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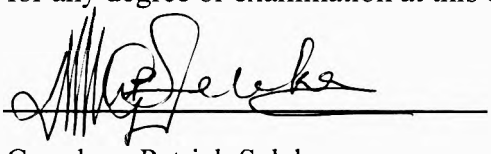
Use of recombinant antigen in the diagnosis of Crimean-Congo haemorrhagic fever virus infection

GOPOLANG PATRICK SELEKA

A dissertation submitted to the Faculty of Health Sciences
University of the Witwatersrand, Johannesburg
For the degree of Master of Science
Johannesburg 2001

Declaration

I declare that this dissertation: Use of recombinant antigen in the diagnosis of Crimean-Congo haemorrhagic fever virus infection, is my own work. It is being submitted to the University of the Witwatersrand, Johannesburg, for the degree Master of Science. It has not previously been submitted for any degree or examination at this or any other University.



Gopolang Patrick Seleka

on this 30th day of January ,2002

Abstract

The Special Pathogens Unit (SPU) of the National Institute for Virology has diagnosed a total of 158 cases of Crimean-Congo haemorrhagic fever (CCHF) from the time that the disease was first recognized in South Africa in 1981 up until the end of 2000. The virus has a propensity to cause nosocomial infections, and consequently rapid diagnosis is important for the isolation of the patient and the institution of barrier-nursing to protect medical staff and the community at large. Thus it is essential that the SPU should have the latest diagnostic and research tools available.

Diagnosis of CCHF is generally confirmed by isolation of the virus, detection of viral RNA amplified by reverse transcriptase polymerase chain reaction (RT-PCR), demonstration of seroconversion or a >4-fold increase in IgG antibody titre, or detection of specific IgM antibody activity. Virus can be isolated in 1-6 days in cell culture, but the method is less sensitive for the isolation of low concentrations of virus than the use of suckling mice which, however, takes 7-9 days. Enzyme-linked immunosorbent assay (ELISA) and indirect immunoflourescence (IF) antibody tests are the most useful for serological diagnosis of CCHF in humans because these tests can distinguish between IgG and IgM in order to confirm that infection has been recent, and to establish that antibody activity detected in the sera of patients does not result merely from the use of immune plasma therapy. However, one of the problems of diagnosing viral haemorrhagic fevers is the biohazardous nature of the test reagents which require biosafety level 4 (BSL4) facilities for preparation and use. Reagents must be inactivated and safety tested before they can be used in other laboratories. A further requirement for safe antigen arises from the need to screen livestock sera for antibody to CCHF virus as a prerequisite to the export of live animals, and in this respect the export of ostriches from southern Africa to Europe has created a particular demand in recent years.

Recombinant antigens are being used more frequently as diagnostic reagents in virology and have the advantage that they are non-hazardous. Because the SPU has a unique collection of sera derived from confirmed CCHF patients and from experimentally infected animals, it was asked to test candidate recombinant CCHF reagents which had been prepared by BSL4 laboratories abroad. The

reagents comprised a baculovirus recombinant expressing CCHF nucleocapsid protein received from one laboratory, plus three recombinant nucleoprotein antigens from another. The initial purpose of the present study, therefore, was to evaluate the reagents received from abroad, but in case none of them proved to be satisfactory it was decided to attempt the production of further recombinant antigen at the same time.

The recombinant baculovirus produced for the present project failed to express antigenically active nucleoprotein, and the 3 recombinant proteins received from a laboratory abroad were weakly reactive and could not be tested as diagnostic antigens. However, the recombinant baculovirus received from the other laboratory abroad expressed antigenically active CCHF nucleocapsid protein, and this produced promising results in IgM and IgG ELISA on 135 serum samples from confirmed human CCHF patients and on 63 sera from experimentally infected sheep, as well as in competitive ELISA (CELISA) on the 135 human sera and on 35 samples from experimentally infected small mammals, plus 271 samples from ostriches. In the various tests on the sera of the different species the sensitivity of the results produced by the recombinant antigen ranged from 82.1-100% in comparison with the sensitivity of the reference antigen prepared by sucrose acetone extraction of infected mouse brain, while the specificity of the results produced by recombinant antigen ranged from 53.3-100%. It appears likely that the sensitivity and specificity of the recombinant antigen can be improved to be comparable to those of the sucrose acetone antigen relatively easily by adjusting the cut off values of the reactions for positivity. However, this will need to be based on prospective studies on serum samples received at the SPU for the investigation of suspected CCHF since definitive determinations of sensitivity and specificity should ideally be conducted in the context of the syndrome under investigation, and not in comparison with random samples from general populations such as blood donors. Nevertheless, the present results based on selected positive and negative sera provide strong evidence in support of proceeding with prospective studies on the recombinant antigen.

Acknowledgements

I am grateful for having been afforded facilities to perform the experimental work in the Special Pathogens Unit, National Institute for Virology, Johannesburg. I thank my supervisors Dr FJ Burt and Professor R Swanepoel for advice, and the Poliomyelitis Research Foundation for a research grant.

