disease during the first 3 days of illness before antibodies become demonstrable. There is an increasing probability of arriving at a rapid serologic diagnosis on specimens submitted from day 4 of illness onwards, and the detection of IgG and/or IgM antibody by IF is virtually certain by day 9 in non-fatal infections. However, it cannot be assumed that the initial history which accompanies specimens is accurate with respect to the date of onset of illness, and it should be routine practice to screen all sera for antibody to the virus.

As a routine, it was found most convenient to screen sera for IgG antibody activity by IF on arrival at the laboratory. Often a positive result may be obtained which allows a tentative diagnosis to be reported in little more than an hour of receiving the specimen. At the same time, confirmatory IgM IF tests are prepared for overnight incubation, cell cultures and mice are inoculated for isolation of virus, and RT-PCR assays are performed. Plates can also be coated in preparation for performance of ELISA on the following day, but ELISA for IgG and IgM antibody are as likely as not to increase the rapidity with which a diagnosis is attained.

Since no antibody response was detected by any of the serological techniques in most of the patients who succumbed to the disease, or the response was at best delayed and weak in fatal infections, it was evident that the demonstration of rising antibody titres constituted a favourable prognostic sign. As a corollary, it was found failure to detect

(Swanepoel *et al*., 1987; Shepherd *et al*., 1989a). ELISA procedures had been described for antigen and antibody detection, but had been applied to comparatively few human serum samples (Donets *et al*., 1982; Smirnova and Karavanov, 1985; Saluzzo and Le Guenno, 1987; Shepherd *et al*., 1989a; Logan *et al*., 1993). Hence, it was decided to compare IF and ELISA techniques on 667 serum samples collected from 101 confirmed CCHF patients from the day of onset of illness up to 59 months later, with particular reference to early detection of antibody.

In brief, the findings were that routine IF tests detected IgM and IgG antibodies to CCHF virus in an increasing proportion of patients from the day 4 of illness onwards, and that all persons who survived the disease had antibodies demonstrable by IF on day 9. Antibody was demonstrable by IF in only 19 patients who died from the disease and had not received immune plasma. The IgM-capture and IgG sandwich ELISAs demonstrated higher antibody titres than did IF, and each detected a response in 4 fatal infections in which no response could be found by IF. Among survivors of the disease, the IgM and IgG ELISA detected an immune response at an earlier stage of infection than did IF tests in 10 and 15 patients respectively (day 3 at the earliest), but the reverse was true in similar numbers of patients (11 and 14 respectively). The failure of the ELISA to produce positive results on occasion with sera which had low IF titres, was possibly related to the higher starting dilutions used in these tests. The CELISA, in which sera were tested at a starting dilution of 1/10, produced lower titres than did the IgM and IgG ELISA, but yielded results which were in close agreement with the findings in IF tests.

procedure should be widely applicable in the diagnosis of CCHF.

Nevertheless, it remains necessary to use the RT-PCR in combination with other diagnostic tests. Early in the course of the disease antigen can be detected by means of an ELISA, a procedure which takes 6-8 hours, or longer if pre-coated test plates are not available, but the assay is often unsuccessful in non-fatal infections (Shepherd et al., 1988). Isolation of virus in cell cultures can occasionally be achieved within 24 hours if viraemia is sufficiently intense, but more often virus is present in low concentrations which can only be isolated in mice, a procedure which usually takes 6-9 days (Shepherd et al., 1986). From day 3-4 of illness onwards antibody response can be detected in an increasing proportion of patients, as discussed below. To conclude the discussion on RT-PCR, it can be noted that combined use of the technique with IF tests permitted a presumptive diagnosis of CCHF to be reported within 8 hours of receiving the first specimen from 18/19 cases of the disease investigated prospectively during the present study. The nineteenth case was confirmed within 48 hours when antibody was detected in a second serum sample, several days before virus could be isolated from the first sample.

Early tests for the detection of antibody to CCHF virus, such as complement fixation, virus neutralization, immunodiffusion, haemagglutination-inhibition and reversed passive haemagglutination-inhibition, lacked sensitivity and/or reproducibility (Casals and Tignor, 1974; Gaidamovich *et al.*, 1974; Swanepoel *et al.*, 1983a). Prior to the present study, the IF test had found widest application as a diagnostic tool, and it had the advantage that it could be used to distinguish IgG and IgM antibody responses

from serum while nucleic acid remains demonstrable in a proportion of patients well into convalescence.

The use of the RT-PCR with ethidium bromide stained gels permits a presumptive diagnosis of CCHF to be reported within 8 hours of receiving a specimen at the laboratory. Sensitivity can be improved by the use of Southern blots and labelled probes, but the improvement is marginal for fresh serum samples, and the time required to obtain a result is increased to 2 days. Moreover, it is difficult to maintain stocks of freshly labelled probe because of the short half-life of the radio-isotope, and despite the use of Southern blots and probes, the RT-PCR fails to detect nucleic acid in a small proportion of sera from which infectious virus can be isolated. The discrepancy may be related to the fact that the volume of serum inoculated into a litter of mice, approximately 300µl, is 3-fold greater than the starting volume used in the RT-PCR. An alternative explanation is that the primers used in the RT-PCR procedure are not universally applicable to strains of CCHF virus circulating in southern Africa. However, the primers were used successfully in the RT-PCR study on 67 serum samples from 44 cases of CCHF which occurred over a period of 14 years in widely separated geographic locations in South Africa and Namibia. Moreover, in 4/9 instances in which the RT-PCR failed to detect RNA in viraemic serum, a positive result was obtained on an earlier sample from the same patient. It seems likely that in some instances there was degradation of RNA in serum to below threshold level required for detection, because it was subsequently confirmed in the investigation of genetic heterogeneity of CCHF virus (Chapter 5) that the primers could be used successfully on cell cultures infected with each of the 57 southern African isolates included in the study. The implication is that the RT-PCR

began to decline towards the end of the observation period, while antigen remained demonstrable in all sera tested up to the termination of the experiment on day 8. Although the infection of infant mice does not necessarily constitute a valid model of CCHF in humans, the findings suggested that the use of RT-PCR for early diagnosis of the disease warranted further investigation.