Table of contents **Page**

Declaration i

Abstract ii

Acknowledgement iv

List of figures x

List of tables xii

1.0. Introduction and review of literature

1.1. History 1

1.2. The Virus 1

1.3. Antigenic relationships 2

1.4. Replication 3

1.5. Distribution 3

1.6. Vectors 4

1.7. Transmission 4

1.7.1. Transmission to humans 4

1.7.2. Transmission to non-human vertebrates 4

1.7.3. Transmission by ticks to birds and the role of birds in dissemination of the virus 5

1.7.4. Transmission of virus from animals to ticks 6

1.8. Pathogenesis 6

1.9. Disease in humans 7

1.9.1. Clinical features 7

1.9.2. Clinical pathology 8

1.9.3. Viraemia and antibody response 8

1.10. Disease in non-human mammals 9

1.11. Diagnosis 10

1.12. Differential diagnosis 11

1.13. Recombinant baculovirus expressed proteins 12

1.14. Objectives 13

2.0. Materials and methods

2.1. Recombinant reagents 15

2.2. Antisera and antigens 16

2.3. Serum samples 17

2.4. Preparation of CCHF baculovirus recombinant 18

2.4.1. Isolation of S segment of CCHF virus genome from pBluebac plasmid 18

2.4.2. Cloning of CCHF virus insert into pFASTBAC 1 donor plasmid 21

2.4.3. Transformation of DH5∝ competent cells 21

2.4.4. Transformation of DH10Bac competent cells 23

2.4.5. Analysis of bacmid DNA 24

2.5. Expression and purification of recombinant antigen 25

2.5.1. Inoculation of cell cultures 25

2.5.2. Detection of *in vitro* expression of CCHF nucleoprotein 25

2.5.2.1. Immunofluorescence tests 25

2.5.2.2. Western blot 26

2.5.3. Purification of expressed proteins 27

2.6. Enzyme-linked immunoassay 28

2.6.1. Evaluation of recombinant antigens 28

2.6.2. IgM-capture ELISA 29

2.6.3. IgG sandwich ELISA 29

2.6.4. Competitive ELISA 30

2.6.5. Calculation of sensitivity and specificity of serological tests with recombinant antigen 30

2.7. Antigenic specificity testing of CCHF recombinant antigen 30

2.8. Stability testing of recombinant antigen 31

3.0 Results

3.1. Preparation of CCHF baculovirus 32

3.2. *In vitro* expression of CCHF recombinant protein 32

3.3. Antigenic reactivity of recombinant proteins 38

3.4. Detection of antibody response to CCHF virus infection in human sera 38
3.4.1. IgM detection by ELISA using sucrose acetone extracted antigen 38
3.4.2. IgM detection by ELISA using recombinant antigen 39
3.4.3. IgG detection by ELISA using sucrose acetone extracted antigen 39
3.4.4. IgG detection by ELISA using recombinant antigen 40
3.4.5. CELISA using the sucrose acetone extracted antigen 40
3.4.6. CELISA using the recombinant antigen 40
3.4.7. Control panel of negative sera 41
3.5. Detection of antibody response to CCHF virus in experimentally infected sheep 41
3.5.1. IgM ELISA using sucrose acetone extracted and recombinant antigen 41
3.5.2. IgG ELISA using sucrose acetone extracted and recombinant antigen 45
3.6. Detection of CCHF antibody in ostrich sera 45
3.7. Detection of antibody by in experimentally infected small mammals 52
3.8. Sensitivity and specificity of serological tests with recombinant antigen 54
3.8.1. IgM-capture ELISA on CCHF patient sera 54
3.8.2. IgG sandwich ELISA on CCHF patient sera 55
3.8.3. CELISA on CCHF patient sera 56
3.8.4. IgM-capture ELISA on experimentally infected sheep sera 56
3.8.5. IgG sandwich ELISA on experimentally infected sheep sera 57
3.8.6. CELISA on ostrich sera 58
3.8.7. CELISA on experimentally infected small mammal sera 58
3.9. Antigenic specificity of CCHF recombinant antigen 58
3.10. Stability testing of recombinant antigen 59

4.0 Discussion 60

5.0 References 65

Appendices

Appendix A: Buffers, reagents, pBlueBac restriction map. 74

Appendix B: IgM antibody titre to CCHF in relation to day after the onset of illness as detected by IgM capture ELISA using sucrose acetone extracted antigen, in serum samples collected from CCHF infected patients from 1986-1999. 79

Appendix C: IgM antibody titre to CCHF in relation to day after the onset of illness as detected by IgM capture ELISA using recombinant antigen, in serum samples collected from CCHF infected patients from 1986-1999. 82

Appendix D: IgG antibody titre to CCHF virus in relation to day after the onset of illness as detected by IgG sandwich ELISA using sucrose acetone extracted antigen, in serum samples collected from CCHF infected patients from 1986-1999. 85

Appendix E: IgG antibody titre to CCHF virus in relation to day after the onset of illness as detected by IgG sandwich ELISA using recombinant antigen, in serum samples collected from CCHF infected patients from 1986-1999. 88

Appendix F: Final antibody titre to CCHF virus in relation to day after the onset of illness as detected by CELISA using sucrose acetone extracted antigen, in serum samples from CCHF virus infected patients collected from 1986-1999. 91

Appendix G: Final antibody titre to CCHF virus in relation to day after the onset of illness as detected by CELISA using recombinant antigen, in serum samples from CCHF virus infected patients collected from 1986-1999. 94

Appendix H: Optical density (OD) values obtained from screening non-CCHF patients by ELISA.97

Appendix I: IgM antibody titre to CCHF virus in relation to day post infection as detected by IgM capture ELISA using sucrose acetone extracted antigen and recombinant antigen in serum samples from experimentally infected sheep number 92. 101

Appendix J: IgM antibody titre to CCHF virus in relation to day post infection as detected by IgM capture ELISA using sucrose acetone extracted antigen and recombinant antigen in serum samples from experimentally infected sheep number 48. 102

Appendix K: IgM antibody titre to CCHF virus in relation to day post infection as detected by IgM capture ELISA using sucrose acetone extracted antigen and recombinant antigen in serum samples from experimentally infected sheep number 37. 103

Appendix L: IgG antibody titre to CCHF virus in relation to day post infection as detected by IgG sandwich ELISA using sucrose acetone extracted antigen and recombinant antigen in serum samples from experimentally infected sheep number 92. 104

Appendix M: IgG antibody titre to CCHF virus in relation to day post infection as detected by IgG sandwich ELISA using sucrose acetone extracted antigen and recombinant antigen in serum samples from experimentally infected sheep number 48. 105

Appendix N: IgG antibody titre to CCHF virus in relation to day post infection as detected by IgG sandwich ELISA using sucrose acetone extracted antigen and recombinant antigen in serum samples from experimentally infected sheep number 37. 106

List of figures **Page**

Figure 1. Histogram showing the number of serum samples included in the study which had been collected from CCHF virus infected patients from 1986 to 1999 at the indicated intervals after the onset of illness. 19

Figure 2. Agarose gel analysis of mini-prep pBluebac DNA. 33

Figure 3. Agarose gel analysis of mini-prep transformed DNA. 34

Figure 4. Agarose gel analysis of mini-prep PCR of transformed DNA. 35

Figure 5. Agarose gel analysis of mini-prep bacmid DNA. 36

Figure 6. Detection of expressed CCHF virus nucleoprotein from the CCHF baculovirus infected Sf9 cells using immunoflourescence. 37

Figure 7. Western blot to determine expressed CCHF nucleoprotein by recombinant baculovirus. Lane 1: molecular weight marker. 37

Figure 8. IgM antibody levels to CCHF in human serum samples collected on different days after the onset of illness, detected by IgM captured ELISA using sucrose acetone extracted antigen (A) and recombinant antigen (B). 42

Figure 9. IgG antibody levels to CCHF in human serum samples collected on different days after the onset of illness, detected by IgG sandwich ELISA using sucrose acetone extracted antigen (A) and recombinant antigen (B). 43

Figure 10. Total antibody levels to CCHF virus in human serum samples collected on different days

after the onset of illness, detected by CELISA using sucrose acetone extracted antigen (A) and recombinant antigen (B). 44

Figure 11. IgM antibody log titre to CCHF virus in relation to day after infection as demonstrated by IgM capture ELISA using two different antigens, in serum samples from experimentally infected sheep number 92. 46

Figure 12. IgM antibody log titre to CCHF virus in relation to day after infection as demonstrated by IgM capture ELISA using two different antigens, in serum samples from experimentally infected sheep number 48. 47

Figure 13. IgM antibody log titre to CCHF virus in relation to day after infection as demonstrated by IgM capture ELISA using two different antigens, in serum samples from experimentally infected sheep number 37. 48

Figure 14. IgG antibody log titre to CCHF virus in relation to day after infection as demonstrated by IgG sandwich ELISA using two different antigens, in serum samples from experimentally infected sheep number 92. 49

Figure 15. IgG antibody log titre to CCHF virus in relation to day after infection as demonstrated by IgG sandwich ELISA using two different antigens, in serum samples from experimentally infected sheep number 48. 50

Figure 16. IgG antibody log titre to CCHF virus in relation to day after infection as demonstrated by IgG sandwich ELISA using two different antigens, in serum samples from experimentally infected sheep number 37. 51

List of tables **Page**

Table 1. Results of screening tests for antibody activity to CCHF virus performed on 271 ostrich serum samples by CELISA using sucrose acetone extracted antigen in comparison with recombinant antigen. 52

Table 2. Total antibody titres in the sera of small mammals experimentally infected with CCHF virus as detected by CELISA using sucrose acetone extracted and recombinant antigens, shown in relation to day after inoculation. 53

Table 3. Summary of the results obtained on 135 serum samples from confirmed CCHF patients in IgM capture ELISA with sucrose acetone extracted and recombinant antigens (antibody titres ≥400 interpreted as positive results). 54

Table 4. Summary of the results obtained on 135 serum samples from confirmed CCHF patients in IgGsandwich ELISA with sucrose acetone extracted and recombinant antigens (antibody titres ≥400 interpreted as positive results). 55

Table 5. Summary of the results obtained on 135 serum samples from confirmed CCHF patients in CELISA with sucrose acetone extracted and recombinant antigens (titres ≥10 are recorded as positive). 56

Table 6. Summary of the results obtained on 63 serum samples from 3 sheep infected with CCHF virus in IgM capture ELISA with sucrose acetone extracted and recombinant antigens (antibody titres ≥400 interpreted as positive results). 57

Table 7. Summary of the results obtained on 63 serum samples from 3 sheep infected with CCHF virus in IgG sandwich ELISA with sucrose acetone extracted and recombinant antigens (antibody titres ≥400 interpreted as positive results). 57

Table 8. Summary of the results obtained on 35 serum samples from small mammals experimentally infected with CCHF virus in CELISA with sucrose acetone extracted and recombinant antigens (titres ≥10 are recorded as positive). 58

1.0. Introduction and review of literature

1.1. History

In 1944 over two hundred cases of acute febrile illness accompanied by severe bleeding manifestation occurred in Russian troops and peasants who were harvesting crops in war devastated Crimea. The disease was given the name Crimean haemorrhagic fever (CHF) (Hoogstraal, 1979) and in the following year, 1945, Russian scientists showed by inoculation of volunteers that the disease was caused by a filterable agent present in blood collected from patients during the acute phase of illness, and that the agent was also present in ticks. In 1969, the use of suckling mice made it possible to isolate and characterize the virus, and it was shown to be antigenically identical to an African virus, Congo virus, isolated in 1956 from the blood of a febrile patient in what is now the Democratic Republic of the Congo (Simpson *et al.*, 1967; Casals, 1969; Casals and Tignor, 1974; Chumakov *et al.*, 1970). It was confirmed by Donets *et al.* (1977) that CHF and Congo viruses have the same physiochemical characteristics. Since the viruses were identical, the use of Crimean haemorrhagic fever-Congo (CHF-C) virus was suggested as a common name (Casals and Tignor, 1974). Even although some Russian writers use only CHF and those working with the virus in Africa prefer the name Congo, the virus is generally known as Crimean-Congo haemorrhagic fever (CCHF) virus.

The first case of CCHF virus infection to be confirmed in South Africa was recorded when the virus was isolated from the blood of a 13 year old boy who died in a haemorrhagic state in Johannesburg on 19 February 1981 (Gear, *et al.*, 1982; Swanepoel *et al.*, 1983a). To date a total of 158 cases of CCHF with 37 fatalities have been confirmed in southern Africa (unpublished laboratory records).

1.2. The Virus

CCHF virus is a member of the family *Bunyaviridae*, genus *Nairovirus* (Casals and Tignor, 1980; Matthews, 1982). Nairoviruses have properties similar to other members of the *Bunyaviridae* in that they are spherical structures 90-120 nm in diameter, with host derived envelope incorporating virus coded glycoprotein (G) surface projections (Donets, *et al.*, 1977; Ellis *et al.*, 1981). The genome

consists of three segments of negative sense, single stranded RNA designated small (S), medium (M) and large (L) with the approximate sizes L: 4.1-4.9 $X10^6$ daltons, M: 1.5-1.9$X10^6$ daltons and S: 0.6-0.7$X10^6$ daltons.(Bishop, *et al.*, 1980; Clerx *et al.*, 1981). The first 8-13 nucleotide bases at the 3' end of the RNA segments tend to have a sequence which is conserved within the viruses of the genus, with a complementary consensus sequence occurring at the 5' end. The ends of the segments are covalently linked so that the RNA occurs in a loosely bound circular configuration within the nucleocapsid. The segmented nature of the genome suggests that the potential exists for reassortment to occur in co-infection, although this has never been recorded (Swanepoel, 1994). The M segment codes for the viral glycoproteins, G1 and G2, which are believed to be responsible for recognition of receptor sites on susceptible cells, manifestation of viral hemagglutinating ability and induction of a protective immune response in the host (Clerx *et al.*, 1981). The S segment codes for the nucleocapsid protein (N). The N protein has a net positive charge and induces production of, and reacts with, complement fixing antibodies (Clerx *et al.*, 1981; Marriott and Nuttall, 1992). The L segment is believed to code for the viral transcriptase (Elliot, 1990).

CCHF virus is highly labile, very sensitive to the effects of lipid solvents and is inactivated by ether, chloroform and ultraviolet light (Bishop, *et al.*, 1980; Smirnova, 1979). The virus is infective in a limited pH range of 6.0-9.5, and has half-life of 2-3 h at 37° C, 10-20 min at 45° C and less than 1 min at 56° C. Ultraviolet radiation inactivates 99% of infectivity in 1 min and within 3 min the virus loses all infectivity (Donets *et al.*, 1977).