It was known prior to applying the RT-PCR to human sera that the infectivity of CCHF virus is labile in material held at 4-20°C, or even in frozen material subjected to repeated freezing and thawing (Shepherd *et al.*, 1986: unpublished laboratory records), and since PCR products were obtained with greater regularity from fresh as opposed to stored sera without recourse to the use of Southern blots and labelled probes, it appears that there was degradation of viral nucleic acid during storage. Nevertheless, it is clear from the results obtained with both fresh and stored sera that RT-PCR can be used as a means of establishing a rapid diagnosis during the first 2 weeks of illness, with positive results being recorded as late as day 16, by which time it can be expected that most patients are either convalescent or have died (Swanepoel et al., 1987: unpublished laboratory records). Although no samples from days 1-2 of illness were analysed, virus has on occasion been isolated at this early stage (unpublished laboratory records), and thus it seems certain that RT-PCR can be used from the time of onset of the disease onwards. It is worth noting that CCHF is apparently seldom recognized or suspected before day 3-5 of illness: the Unit has received specimens taken on days 1-2 of illness in only 7/141 cases of the disease investigated (unpublished laboratory records). Early in the disease there is relatively good correlation between the results obtained by RT-PCR and virus isolation, but after the first week it appears that infective virus is progressively cleared

common infections, does not alter the urgency with which diagnoses are required. Even false alarms can prove to be highly expensive and disruptive of normal medical services. The institution of barrier-nursing procedures in hospital wards often involves the emptying of surrounding wards, with the referral of patients resulting in the overloading of neighbouring hospitals, while public health services may be strained in tracing and placing under observation the contacts and cohorts of patients. Thus, it becomes almost equally important to eliminate an erroneous clinical diagnosis of viral haemorrhagic fever rapidly, or to establish an alternative diagnosis, as it is to confirm genuine haemorrhagic fever.

The viral haemorrhagic fevers are commonly characterized by sudden onset following a short incubation period, and run a peracute or acute course, so patients inevitably lack markers of immune response for at least the first few days of illness. Improvements in ability to make a diagnosis very early in the course of disease, therefore, must stem from advances in virological methods. Rapid detection of viraemia and antigenaemia in CCHF have previously been investigated in the Special Pathogens Unit (Shepherd *et al.*, 1986, 1988), and hence it was considered logical to evaluate the utility of RT-PCR as a diagnostic method for detecting viral RNA early in the disease.

The comparison of RT-PCR, virus isolation and antigen detection procedures in experimentally infected mice, was designed to explore the feasibility of demonstrating viral RNA in serum. It was found that viral RNA could be detected in a proportion of mouse sera from day 1 post-infection onwards, earlier than infectious virus could be isolated or antigenaemia detected, but the presence of RNA and infectious virus in sera

8. Discussion

Laboratory confirmation of the diagnosis of systemic virus infections in live patients is generally achieved in 2 ways: either by detecting evidence of the presence of the etiological agent, or by demonstrating a specific immune response, and less often by other methods such as demonstrating pathognomonic lesions. Although the presence of virus can be detected by immunological means, and although not all tests of immune response relate to humoral immunity, the 2 main approaches to establishing a diagnosis are commonly referred to as virological and serological investigation, which also alludes to the fact that serum is the most commonly investigated specimen. In subacute and chronic virus diseases, such as hepatitis A and B, or the acquired immunodeficiency syndrome, specific markers of the infection are usually present in serum by the time that the disease is suspected, and this facilitates rapid laboratory confirmation of the diagnosis. Despite the importance of these diseases, however, any delay experienced in the actual performance of laboratory tests is not necessarily critical to the management or treatment of the patient; it is more important that the tests should be sensitive and specific rather than capable of being performed ultra-rapidly.

In contrast, it is of great importance that the so-called formidable viral haemorrhagic fevers should be diagnosed as early in the course of the disease and as rapidly as possible. The high death rates and propensity for nosocomial spread associated with the viruses, renders rapid diagnosis important for treatment of the patient, particularly where specific therapies are available, and for protection of medical staff. The fact that the vast majority of suspected cases of haemorrhagic fever prove to be severe cases of more

(McIntosh, 1980). Certain other viruses should probably have been included in the study, but viable stocks were not available.

The fact that the findings on CCHF antibody in cattle sera were essentially in agreement with results obtained previously by RPHI, suggests that the ELISA procedure used in the present study constituted a valid and sensitive technique, and confirms that the virus is widely prevalent in South Africa. In contrast, antibodies to nairovirus antigens other than CCHF were detected only in 7 herds and 4 human patients from locations within the known distribution range of the tick *A. hebraeum*, with the exception of a single reaction to NSD antigen recorded in a herd in the Orange Free State. Since 532/571 (93,2%) cattle with antibody to CCHF virus and 69/72 (95,8%) patients with confirmed CCHF infection lacked antibody activity to the other nairoviruses tested, it can be surmised that primary or uncomplicated infection with CCHF virus in cattle and humans does not appear to induce the production of antibody which is cross-reactive with other nairoviruses. It follows that the suggested use of conventional or recombinant antigens prepared from heterologous, antigenically related viruses (Ward *et al.*, 1992) is unlikely to be satisfactory for the serodiagnosis of CCHF infection.

It nevertheless remains clear that 1 or more nairoviruses other than CCHF are active in certain parts of South Africa, and the indications are that this includes Dugbe virus: 31/70 (44,3%) cattle which had antibody activity to Dugbe virus antigen, and the human patient monitored over a period of 34 months, reacted monospecifically with the antigen. The diagnosis of Dugbe virus infection in the human patient is supported particularly by the failure to detect antibody activity to NSD virus during the extended period of

1983a; Shepherd *et al.*, 1987b). The larger mammals have much longer lifespans than hares, and this would be conducive to the occurrence of greater cumulative prevalences of antibody, particularly in locations where the challenge rate is inherently low.

The ability to detect antibodies in different species by CFI.ISA, has important implications for many other epidemiologic studies, including those on Marburg and Ebola haemorrhagic fever viruses for which the natural hosts are unknown.