1.3. Antigenic relationships

The *Bunyaviridae* is composed of 4 recognised genera of animal viruses, *Bunyavirus*, *Nairovirus*, *Phlebovirus* and *Hantavirus* plus a genus of plant viruses (Bishop *et al.*, 1980, Murphy *et al.*, 1995). The *Nairovirus* genus is composed of seven antigenic serogroups, and CCHF virus belongs to a serogroup of the same name. There are two other member of the CCHF serogroup, Hazara and Khasan, neither of which have been shown to be pathogenic for humans. Other members of the genus known to be pathogenic for human are Nairobi sheep disease and Dugbe viruses (Clerx, *et al.*, 1981; Watts *et al.*, 1989). Nairobi sheep disease virus of East Africa is believed to be identical to

Ganjam of India, and is a tick borne pathogen of sheep and goats which causes benign illness in humans. Dugbe is a tick borne virus which is commonly associated with infection of cattle and sheep in West Africa, and causes benign disease in human (Swanepoel, 1994). Hazara virus is most closely related antigenically to CCHF virus as shown by haemagglutination inhibition (HAI), complement fixation (CF) and indirect immunofluorescence (IF) techniques. Even although members of the Nairobi sheep disease serogroup are related to the CCHF serogroup they show weak HAI, IF and CF cross-reactions (Casals and Tignor, 1980). Marriott and Nuttall (1992) showed that the genetic sequences of the N proteins of CCHF and Hazara viruses are more closely related to each other (60% amino acid identity) than to the N protein of Dugbe (55.4% identity with CCHF, and 53.0% identity with Hazara), and 39.5% amino acids are conserved across all three viruses. Both CCHF and Hazara N protein genes are larger than that of Dugbe virus, having extra sequences at their carboxyl termini.

1.4. Replication

Replication strategies of CCHF virus are still unknown (Bishop *et al.*, 1980) and the early events in the infection process of members of the family *Bunyaviridae* are not well defined. Like other enveloped viruses, one or both integral viral envelope proteins mediate attachment to host cell receptors. The main stages of CCHF virus morphogenesis include assembly of virion on the membrane of the Golgi complex, transportation of virus containing vacuoles to the cell surface and virus release by exocytosis. It seems that the final differentiation of the virion envelope occurs after budding as the vacuole moves to the surface. Virus release from the cell may also occur upon lysis of the infected cell (Donets, *et al.*, 1977; Elliot *et al.*, 1990).

1.5. Distribution

CCHF virus is widely distributed in Asia, eastern Europe and Africa (Hoogstraal, 1979). The list of countries where the virus or the antibody is known to occur has grown over the past four decades, probably due to an increased awareness rather than the spread of the infection to new areas. The strongest evidence to support this conclusion relates to the coincidence in distribution of the virus and its main vectors, ticks of the genus *Hyalomma* (Swanepoel *et al.*, 1987). Human cases have been reported in Bulgaria, Burkina Faso, Greece, Iraq, Mauritania, Namibia, Oman, Pakistan, South

Africa, Tanzania, Yugoslavia, Uganda and United Arab Emirates (Hoogstraal, 1979; Antoniadis, *et al.*, 1982; Butenko and Chumakov, 1990; Burney, *et al.*, 1980; Chumakov *et al.*, 1970; Gear, *et al.*, 1982; Hoogstraal, 1979; Saluzzo, *et al.*, 1985; Schwartz, *et al.*, 1995; Scrimgeour, 1996; Simpson, *et al.*, 1967; Suleiman, 1980; Swanepoel *et al.*, 1983a; Swanepoel *et al.*, 1987). The virus has been isolated from ticks and non-human mammals in Ethiopia, Greece, Kenya, Madagascar, Nigeria and Senegal, (Camicas, *et al.*, 1990; Causey, *et al.*, 1969; Hoogstraal, 1979; Morrill *et al.*, 1991; Wood *et al.*, 1978). Furthermore, serological evidence of the presence of the virus has been reported from Afghanistan, Egypt, Hungary, India, Iran, Turkey and Portugal (Hoogstraal, 1979; Morrill *et al.*, 1990; Saidi *et al.*, 1975).

1.6. Vectors

The virus has been isolated from 31 species of ticks which include 2 argasids and 29 ixodids (11 *Hyalomma,* 9 *Rhipicephalus*, 4 *Boophilus*, 2 *Dermacentor,* 1 *Amblyomma*, 1 *Haemaphysalis* and 1 *Ixodes)*, although very few species have been proved experimentally to be capable of transmitting infection (Camicas *et al.*, 1990; Hoogstraal, 1979; Shepherd *et al.*, 1989a; Watts *et al.*, 1989).

1.7.Transmission

1.7.1. Transmission to humans

Like most of the arthropod-borne agents causing human disease, CCHF virus is generally a zoonosis circulating unnoticed in nature in a tick and non-human vertebrate cycle (Hoogstraal, 1979). A common mode of transmission of CCHF virus to humans is by tick-bite, as in the first confirmed case in South Africa; but humans also acquire infection from crushing infected ticks, or through contact with infected human or animal blood and other tissues (Hoogstraal, 1979; Burney *et al.*, 1980; Khan, *et al.*, 1997; Shepherd *et al.*, 1985; Swanepoel *et al.*, 1983a; Swanepoel et al., 1985a). Adult males are most commonly infected because of the occupational risk factor of those employed in the livestock industry.

1.7.2. Transmission to non-human vertebrates

The host preferences of the tick vectors of the virus play an important role in determining which

vertebrates become infected. Species of the genus *Hyalomma*, considered to be the main vectors of the virus, feed exclusively on small mammals and ground frequenting birds as immature ticks, while as adult ticks they feed on large mammals such as cattle, sheep, and goats, in preference to man and small mammals (Swanepoel *et al.*, 1985b). Antibodies to CCHF virus are widely distributed in cattle sera in South Africa, and the only place that appears to have a consistently low prevalence of antibodies is along the southern coast, where *Hyalomma marginatum rufipes* is absent. However, given the mobility of man and his livestock today, clinicians can expect to encounter CCHF infection virtually anywhere in South Africa (Swanepoel *et al.*, 1985a). To date a total of 158 cases of CCHF have been confirmed in southern Africa from areas distributed throughout the region(unpublished laboratory records). The occurrence of infections can be expected to occur in other geographic regions where the vector of this virus is distributed however data from serological surveys is not available as the ability to perform these tests is restricted to a limited number of laboratories partly because of the lack of safe reagents (Swanepoel, 1998). Recent outbreaks have been documented in the United Arab Emirates in 1994-1995 (Rodriguez *et al.*, 1997; Khan *et al.*, 1997) and more recently an outbreak occurred in Kosovo 2000 (unpublished).

Serological evidence of CCHF infection has been detected in many wild vertebrates, and the occurrence of viraemia has been demonstrated experimentally in several species (Hoogstraal, 1979; Saluzzo *et al.*,1985; Shepherd *et al.*, 1989b).

1.7.3. Transmission by ticks to birds and the role of birds in dissemination of the virus

Birds play a role in the epidemiology of CCHF through serving as hosts for immature *Hyalommas*, and may help to disseminate infected ticks both locally and on long migration routes (Hoogstraal, 1979; Zeller *et al.,* 1994). Most birds do not develop demonstrable viraemia (Hoogstraal, 1979; Zeller *et al.,* 1994; Shepherd *et al.*, 1987a), but following the occurrence of an outbreak of CCHF among workers at an ostrich slaughterhouse it was found that experimentally infected ostriches developed viraemia that was detectable on days 1-4 following subcutaeous inoculation of virus (Swanepoel *et al.,* 1998). As a consequence of this incident the European Union requires that imported ostrich meat must be derived from birds that have been treated and kept tick-free for 14

days prior to slaughter, and that imported live ostriches must be free of antibody to CCHF virus (Swanepoel *et al.*, 1998; Capua, 1998).

1.7.4. Transmission of virus from animals to ticks

Although livestock and small mammals develop viraemic infection, humans and suckling mice are the only species for which CCHF virus is pathogenic (Watts *et al.*, 1989). Many species of ticks have been shown to be capable of transmitting CCHF experimentally (Hoogstraal, 1979). In a study done by Shepherd *et al.* (1991) it was shown that infected cattle and scrub hares could serve as a source of virus for infection of five South African species of ticks through the ingestion of viraemic blood meals (*H. truncatum*, *H. m. rufipes*, *Amblyomma hebraeum*, *Boophilus decoloratus*, *Rhipicephalus evertsi evertsi* and *R. appendiculatus*), but subsequent transmission of infection to a susceptible host was demonstrated for only one species, *H. m. rufipes*. Shepherd *et al.* (1991) failed to demonstrate transovarial passage of virus from one generation of ticks to the next, a mechanism which can ensure perpetuation of the virus even in the absence of viraemic hosts. However, regardless of whether they transmit virus transovarially, infected ticks survive for long periods away from hosts, and can thus ensure perpetuation of virus in the environment from one season of tick activity to the next.

1.8. Pathogenesis

The pathogenesis of the disease is incompletely understood (Joubert, *et al.*, 1985; Swanepoel *et al.*, 1989), but by analogy with other arthropod-borne virus infections it can be surmised that peripherally introduced CCHF virus must undergo some replication at the site of inoculation, and that heterogenous and lymph-borne spread of infection occurs to major sites of replication. It has been found that the major targets of infection are macrophages, hepatocytes and endothelial cells (Burt *et al.*, 1997), with consequent platelet activation and triggering of the intrinsic coagulation cascade leading to the development of disseminated intravascular coagulopathy (DIC). Formation of circulating immune complexes with complement activation probably accounts for the occurrence of a rash and further contributes to the damage of capillary bed and the genesis of renal and pulmonary failure (Swanepoel, 1994; Joubert *et al.*, 1985). Widespread tissue damage in organs such as the liver results in the release of procoagulants into the bloodstream and further impairment of the circulation

through promoting DIC. Liver failure results in limited clearance of fibrin degradation products and impaired synthesis of coagulation factors to replace those which are consumed (Swanepoel, 1984).

1.9. Disease in humans

1.9.1. Clinical features

The incubation period between tick-bite and onset of illness is generally very short, 2-3 days, and 5-6 days (maximum 13 days) following contact with infected tissues of livestock or human patients.

The onset of illness is extremely sudden, with a severe headache usually being the earliest symptom, frequently accompanied by dizziness, neck pain and stiffness, sore eyes and photophobia (Swanepoel *et al.*, 1987; 1989). Fever is constant, diphasic or irregular, with rigors and chills evident at the same time or shortly thereafter. Patients rapidly develop general myalgia and malaise, with intense backache and leg pain. Nausea, sore throat and vomiting not associated with eating, liquid stools and loss of appetite are common at the onset of illness and about half of the patients experience non-localised abdominal pain. Patients experience sharp change of mood, with feeling of confusion and aggression, some even have bouts of violent behaviour (Swanepoel *et al.*, 1987; 1989). After 2-4 days of illness patients often exhibited lassitude, depression and somnolence. Hyperaemia of the face, neck and chest, congested sclerae, conjuctivitis, a slightly hyperaemic pharynx and spotted enanthemas on the soft and hard palate are common. A macular rash appears on the trunk about 5 days after onset of illness. Tachycardia is common and there is a tendency for patients to be slightly hypotensive. Lymphadenopathy is recorded in some patients, with moderate swelling of inguinal, axillary and or cervical glands. Right upper quadrant tenderness of the abdomen and hepatomegaly is discernible in about half of patients. Haemorrhage can present as petechia to large haematomas on the mucous membranes and the skin, and at injection and pressure sites. Intestinal haemorrhage (melaena) and uterine haemorrhage can occur, and sometimes haematemesis. The more severely ill patients enter a state of hepatorenal failure from about day five onwards and progressively become drowsy, stuporous and comatose. Jaundice is noted in the second week in some patients (Swanepoel *et al.*, 1987; 1989; 1998).

Reported death rates range from 25-50%, and deaths occur from day 5-14 of illness. Factors contributing to the fatal outcome include cerebral haemorrhage, severe anaemia, severe dehydration and shock associated with protracted diarrhoea, lung oedema and pleural effusion. Patients who die are reported to have developed multiple organ failure including cerebral, liver and kidney failure, and cardiac and pulmonary insufficiency (Swanepoel *et al.*, 1987). In patients that recover convalescence usually progresses rapidly from about day 10-15 after onset of illness.

1.9.2. Clinical pathology

Leucocyte counts fall progressively during the period from day 3-12 of illness, with absolute lymphopenia tending to precede neutropenia. Thrombocytopaenia is a consistent feature of the infection and counts fall below $100X10^9$ platelets/l in most patients at some stage during the period from day 3 to 15 of illness. Most patients show a decline in haemoglobin level during the second week of illness, even in instances where overt haemorrhage is not a marked feature of the disease. Fibrinogen levels decline and become very low in fatal cases. The most noteworthy findings in the study by Joubert *et al.* (1985) relate to the grossly abnormal prothrombin ratio (PR), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen, and fibrinogen degradation products (FDP) recorded from an early stage of illness in fatal infection.

Patients have elevated serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (γGT) values early in the disease. AST and ALT values >1000 U/l are associated with fatal outcome. Hyperbilirubinaemia and in some instances jaundice, became evident from day 9 onwards and there is an increase in both total and direct bilirubin readings. Serum creatinine kinase (CK) levels are markedly elevated in the second week of illness (Swanepoel, *et al.*, 1987; 1989). Total serum protein and albumin levels are moderately depressed during the first week of illness, but are elevated from the end of the second week onwards. Irregular increase in blood urea level is observed from day three onwards, and there is a clear tendency for the level to be elevated from the end of the second week (Swanepoel *et al.*, 1987).