Among the patients investigated in the Special Pathogens Unit over the years, there were some with a history of having been bitten by ticks for whom no cause of illness could be found, and it was decided to investigate the possibility that tick-borne viruses other than CCHF were involved. Twelve viruses, including CCHF, were selected for the investigation, and ELISA were developed to test for antibodies to the viruses. An antibody survey was conducted on cattle sera to determine whether there was evidence that the selected viruses occur in South Africa. Sera taken during convalescence from patients with confirmed CCHF infection were tested to determine whether the causative agent of the disease induces production of antibody which is cross-reactive with antigenically related viruses, and to check the possibility that dual infections may have occurred in some instances. Finally, the tests for antibodies to the selected viruses were applied to convalescent phase sera from patients who lacked evidence of CCHF infection, but either had a history of being bitten by ticks, or lived in or visited a rural location where exposure to ticks was possible.

The prevalence of antibody activity in cattle sera was found to be 90% (116 of 128) in

domestic and wild animals encountered in epidemiologic studies. For this reason, a competitive ELISA, using rabbit anti-CCHF HRPO which had initially been developed for use on human sera as discussed above, was also examined for its applicability to livestock sera, as well as to the sera of wild vertebrates, using serial samples from experimentally infected animals to evaluate the technique. The CELISA was then applied to the sera of 960 wild vertebrates collected in a nature reserve.

The findings indicated that IgM antibody to CCHF virus can be demonstrated in sheep sera by IgM-capture ELISA with either of detection methods, using the rabbit anti-CCHF conjugate or a sandwich technique with anti-CCHF serum and a commercial anti-species immunoglobulin conjugate. Titres detected by use of the anti-CCHF conjugate were slightly but not significantly lower than those detected by the sandwich method. Furthermore, it was confirmed that the technique using anti-sheep IgM-capture antibody can also be used on cattle sera, as has been demonstrated previously with antibody to Rift Valley fever virus (Ksiazek *et al.*, 1989). The fact that IgM antibody titres in sheep and cattle sera only remained demonstrable for up to 3-7 weeks respectively following infection is in contrast to the 3-5 months recorded in human patients (Donets *et al.*, 1982), and is probably a function of the relative lack of susceptibility of these animals to infection with the virus. The experimental infections in the sheep and cattle resulted in only transient low-titred viraemia and mild fever (Shepherd, 1987). In contrast, the virus is highly pathogenic for humans, causing illness with a 30% fatality rate. In guinea fowl, of which only a proportion of individuals develop low-grade viraemia or demonstrable antibody response following infection, total antibody titres decline to undetectable levels in about 7 weeks, while domestic chickens fail to develop either

It must be allowed that infected immature ticks could detach from migrant birds, moult to the next instar, and transmit infection to a second host in the new environment, but the low infection rate inherent in ticks, and the high attrition rate in the life cycle, would limit the possibility of this occurring. Moreover, the relatively short period for which immature ticks remain attached to hosts, generally less than a week (Hoogstraal, 1956), would limit the penetration of species from 1 continent into the other on migrating birds. This is reflected in the fact that *H. m. marginatum* of Asia has established on the north African littoral, but has not penetrated the continent further south, although isolated specimens have been collected in the Sudan and Kenya (Hoogstraal, *et al.*, 1963). Similarly, *H. m. rufipes* is endemic to Africa but isolated specimens have been reported in Tadzhikistan and Transcaucasia (Hoogstraal *et al.*, 1961). Although large numbers of ticks may be carried between the continents by migrating birds and slaughter animals, the establishment of a tick species in a new location depends on the availability of a suitable microclimate, sufficient numbers of ticks to ensure breeding, and the presence of suitable hosts. In contrast, ticks dispersed within a continent by migrating birds or movement of domestic and wild animals are being circulated within their endemic distribution range.

Despite the potential for dispersal of the virus between the continents which has existed for millenia, therefore, it appears from the phylogenetic analysis of CCHF isolates that the circulation of the virus is largely compartmentalized within the 2 continents, with occasional translocations of virus adding to diversity within the circulation pools. The uniqueness of the Greek isolate included in the phylogenetic analysis suggests that isolating mechanisms, possibly relating to bird migration paths, have operated in the

2 or more antigenically related members of the *Bunyaviridae* (Calisher and Karabatsos, 1989; Peters and Le Duc, 1991), it can be postulated that the reactivity for Hazara and NSD antigens observed in the sera of the 3 confirmed CCHF patients could have stemmed from the occurrence of CCHF infection in subjects who had previously experienced Dugbe infection. At least there is no evidence apart from the antibody reactions in the 3 CCHF patients that Hazara virus, known only from Asia, occurs in southern Africa. The possibility that NSD virus occurs in the subcontinent, however, cannot be discounted entirely. It is well documented that NSD virus causes disease of sheep and goats in a belt extending 80-1,200 km north and south of the equator from Kenya to Zaire, and antibody has been found in livestock as far north as Somalia and Ethiopia (Terpstra, 1994; Davies *et al.*, 1974; Edelsten, 1975). There has also been brief reference to the detection of antibody as far south as Botswana, Mozambique and Natal-KwaZulu province in South Africa (Karabatsos, 1975; Weinbren, 1967), but the evidence is no more conclusive than that obtained in the present study. Examination of the original experimental records at the National Institute for Virology reveals that undiluted human serum samples collected in 1957-1959 were screened for ability to neutralize 100 mouse intracerebral 50% lethal doses (MICLD$_{50}$) of NSD virus by inoculation of 6 mice per specimen, and that positive results (survival of 5-6/6 inoculated mice) were obtained for 1/131 specimens from South Africa (Natal KwaZulu), 1/450 from Mozambique, 0/22 from Namibia (Caprivi Strip), and 0/36 from Botswana, while inconclusive results (survival of 3-4/6 mice) were obtained for 11 specimens from these countries (unpublished records). Moreover, the serum samples were not treated for the removal of non-specific inhibitors of nairovirus infectivity (Casals and Tignor, 1974). Definitive evidence of the occurrence in South Africa of nairoviruses other than CCHF can

and the Central African Republic, including a laboratory infection (Moore *et al.*, 1975; Georges *et al.*, 1980). One patient had meningitis and virus was isolated from cerebrospinal fluid. The mechanisms involved in the pathogenesis of the thrombocytopenia observed in our patient remain undetermined, but the failure of the administration of large doses of platelets to lead to immediate elevation of thrombocyte counts suggests that at least during the acute illness there was consumption of platelets. The performance of virological and RT-PCR tests on biopsy specimens may have revealed whether there was infection of bone marrow. It was not established whether platelet counts were inherently low in the patient for other reasons, but depression of thrombopoiesis due to parvovirus infection was excluded, and mumps was eliminated as a cause of the orchitis seen in the patient.

It can be concluded that although Dugbe virus infection of humans may occur infrequently, the condition should be borne in mind in the differential diagnosis of suspected cases of viral haemorrhagic fever in Africa, particularly when there is a history of tick bite. In South Africa, the potential exists for the infection to occur within the distribution range of the tick *A. hebraeum*, at least as far south as Natal-KwaZulu. Dugbe infection should also be investigated as 1 of the possible causes of onyalai, a syndrome characterized by thrombocytopenia and a haemorrhagic tendency which has been reported from widely scattered locations in sub-Saharan Africa, largely within the distribution range of the tick *A. variegatum* (Huard *et al.*, 1978; Walker and Olwage, 1987).

Since the cross-reactivity of humane serum broadens following sequential infection with

monitoring since this is the closest known antigenic relative of Dugbe virus (Ganjam virus of India is regarded as synonymous with NSD) (Calisher and Karabatsos, 1989; Casals and Tignor, 1980). Although the weak and transient virus neutralizing activity demonstrated in the serum of the patient would seem to militate against a diagnosis of Dugbe infection, it is well known that nairoviruses induce poor neutralizing antibody responses (Casals and Tignor, 1974; Davies *et al.*, 1976; Peters and Le Duc, 1991), and it is notable that investigators in West Africa were unable to detect the response in patients from whom they had isolated Dugbe virus (Moore *et al.*, 1975; Georges *et al.*, 1980).

Dugbe virus has been isolated in Nigeria, Central African Republic and Ethiopia, and antibody has been found in Senegal and Uganda (Karabatsos, 1985). Although there have been no reports of the presence of the virus further south in Africa, there do not appear to have been specific investigations. The virus has been isolated on at least 598 occasions from ixodid ticks in West Africa, but it appears to have a particular relationship with *A. variegatum* from which the majority of the isolations were made (Karabatsos, 1985; Hoogstraal *et al.*, 1978). This tick is the most widely distributed *Amblyomma* in Africa and it overlaps in distribution with *A. hebraeum* in Zimbabwe (Walker and Olwage, 1987), so it is theoretically possible that the dissemination of the virus historically extended to South Africa.

Despite the large number of isolations of Dugbe virus made from ticks in West Africa, serosurveys did not reveal widespread human infection, but 7 isolations of the virus were made from the blood of persons, mainly children, with benign febrile illness in Nigeria

induced by CCHF infection is cross-reactive with other nairoviruses. Dhori, a tick-borne virus belonging to the Orthomyxoviridae is morphologically and genetically related to Thogoto virus and although it has not been associated with human diseases it has been isolated from ticks and vertebrates in Asia, Europe and North Africa (Karabatsos, 1985).

Louping ill antigen was included in order to seek evidence that the tick-borne encephalitis complex of flaviviruses is represented in southern Africa: these viruses of the northern hemisphere are difficult to distinguish from each other in serological tests, but vary in pathogenicity and 2 of them, Kyasanur Forest disease and Omsk haemorrhagic fever, are known to be associated with haemorrhagic syndromes in humans. Kadam, a tick-borne flavivirus from Uganda, was included as a check on the specificity of any reactions recorded with louping ill antigen. West Nile virus served as a further check for flavivirus cross-reactions. However, this well known mosquito-borne flavivirus of Africa, Europe and Asia, causes a few human infections each year in South Africa, and has been associated with a few cases of fulminant hepatitis resembling haemorrhagic fever in West Africa (Georges *et al.*, 1987). Moreover, the virus has been isolated from ticks on occasion, also in South Africa (Blackburn *et al.*, 1990), and thus it was considered possible that the infection could account for some of the unexplained illnesses observed in patients bitten by ticks. Bahig, a bunyavirus associated with birds in Egypt and southern Europe, was included in the study merely because it had been isolated from *Hyalomma* ticks of the same species as are involved in the transmission of CCHF in South Africa. Chenuda virus was originally isolated from argasid ticks of birds in Egypt, but was included in the study because antibody had been found in livestock in Egypt, and because there had been multiple isolations of the virus in South Africa

CCHF virus antigen, 70/1358 (5.2%) for Dugbe, 21/1358 (1.5%) for Looping ill, 6/450 (1.3%) for West Nile, 7/1358 (0.5%) for NSD, 3/625 (0.5%) for Kadam and 2/450 (0.4%) for Chenuda, while no reactions were recorded with Hazara, Bahig, Bhanja, Thogoto and Dhori antigens. The findings on CCHF confirmed previous observations that the virus is widely prevalent within the distribution range of ticks of the genus *Hyalomma*. Antibody activity to Dugbe antigen was detected only in locations within the distribution range of the tick *Amblyomma hebraeum*. Cross-reactivity for the antigens of the nairoviruses, Hazara, Nairobi sheep disease and Dugbe, was detected in serum samples from 3/72 human patients with confirmed CCHF infection, while serum from 1/162 other patients reacted monospecifically with Dugbe antigen. The latter patient suffered from febrile illness with transient encephalitis, petechial rash, bruising tendency and orchitis, plus prolonged thrombocytopenia after being bitten by larval ticks, and observations were made on his haematological and antibody status at intervals over a peri[illegible]34 months following the onset of illness.