1.9.3. Viraemia and antibody response

Viraemia has been detected in patients from the time of onset up to day 13 of illness, with highest titres occurring during the first 5 days, and a maximum recorded intensity of $10^{6.2}$ mouse intracerebral LD_{50} (Swanepoel, 1998). Antibody, both IgG and IgM, become demonstrable by indirect immunofluorescence (IF) from about day 7 of illness, and are present in the sera of all survivors of the disease by day 9 at the latest, while antibody response is generally not demonstrable in fatal cases (Burt, *et al.*,1993; Shepherd *et al.*, 1989c). Antibodies are occasionally demonstrable slightly earlier than day 7, particularly by enzyme-linked immunosorbent assay (ELISA). The IgM antibody activity declines to undetectable levels by the fourth month after infection, and IgG titre may begin to decline gradually at this stage, but remains demonstrable for at least 5 years. Recent or current infection is confirmed by demonstrating seroconversion or a four fold or greater increase in antibody titre in paired serum samples, or IgM antibody activity in a single sample. Although patients with fatal infections often fail to mount a detectable antibody response it should be noted that they sometimes succumb before the seventh day of illness when antibody is first demonstrable in survivors. Among fatal cases in which antibody was detected, some patients had received multiple transfusions of immune plasma and developed moderate levels of IgG antibody, but exhibited nil or weak and transient IgM antibody activity so that it appears unlikely that endogenous antibody response had occurred (Swanepoel *et al.*, 1987).

1.10. Disease in non-human mammals

Although humans can acquire infection with CCHF virus after contact with infected tissues of livestock and wild animals there is no evidence that the virus produces disease in these animals. Antibody surveys among livestock in endemic areas have shown high prevalences among both cattle and sheep (Swanepoel *et al.*, 1987). Experimental studies have confirmed that sheep and cattle develop mild infection (Shepherd *et al.*, 1987b), but they are briefly viraemic and hence can act as a source of infection for man. The danger is increased if animals are slaughtered or die from other causes during CCHF infections and are then butchered. The virus does not survive well in tissues after death and there has been no indication that CCHF constitutes a hazard in meat processed and sold according to normal health regulations (Swanepoel *et al.*, 1987).

Experimental viraemic CCHF infection has been demonstrated in small wild vertebrates that are hosts for the immature stages of the tick vectors, and viral and serological surveys suggest that hares and hedgehogs become infected (Shepherd *et al.*, 1987b).

1.11. Diagnosis

Rapid diagnosis of CCHF infection is important so that appropriate therapy can be instituted quickly, and the patient can be placed in isolation and treated under conditions of barrier-nursing for the protection of health workers and the community at large. Etiologic investigation of the disease is undertaken in maximum security (biosafety level 4, or BSL4) laboratories in countries which have appropriate biosafety regulations and facilities.

Until comparatively recently laboratory diagnosis of the infection in live patients consisted solely of the isolation of the virus from serum or plasma samples taken early in the disease, or demonstration of antibody seroconversion or a four-fold increase in antibody titre in paired samples, or demonstration of IgM antibody activity. However, certain problems are encountered in attempts to achieve a specific diagnosis rapidly by conventional techniques. Most patients that succumb to infection fail to develop an antibody response, and isolation of virus by inoculation of infant mice is generally slow, with incubation periods of 7-9 days (Shepherd *et al.*, 1986). Isolation of virus can be achieved more rapidly by inoculation of cell cultures (1-6 days) if viraemia is intense, but more often virus is present in low concentration and can only be isolated by inoculation of mice which are more sensitive than cell cultures (Shepherd, *et al.*, 1986; Swanepoel *et al.*, 1987).

Problems encountered in the isolation of virus can be overcome by the use of sensitive techniques, such as ELISA, or reversed passive haemagglutination, to detect viral antigens in clinical specimens from CCHF patients (Shepherd *et al.*, 1988). However, more recently rapid diagnosis in the acute stage of the disease has been achieved by detection of viral RNA amplified by reverse transcriptase polymerase chain reaction (RT-PCR) (Schwartz, *et al.*, 1995; Burt *et al.*, 1998).

Among the methods used historically for demonstrating antibody to arthropod-borne viruses CF and

immunodiffusion tests lack sensitivity and are unsuitable for detecting early antibody response, while production of haemagglutinin for HAI tests has proved to be difficult for CCHF virus and the tests lack reproducibility (Casals and Tignor, 1974; Swanepoel *et al.*, 1983b). Reversed passive haemagglutination inhibition has been used for demonstrating antibody to CCHF virus, but the test also lacks reproducibility and amenability to automation, and has not found wide application (Gaidamovich *et al.*, 1974; Swanepoel *et al.*, 1983b). Nairoviruses in general, including CCHF, do not induce strong neutralizing antibody responses, and the sera of many species contain non-specific inhibitors of virus infectivity which complicate neutralization tests (Swanepoel *et al.*, 1983b; Swanepoel, 1998). Moreover, neutralization tests take too long to yield useful results when a rapid diagnosis is required. The IF test can be used to distinguish between IgG and IgM antibodies, and is useful for making rapid diagnoses in individual or small numbers of suspected cases of the disease (Swanepoel *et al.*, 1987; Shepherd *et al,* 1988; Burt *et al.*, 1994), but the reading of the test is subjective, and the technique is not well suited to conducting large serosurveys. These problems were potentially overcome by the development of a solid-phase radioimmunoassay and indirect or sandwich ELISA, and the ELISA has found practical application for the demonstration of antibodies in human sera for diagnostic and survey purposes (Donets *et al.*, 1982; Shepherd *et al.,* 1988; Burt *et al.*, 1994). Moreover, the use of commercially available anti-species immunoglobulin peroxidase conjugates makes it possible to apply the ELISA to serosurveys on livestock sera, while a competitive or blocking ELISA (CELISA) can be used to test sera of species such as ostriches for which no conjugates are available (Burt *et al.*, 1993; Swanepoel *et al.*, 1998).

1.12. Differential diagnosis

The disease should be distinguished from tick-borne typhus (rickettsial infection) which has an incubation period of 7-10 days and more insidious onset than CCHF. Rift Valley fever can also be acquired from contact with the tissue of livestock in Africa, but this usually occurs in the context of a massive epizootic involving abortion and death of sheep and cattle at irregular intervals of years when heavy rains favour the breeding of the mosquito vector of the virus (Swanepoel, 1994). Many other infectious conditions of humans may present as haemorrhagic disease resembling CCHF, including bacterial septicemias, but of special interest are the so-called viral haemorrhagic fever

(VHF) of Africa and Eurasia. They include Marburg and Ebola fever caused by members of the family *Filoviridae*, Lassa fever caused by a virus of the family *Arenaviridae*, haemorrhagic fever with renal syndrome (HFRS) caused by members of the *Hantavirus* genus of the family *Bunyaviridae*. Distinguishing between the various possible causes of suspected cases of VHF is specialised task, normally undertaken in laboratories which are equipped for the purpose (Swanepoel, 1994).