At least 24 viruses which occur in Africa are known or suspected to be tick-borne, while a few additional viruses which are transmitted by other vectors have been isolated from ticks on occasion (Karabatsos, 1985; Calisher and Karabatsos, 1989). Four viruses, CCHF, NSD, Dugbe and Thogoto, were included in the study because they were known to have been associated with naturally occurring disease of humans, while a fifth, Bhanja, was known to have caused febrile illness following laboratory infection. Hazara virus was isolated from ixodid ticks in Pakistan and is not known to be pathogenic for humans, but it is the closest known antigenic relative of CCHF virus and it was included in the study along with NSD and Dugbe viruses in order to assess the extent to which antibody

demonstrable viraemia or an antibody response (Shepherd *et al.*, 1987b).

It was further demonstrated that total antibody activity or IgG antibody activity in sheep sera can be detected by CFT, ELISA or a sandwich method respectively, using commercial anti-species conjugate, and that the CFT, ELISA can also be used to detect antibody in the sera of cattle and small and large wild vertebrates.

The prevalence of antibody to CCHF virus detected in serum samples from the Kruger National Park is lower than was found previously in wild vertebrates from other locations in southern Africa (Swanepoel *et al.*, 1983a; Shepherd *et al.*, 1987a) and this may be related to the fact that *H. truncatum* is the only species of *Hyalomma* believed to occur in the Park (Howell *et al.*,1978; Horak *et al.*,1983a; Horak *et al.*,1983b; Horak *et al.*,1984; Spickett *et al.*,1991). *Hyalomma* ticks are considered to be the principal vectors of CCHF virus and it had been observed earlier (Swanepoel *et al.*, 1987) that there was a much lower prevalence of antibody to the virus in cattle sera in the south-western portion of the Cape province of South Africa, where *H. truncatum* is also the only representative of the genus, than elsewhere in southern Africa. The fact that the highest prevalences of antibody occurred in the sera of animals such as the rhinoceros, giraffe and buffalo in the Kruger National Park is in accordance with the previous observation that CCHF infection appears to occur most frequently in larger mammals which are the preferred hosts of adult *Hyalomma* ticks (Shepherd *et al.*, 1987a). Immature *Hyalommas* feed on ground-frequenting birds and small mammals and although antibody was found in guinea fowl sera, the lack of CCHF antibody in hares in the Kruger National Park is in contrast to the situation observed elsewhere in the subcontinent (Swanepoel *et al.*,

Balkans for centuries. It is interesting to note that the isolate from Madagascar included in the phylogenetic analysis was obtained from a tick, *Boophilus microplus* which is found primarily in Pakistan and India, but was known to have been imported into Madagascar on cattle (Hoogstraal, 1979). This could explain the close relationship observed between isolates from Madagascar and Pakistan.

As discussed below, certain diagnostic problems encountered in the Special Pathogens Unit suggested that there was a need to investigate the possibility that tick-borne viruses other than CCHF were causing human disease in South Africa, and it was decided to conduct surveys on cattle sera for antibodies to selected viruses in order to seek evidence that the viruses were present in the country. Serological techniques such as immunodiffusion, complement-fixation, IF, haemagglutination-inhibition, passive haemagglutination-inhibition and virus neutralization used to investigate CCHF in the past, lacked sensitivity, reproducibility and amenity to automation (Woodall *et al.*, 1967; Casals, 1969; Casals, 1978; Zavodova *et al.*, 1971; Casals and Tignor, 1974; Buckley, 1974; Gaidamovich *et al.*, 1974; Saluzzi *et al.*, 1975; Tignor *et al.*, 1980; Swanepoel *et al.*, 1983b; 1987; Shepherd *et al.*, 1987a) The problems were potentially overcome by the development of an ELISA for CCHF (Dowdle *et al.*, 1982), and as a preliminary to the proposed survey on cattle sera it was considered logical to perform a feasibility study by developing CCHF sandwich IgG antibody and IgM-capture antibody ELISA for sheep and cattle sera, using commercially available anti-species immunoglobulin peroxidase conjugates, and the sera of experimentally infected sheep and cattle. Although anti-species immunoglobulin conjugates are commercially available for performing IF and ELISA tests on the sera of various domestic animals, they are not applicable to all

of ixodid ticks, and although the importance of some of these ticks as vectors is uncertain, members of the genus *Hyalomma* are believed to be the principal vectors (Watts *et al.* 1989a; Carniel *et al.*, 1990). The broad distribution of the virus correlates with that of *Hyalomma* ticks of which there are currently about 30 recognized species (Hoogstraal, 1979; Keirans, 1992).

One way in which arthropod-borne viruses are dispersed within and between continents is by migrating birds, which can either harbour the virus themselves, or transport infected vectors. Originally it was believed that birds were refractory to infection with CCHF virus. However, it was recently shown that experimentally infected guinea fowl developed transient and low-titred viraemia (Shepherd *et al.*, 1987b), and ostriches exhibited viraemia of up to 4 days duration (Swanepoel *et al.*, 1997).

Ticks which parasitize birds include the immature stadia of certain proven or potential vectors of CCHF virus: *H. m. marginatum* and *H. m. turanicum* of eastern Europe and Asia, and *H. m. rufipes*, *H. truncatum*, *H. nitidum* and *Amblyomma variegatum* from Africa. It was found in Egypt that vast numbers of birds migrating from the northern hemisphere in autumn were most heavily infested with immature *H. m. marginatum*, whereas spring migrants going north were generally infested with immature *H. m. rufipes* (Hoogstraal, *et al.*, 1961; Hoogstraal, *et al.*, 1963), both proven vectors of CCHF virus. Another vehicle for the movement of ticks between the 2 continents relates to trade in infested livestock for slaughter, and this has resulted in known introduction of CCHF infection from Africa to the Near East on occasion (Rodriguez *et al.*, 1997).

Appendix G: (continued)

Day	Species	Animal no	Total antibody titres to CCHF virus
28	*Oryctolagus cuniculi* (New Zealand giant rabbit)	1	40
		2	80
		3	80
		4	10
		5	160
		6	0
		7	40
		8	40

Appendix G: (continued)

Day	Species	Animal no	Total antibody titres to CCHF virus
1	*Lepus saxatilus* (Scrub hare)	1	0
		2	0
		3	0
		4	0
2-6		3	0
		4	0
7		3	80
		4	10
14		1	40
		2	320
		4	20
21		1	320
		2	320
27		1	160
		2	160
35		1	80
		2	80
48		2	20
		3	40
		4	40

Appendix G: (continued)

Day	Species	Animal no	Total antibody titres to CCHF virus
1	*Mystromys albicaudatus* (White tailed rat)	1	0
		2	0
		3	0
14		1	80
		2	20
		3	0
21		1	160
		2	20
		3	20
28		1	40
		2	40
		3	20
1	*Aethomys chrysophilus* (Red veld rat)	1	0
		2	0
14		1	80
		2	80

Appendix G: Total antibody titres to CCHF virus in relation to day post infection demonstrated by CELISA in experimentally infected small mammals.

Day	Species	Animal no.	Total antibody titres to CCHF virus
21	*Cavia porcellus* (Guinea pig)	1	320[1]
		2	320
		3	320
		4	320
		5	320
		6	320
		7	320
		8	160
		9	320
1	*Tatera brantsii* (Bushveld gerbil)	1	0
		2	0
21		1	160
		2	160
35		1	20
1	*Tatera leucogaster* (Highveld gerbil)	1	0
		2	0
		3	0
		4	0
28	*Tatera leucogaster* (Highveld gerbil)	1	320
		2	320
		3	0

[1] Reciprocal titre

Appendix F: Total antibody titres to CCHF virus in relation to day post infection detected by CELISA in serum samples from experimentally infected cattle (cattle numbers 34, 30, 17, 29, 17 (calf), 25, 64, 60, 69, 82, 97).

	Total antibody titres to CCHF virus in cattle serum samples										
Day	34	30	17	29	17	25	64	60	69	82	97
1	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0
4	NA	NA	NA	NA	0	0	0	0	0	0	0
5	NA	NA	NA	NA	0	0	0	0	0	0	0
6	NA	NA	NA	NA	0	0	0	0	0	0	200
7	NA	NA	NA	NA	0	200	0	0	200	0	200
8	NA	NA	NA	NA	0	200	0	200	200	200	NA
9	NA	NA	NA	NA	0	200	0	800	800	200	200
10	NA	NA	NA	NA	0	400	0	200	400	800	800
11	NA	NA	NA	NA	0	400	0	200	800	800	800
12	NA	NA	NA	NA	0	800	0	200	400	400	800
13	NA	NA	NA	NA	0	400	0	200	400	400	800
14	NA	NA	NA	NA	0	400	0	200	400	800	800
20	1,600[1]	1,600	0	400	NA	NA	NA	NA	NA	NA	NA
28	1,600	1,600	0	400	NA	NA	NA	NA	NA	NA	NA
35	1,600	1,600	0	400	NA	NA	NA	NA	NA	NA	NA
42	1,600	1,600	0	400	NA	NA	NA	NA	NA	NA	NA
49	1,600	NA	0	400	NA	NA	NA	NA	NA	NA	NA
56	1,600	NA	0	800	NA	NA	NA	NA	NA	NA	NA

[1]Reciprocal titre
[2]Not available

Appendix E: IgM total antibody titres to CCHF virus in relation to day post infection detected by ELISA using anti CCHF conjugate, in serum samples from experimentally infected cattle (cattle numbers 34, 30, 17, 29, 17 (calf), 25, 64, 60, 69, 82, 97).

	IgM antibody titres to CCHF virus in cattle serum samples										
Day	34	30	17	29	17	25	64	60	69	82	97
1	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0
4	NA	NA	NA	NA	0	0	0	0	0	0	0
5	NA	NA	NA	NA	0	0	0	0	0	0	0
6	NA	NA	NA	NA	0	0	0	0	0	0	0
7	NA	NA	NA	NA	0	0	400	0	0	0	0
8	NA	NA	NA	NA	6,400	6,400	6,400	0	0	0	0
9	NA	NA	NA	NA	12,800	25,600	25,600	0	0	0	0
10	NA	NA	NA	NA	12,800	25,600	25,600	0	0	0	0
11	NA	NA	NA	NA	12,800	25,600	25,600	0	0	0	0
12	NA	NA	NA	NA	12,800	25,600	25,600	0	0	0	0
13	NA	NA	NA	NA	25,600	25,600	25,600	0	0	0	0
14	NA	NA	NA	NA	25,600	25,600	25,600	0	0	0	0
20	3,200	0	0	3,200	NA	NA	NA	NA	NA	NA	NA
28	800	1600	0	1,600	NA	NA	NA	NA	NA	NA	NA
35	1,600	0	0	800	NA	NA	NA	NA	NA	NA	NA
42	800	0	0	0	NA	NA	NA	NA	NA	NA	NA
49	0	0	0	0	NA	NA	NA	NA	NA	NA	NA
56	0	0	0	0	NA	NA	NA	NA	NA	NA	NA

[1]Reciprocal titre
[2]Not available

Appendix D: Total antibody titres to CCHF virus in relation to day post infection, detected by CELISA, in serum samples from experimentally infected sheep (sheep numbers 08, 24 and 37).

	Total antibody titres in sheep serum samples		
Day	8	24	37
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	20	20
6	0	0	40
7	20[1]	40	40
8	40	40	20
9	80	80	20
10	40	40	40
11	160	40	40
12	160	80	640
13	640	640	640
14	640	640	640
21	640	640	640
28		80	80
35		40	40
42		40	20
49		80	320
70		160	32

[1]Reciprocal titre

Appendix C: IgG antibody titres to CCHF virus in relation to day post infection, detected by ELISA using anti-sheep conjugate, in serum samples from experimentally infected sheep (sheep numbers 08, 24 and 37).