1.13. Recombinant baculovirus expressed antigen

Recombinant polypeptides are being used with increasing frequency as diagnostic antigens and have the advantage that they are non-hazardous, and indeed there have already been attempts to use recombinant antigens for detecting antibodies to CCHF and a related nairovirus, Dugbe, but the CCHF reagents lacked sensitivity and have not found application (Marriott, *et al.*, 1992; Ward *et al.*, 1992). Baculoviruses (of the family *Baculoviridae*) are insect-specific viruses which have become of great interest due to their usefulness as biopesticides and recombinant protein expression vectors. They are widely used as eukaryotic vectors for the expression of a variety of foreign genes. In most cases such vectors have resulted in high levels of protein being produced which, with few exceptions, are correctly processed and biologically active. The expression system is based on replacing the highly expressed viral polyhedrin gene with a recombinant gene consisting of the polyhedrin promoter fused upstream to the desired foreign coding sequence. Since the polyhedrin gene is dispensable for viral replication, the ensuing recombinant virus is fully infectious and at late times of infection the activated polyhedrin promoter directs efficient transcription of the foreign gene (Patel *et al.*, 1991). Expression of the foreign gene is usually driven by the polyhedrin promoter of the autographa californica nuclear polyhedrin virus (AcNPV), which is highly transcribed during the late stages of infection. AcNPV has a large circular double stranded DNA genome with multiple recognition sites for many restriction endonucleases, and as a result, recombinant baculoviruses are traditionally constructed in a two stage process. First, a foreign gene is cloned into a plasmid transfer vector down-stream from a baculovirus promoter and flanked by baculovirus DNA derived from a non-essential locus, usually the polyhedrin gene. Second, this plasmid DNA is introduced into insect cells along with wild-type genomic viral DNA. Typically, 0.1-1% of the resulting progeny are

recombinants with homologous recombination *in vivo*. Recombinant viruses containing the foreign gene inserted into the polyhedrin locus can be identified by an altered plaque morphology, which is characterised by the absence of occluded virus in the nucleus of the infected cells, and the recombinant virus is purified to homogeneity by sequential plaque assay (Luckow *et al.*,1993).

Recombinant antigens are being used more frequently as diagnostic antigens, and it was decided to evaluate the use of a recombinant antigen as a diagnostic tool for CCHF virus infection in human patients, and for serological surveys of domestic livestock and wild animal sera for antibody to the virus. It had previously been shown using monoclonal antibody studies that the nucleocapsid is the most highly conserved viral protein (Blackburn *et al.*, 1987), and is therefore the most useful for diagnostic assays. Hence it was decided to investigate baculovirus systems expressing the CCHF nucleocapsid protein as the antigen of choice.

1.14 Objectives

Due to the biohazardous nature of VHF viruses all diagnostic reagents must be prepared within the confines of BSL4 laboratories, and inactivated and safety tested before they can be used in other laboratories. There are a limited number of BSL4 laboratories worldwide and for this reason reagents that are safe to produce and use would be of great value, not only as diagnostic tools, but also for use in serosurveys and for the testing of livestock and wild animal sera as a prerequisite to the export of live animals. In recent years the SPU has diagnosed cases of the disease or found serological evidence of the presence of the virus in several countries in Africa and in the Middle East, including Iraq, Iran, Afghanistan, and Pakistan (unpublished laboratory records). Few, if any, of these countries can afford to establish BSL4 laboratories of their own, and the SPU has received several requests from health authorities in these countries, and the World Health Organization in Geneva, to be of assistance in developing safe diagnostic procedures which can be used in laboratories which are less sophisticated than BSL4 laboratories, but which could nevertheless provide adequate protection for laboratory personnel. Hence, it was decided to explore the use of recombinant antigen for the diagnosis of CCHF virus infection in human patients, and for the screening of livestock and wild animal sera for antibody to the virus. For purposes of determining the sensitivity and specificity of tests for antibody to CCHF virus using recombinant antigen, the tests using sucrose acetone antigen were regarded as

the gold standard. A previous publication investigating several methods for demonstrating antibody to CCHF virus in serum samples from confirmed patients showed that the ELISA was a sensitive test for detecting an antibody response (Burt *et al.*, 1994). It is extremely difficult to provide the perfect cohort of specimens with an infection such as CCHF in humans. The number of infections annually is very small, and the specimens submitted from patients is limited, hence the evaluation is based on availability of specimens. Statistically significant numbers are unrealistic in a laboratory dependent on natural infections of CCHF in humans and experimental infection of sheep and mammals with a highly infectious agent such as CCHF. For experimental infections the animals must be confined for the duration of the experiment and hence logistics do not allow large numbers to be of these animals to be analysed.

The SPU is in a particularly favourable position to conduct research on assays for antibody to CCHF virus because it has a unique collection of serum samples derived from confirmed CCHF patients, as well as from experimentally infected livestock and small mammals. For this reason, BSL4 laboratories abroad which had produced candidate recombinant CCHF antigens asked the SPU to evaluate the antigens: the Virology Division, United States Army Medical Research Institute for Infectious Diseases (USAMRIID), Fort Detrick, Maryland, USA, supplied a baculovirus recombinant expressing an insert from the S segment of the CCHF genome coding for the nucleocapsid protein, while the Special Pathogens Branch, Centres for Disease Control (CDC), Atlanta, Georgia, USA, supplied three recombinant nucleoprotein antigens. The initial purpose of the present study, therefore, was to evaluate the reagents received from abroad, but in case none of them proved to be satisfactory it was decided to attempt production of further recombinant antigen at the same time. Consequently, the study comprised:

1. Expression of the nucleoprotein of CCHF virus as a recombinant for use as a non-hazardous diagnostic reagent.

2. Development of IgG and IgM ELISA for the serodiagnosis of CCHF infection, using recombinant antigens.

3. Evaluation of ELISA for detection of IgG and IgM antibody to CCHF virus using stored serum samples from confirmed CCHF patients and experimentally infected small mammals and livestock.

2.0 Materials and methods

2.1. Recombinant reagents

A baculovirus transfer plasmid, designated CCHF pBlueBac, containing a 1510 base pair (bp) segment of the CCHF S segment inserted at an Nhe1 restriction site was supplied as a glycerol stock of INV alphaF' *Escherichia coli* by Dr J. Smith of the Virology Division, United States Army Medical Research Institute for Infectious Diseases (USAMRIID).

A recombinant baculovirus, designated BacCCHF1, expressing the nucleocapsid protein of CCHF virus was also supplied by Dr Smith of USAMRIID. The CCHF nucleocapsid protein expression and purification is described below in Section 2. Autographa californica nuclear polyhedrosis virus (AcNPV) was used as the control baculovirus.