	IgG antibody titres to CCHF virus in sheep serum samples		
Day	08	24	37
1	0	0	0
2	0	0	0
3	0	0	0
4	0	800	0
5	0	2,600	1,600
6	400[1]	3,200	1,600
7	400	6,400	3,200
8	800	6,400	3,200
9	1,600	6,400	3,200
10	1,600	25,600	6,400
11	3,200	51,200	3,200
12	3,200	25,600	1,600
13	3,200	6,400	800
14	3,200	6,400	NA[2]
21	1,600	6,400	NA
28	1,600	6,400	NA
35	800	12,800	NA
42	800	6,400	NA
49	1,600	12,800	NA
70	800	6,400	NA

[1]Reciprocal titre
[2]Not available

Appendix B: IgM antibody titres to CCHF virus in relation to day post infection, detected by ELISA using anti-CCHF conjugate, in serum samples from experimentally infected sheep (sheep numbers 08, 24 and 37).

	IgM antibody titre to CCHF virus in sheep serum samples		
Day	08	24	37
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	400
6	0	400	200
7	400[1]	400	400
8	400	3,200	3,200
9	3,200	3,200	3,200
10	1,600	1,600	1,600
11	1,600	800	3,200
12	1,600	1,600	1,600
13	1,600	400	400
14	3,200	800	800
21	800	0	0
28	0	0	0
35	0	0	0
42	0	0	0
49	0	0	0
70	0	0	0

[1]Reciprocal titre

Appendix A: IgM antibody titres to CCHF virus in relation to day post infection, detected by ELISA using anti-sheep conjugate, in serum samples from experimentally infected sheep (sheep numbers 08, 24 and 37).

	IgM antibody titres to CCHF virus in sheep serum samples		
Day	08	24	37
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	1,600
6	0	1,600	1,600
7	0	1,600	3,200
8	1,600[1]	3,200	3,200
9	3,200	3,200	3,200
10	3,200	1,600	6,400
11	3,200	1,600	3,200
12	3,200	1,600	1,600
13	6,400	400	800
14	12,800	200	0
21	6,400	0	0
28	0	0	0
35	0	0	0
42	0	0	0
49	0	0	0
70	0	0	0

[1]Reciprocal titre

Although there have been 38 isolations of Chenuda virus from argasid tick parasites of swallows in South Africa (McIntosh, 1980), the present findings suggest that livestock and humans rarely encounter the infection, and no evidence was obtained to indicate that the remaining viruses in the study, Bahig, Bhanja, Thogoto and Dhori, occur in the country.

It can be concluded that serologic evidence was obtained to indicate that certain tick-borne viruses, including Dugbe, may be present in South Africa, but that definitive evidence can probably best be obtained through systematic attempts to isolate the viruses from ticks collected in locations where antibodies were detected in cattle sera.

probably best be obtained through systematic attempts to isolate the viruses from ticks collected in the locations where antibodies were detected in cattle sera.

Cross-reactivity of immune sera is a particular problem among flaviviruses (Calisher and Karabatsos, 1989), and the lack of antibody activity to West Nile, Kadam and louping ill antigens in the sera of the patients selected for inclusion in the study, plus the paucity of reactions recorded in cattle sera, are possibly related to the fact that the drought conditions which have prevailed over large parts of South Africa in recent years are not conducive to epidemic transmission of indigenous mosquito-associated flaviviruses such as West Nile, Wesselsbron, Banzi, Usutu and Spondweni. West Nile virus is believed to be maintained principally by circulation in birds and mosquitoes, but high antibody rates have been recorded in human and livestock sera in various parts of South Africa in the past, particularly when epidemics have followed heavy rains, as occurred in the northern Cape Province in 1974 (McIntosh, 1980). Had there been more evidence of flavivirus activity it would have been necessary to broaden the range of antigens included in the study in order to determine the specifity of the antibody reactions. In the event, no cross-reactions were observed among the 3 flaviviruses included in the study, and although this does not prove that the comparatively few reactions recorded in cattle sera with louping ill antigen were specific, the apparent coincidence in distribution of the antibody and ticks of the genus *Ixodes* suggests that attempts to isolate virus from ticks may be warranted. Most viruses of the tick-borne encephalitis complex in the northern hemisphere are associated with *Ixodes* ticks (Karabatsos, 1985). The 3 isolated reactions recorded with Kadam antigen were either non-specific, or at the most constitute weak evidence of the circulation of the virus in South Africa.

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RECEIVED 10/10/95

Ms FJ Burt
National Institute for Virology
Private Bag X4
Sandringham 2131

4 October 1995

Professor M Markus
Chairman, Animal Ethics Committee
University of the Witwatersrand

Dear Professor Markus,

re: Animal ethics clearance

Please could you advise me as to whether animal ethics clearance is required for the preparation of reagents (hyperimmune mouse ascitic fluids, antigens and control sera) by another department for use in enzyme-linked immunoassays, and for stocks of rabbit serum and harvested mouse brains from previous unrelated projects which were stored at -70°C. The following is a list of the reagents:
12 hyperimmune mouse ascitic fluids against tick-borne viruses;
11 antigens prepared from mouse brain suspensions from infected suckling mice;
9 positive control sera and 1 negative control serum from guinea pigs;
serum from a rabbit hyperimmunised with Crimean-Congo haemorrhagic fever (CCHF);
preparation of CCHF antigen from a stock of harvested mouse brains from suckling mice that were inoculated with CCHF virus.
Thank-you for your assistance

Yours sincerely

FJ Burt

YOU DO NOT REQUIRE ANIMAL ETHICS CLEARANCE FOR THE ABOVE.

10 October 1995 M. B. MARKUS

AESC 3

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:	95	94	3

APPLICANT: Ms F J Burt

DEPARTMENT: Virology

PROJECT TITLE: Evaluation of PCR for the diagnosis of Crimean-Congo haemorrhagic fever

Species	Number	Expiry Date
Mice	580	30 August 1997

First-time users of the CAS should contact the Director of the CAS in order to familiarise themselves with the facilities available.

Approval given by the AESC for category 5 and 6 experiments is subject to ratification by the Animal Ethics and Control Committee.

The use of these animals is subject to AESC Guidelines for the use and care of animals, to the procedures specified in the application form, and to:

Nil.

SIGNED ..... DATE : 30 August 1995

(Chairman: Animal Ethics Screening Committee)

Please see attachment:

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO: 90 / 6

APPLICANT: Ms P J Burt

DEPARTMENT: National Institute of Virology, Wits Medical School

PROJECT TITLE: Preparation of stock of CCHF virus

SPECIES	NUMBER	DATE OF EXPIRY
Day old mice	150	Sept. 1992

The use of these animals is subject to AESC Guidelines for the use and care of animals, to the procedures specified in the application form, and to:

Signed _____ Date _____
(Chairman: Animal Ethics Screening Committee)

Appendix I: Animal ethics clearance certificates

Appendix II:(continued)

SPU	District	No of sera	No of positive sera					
Eastern Transvaal			Dolping ill	West Nile	Kadam	Chenuda	Thogoto	Dho
37/83	Witbank	50	1				0	
46/83	Ermelo	50	0				0	0
26/83	Lydenburg	50	1				0	0
Kwazulu/Natal								
105/83	Estcourt	50	1	1	0	1	0	0
56/84	Mt Currie	50	0				0	
101/83	Ixopo	48	0		0		0	0
105/84	Eshowe	NA						
191/84	Hlabisa	50	1	1	0		0	0
441/84	Umvoti	50	0			0	0	0
Orange Free State								
88/83	Harrismith	50	0		0		0	0
86/83	Lindley	40			0			
175/83	Viljoenskroon	50						
40/83	Theunissen	50	1	1	1	0	0	0
234/84	Petrusburg	50						
33/84	Aliwal North	50	0	3	1	0	0	0
168/83	Ladybrand	50						

Appendix H (continued)

SPU	District	No of sera	No of positive sera					
Eastern Cape			Lumping ill	West Nile	Kadam	Chenuda	Thogoto	Dho
81/83	Somerset East	50						
64/83	Albany	50	0				0	
202/83	Port Elizabeth	50	0	0	0	0	0	0
32/84	Stutterheim	50	1				0	
104/83	Maclear	50						
308/84	Steynsburg	50						
North West								
65/83	Vryburg	50						
37/84	Christiana	50						
94/83	Klerksdorp	50	0	0	0	0	0	0
24/83	Marico	50	0				0	0
Northern Transvaal								
33/83	Thabazimbi	50	2				0	0
27/83	Tzaneen	50	0	0	1	1	0	0
74/83	Louis Trichardt	50	0				0	
76/83	Messina	48	0				0	
123/83	Potgietersrus	50	0				0	

Appendix H: (continued)

SPU	District	No of sera	No of positive sera					
Western Cape			Looping ill	West-Nile	Kadam	Chenuda	Thogoto	Dhu
299/84	van Rhynsdorp	18						
220/84	Clanwilliam	37	7		0		0	0
436/84	Vredenburg	50						
163/83	Malmesbury	50						
18/84	Wynberg	50						
43/83	Caledon	50	1	0	0	0	0	0
42/83	Worcester	50						
190/83	Heidelburg	50	1				0	
167/83	Knysna	50	1				0	
294/84	Oudtshoorn	50						
309/84	Beaufort West	50						
Northern Cape								
236/84	Richmond	50	0				0	
39/83	Herbert	25	0				0	
186/83	Kuruman	50						
187/83	Postmasburg	50	1	0	0	0	0	0

Appendix H:(continued)

SPU	District	No of sera	No of positive sera					
	Eastern Transvaal		CCHF	Hazara	NSD	Dugbe	Bahig	Bhanja
37/83	Witbank	50	3		0	0		
46/83	Ermelo	50	20		0	0		
26/83	Lydenburg	50	31	0	0	16	0	0
	Kwazulu Natal							
105/83	Estcourt	50	33	0	0	0	0	
56/84	Mt Currie	50	31		0	0		
101/83	Ixopo	48	25	0	0	0		0
105/84	Eshowe	NA						
194/84	Hlabisa	50	7	0	3	11	0	
441/84	Umvoti	50	36	0	0	0		0
	Orange Free State							
88/83	Harrismith	50	18	0	0	0		0
86/83	Lindley	40	15					
175/83	Viljoenskroon	50	10					
40/83	Theunissen	50	36	0	1	0	0	0
234/84	Petrusburg	NA						
33/84	Aliwal North	50	20	0	0	0		
168/83	Ladybrand	50	5					

Appendix H:(continued)

SPU	District	No of sera	No of positive sera				
Eastern Cape			CCHF	Hazara	NSD	Dugbe	Babig	Bhanja
81/83	Somerset East	50	1					
64/83	Albany	50	13		0	0		
202/83	Port Elizabeth	50	0	0	0	0	0	
32/84	Stutterheim	50	5		0	0		
104/83	Maclear	50	44					
308/84	Steynsburg	50	7					
North West								
65/83	Vryburg	50	18					
37/84	Christiana	50	46					
94/83	Klerksdorp	50	32	0	0	0	0	
24/83	Marico	50	41	0	0	0		0
Northern Transvaal								
33/83	Thabazimbi	50	39	0	0	10	0	0
27/83	Tzaneen	50	7	0	3	15	0	
74/83	Louis Trichardt	50	17		0	1		
76/83	Messina	48	44		0	3		
123/83	Potgietersrus	50	21		0	14		

Appendix H: Detection of IgG antibody to tick-borne viruses in cattle sera collected in South Africa

SPU	District	No of sera	No of positive sera					
Western Cape			CCHF	Hazara	NSD	Dugbe	Bahig	Bhanja
299/84	van Rhynsdorp	18	12					
220/84	Clanwilliam	37	30	0	0	0		0
436/84	Vredenburg	50	33					
163/83	Malmesbury	50	3					
18/84	Wynberg	50	0					
43/83	Caledon	50	2	0	0	0		0
42/83	Worcester	50	35					
190/83	Heidelburg	50	0		0			
167/83	Knysna	50	5		0	0		
294/84	Oudtshoorn	50	13					
309/84	Beaufort West	50	29					
Northern Cape								
236/84	Richmond	50	5		0	0		
39/83	Herbert	25	14		0	0		
186/83	Kuruman	50	30					
187/83	Postmasburg	50	39	0	0	0		0

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