Three CCHF virus recombinant antigens were supplied by Dr S. Nichol, Special Pathogens Branch, Centres for Disease Control (CDC), Atlanta, Georgia. The antigens had been characterised but had not been evaluated because of lack of suitable reagents, hence they were donated as a gift for evaluation in ELISA for detection of specific antibody using clinical sera from confirmed CCHF patients. The three recombinant antigens supplied were as follows:

1. a full length CCHF nucleocapsid protein that was expressed in *E. coli* M15 (Qiagen, Hilden, Germany),

2. a full length CCHF nucleocapsid protein that was expressed in *E. coli* SG 13009 (Qiagen),

3. a 63 amino acid fragment of nucleocapsid protein coded by nucleotides 689-879 of the S segment of the genome of CCHF strain 10200, that was expressed in *E.coli*. The nucleotide was expressed behind dihydrofolate reductase carrier protein to protect the peptide from degradation in *E. coli*.

2.2. Antisera and antigens

Monoclonal antibody, 5G2, specific for viral nucleocapsid protein was used as a coating antibody in the ELISA (Blackburn *et al.*,1987). The antibody was stored freeze dried at -70°C. Anti-CCHF hyperimmune mouse ascitic fluid (HMAF) previously prepared for unrelated studies was stored freeze dried at -70^0 C and used as a detection antibody in the IgM-capture ELISA.

Guinea pig immune sera from animals hyperimmunised against Dugbe, Hazara and Nairobi sheep disease viruses were previously prepared for unrelated studies and stored freeze dried at -70°C. These immune sera were used to determine the antigenic specificity of the recombinant antigen.

Sucrose acetone extracted antigen was previously prepared for unrelated studies from suckling mouse brain infected with a South African human CCHF isolate 4/81 (Swanepoel *et al.*,1983a), inactivated with 0.1% beta-propriolactone (Clarke *et al.*,1958) and stored freeze dried at -70°C. Control antigen was prepared from non-infected mouse brain.

Anti-CCHF horseradish peroxidase (HRPO) conjugate was prepared by the method of Wilson and Nakane (1978) from serum which had been collected from rabbits hyperimmunised with CCHF virus for an unrelated project, and stored at -70^{0}C. The immunoglobulin fraction was isolated from pooled immune rabbit serum by ammonium sulphate precipitation (31%, pH 7.4) of the IgG fraction. The serum was diluted 1:2 with distilled water, 31% ammonium sulphate was added and the solution left to stand for 30 minutes at room temperature. The solution was centrifuged for 30 minutes at 5000x g in a high speed Beckman centrifuge, model J2-21, rotor JA 21 (Beckman, California, USA), the supernatant discarded and the precipitate dialysed against carbonate buffer, pH 9.6, for seven days at 4°C. Four micrograms of HRPO Sigma type IV (Sigma Chemical Company, St. Louis, USA) was dissolved in 1 ml of distilled water. A 200μl aliquot of freshly prepared 0.1 M sodium periodate (Sigma) was added and incubated at room temperature for 30 minutes. The HRPO-aldehyde solution was dialysed against 1 mM sodium acetate, pH 4.4, overnight at 4°C. Sufficient carbonate buffer was added to increase the pH of the solution to 9.5. The pH was determined using Merck Neutralit pH paper, range of pH 5-10 (Merck, Frankfurter, Darmstedt, Germany). A 1 ml aliquot of purified rabbit

immunoglobulin (previously dialysed against carbonate buffer) was immediately added and left at room temperature for 2 hours. The solution was dialysed against phosphate buffered saline (PBS) pH 7.4 (Appendix A), for 7 days and the anti-CCHF HRPO conjugate was stored at -70^{0}C.

2.3. Serum samples

A total of 295 human sera tested in the study included: 135 human serum samples from cases of CCHF diagnosed between 1981 and 1999 in SPU, collected at various intervals from the day of illness as shown in Figure 1, and 160 sera from non-CCHF patients submitted to the Special Pathogens Unit that had previously been tested routinely on submission to the laboratory for antibody activity to CCHF and virus isolation attempts were performed by inoculation of suckling mice and cell culture. No evidence of CCHF infection was obtained in any of the sera from non-CCHF patients. All serum samples were stored at -70^0 C. The sera were tested by ELISA for IgG and IgM antibody and by competitve ELISA (CELISA) for total antibody activity to CCHF virus using both a sucrose acetone extracted antigen and a recombinant antigen as described below.

A total of 271 ostrich serum samples were included in the study. The ostrich sera were submitted to SPU from 1981 to 1999 to test for antibody to CCHF virus as one of the requirements for permission to export the ostriches. The sera were stored at -70°C. Ostrich serum samples were tested for antibody activity to CCHF virus by CELISA using each antigen to evaluate the recombinant CCHFantigen in the ELISA.

A total of 63 sera from 3 experimentally infected sheep were tested by ELISA for IgG and IgM antibody to CCHF virus. The sheep had been experimentally infected for an unrelated study and sampled daily from day 0, the day of inoculation with CCHF virus until day twenty-eight, and monthly from month four to month eight. Samples were collected on day 0, prior to inoculation, in order to be used as controls and provide baseline results for each individual sheep. The sera were stored at -70°C.

Total antibody screening was performed on 35 sera from experimentally infected small mammals.

The serum samples were collected from the following mammals: *Mystromys albicaudatus* (White-tailed rat), *Tatera brantsii* (Bushveld gerbil), *Tatera leucogaster* (Highveld gerbil) and *Aethomys namaqueisis* (Namaqua rock mouse) on days after inoculation as indicated in the results section. The mammals were experimentally infected with CCHF virus for an unrelated study and the serum samples were stored at -70°C.

All serum samples listed in Section 2.3 were collected for unrelated studies and were stored wet at -70°C.

2.4. Preparation of CCHF baculovirus recombinant

An attempt to prepare a recombinant baculovirus system for expression of the CCHF nucleocapsid protein was performed using the Bac-To-Bac Baculovirus Expression System (Bac-To-Bac™ Baculovirus Expression System, GibcoBRL, Paisley, UK) according the manufacturer's protocol. The 1510 bp region of the CCHF S segment coding for the nucleocapsid protein excised from the pBluebac baculovirus transfer plasmid by digestion with Nhe I (GibcoBRL) restriction enzyme was cloned into a pFastBac 1 (GibcoBRL) donor plasmid. A restriction map illustrating the pBluebac plasmid is provided in Appendix A.

2.4.1. Isolation of S segment of CCHF virus genome from pBluebac plasmid

The CCHF pBlueBac plasmid was initially grown in broth to obtain a large volume from which the DNA was purified to remove broth constituents such as salts and concentrated to a volume suitable for digestion with the restriction enzyme to obtain the CCHF insert. The plasmid was grown by inoculation of 5 ml Luria-Bertani (LB) broth (Appendix A) containing 100 μg/ml ampicillin, incubated overnight at 37 °C and the following day a 0.5 ml aliquot was inoculated into 150 ml of LB broth containing 100 μg/ml ampicillin and incubated overnight at 37 °C. The cells were harvested by centrifugation of the suspension at in a high speed centrifuge 3000x g (Beckman) for 30 min and lysed in 8 ml of lysosome solution (Appendix A) on ice for 30 minutes. Two volumes of 0.2M

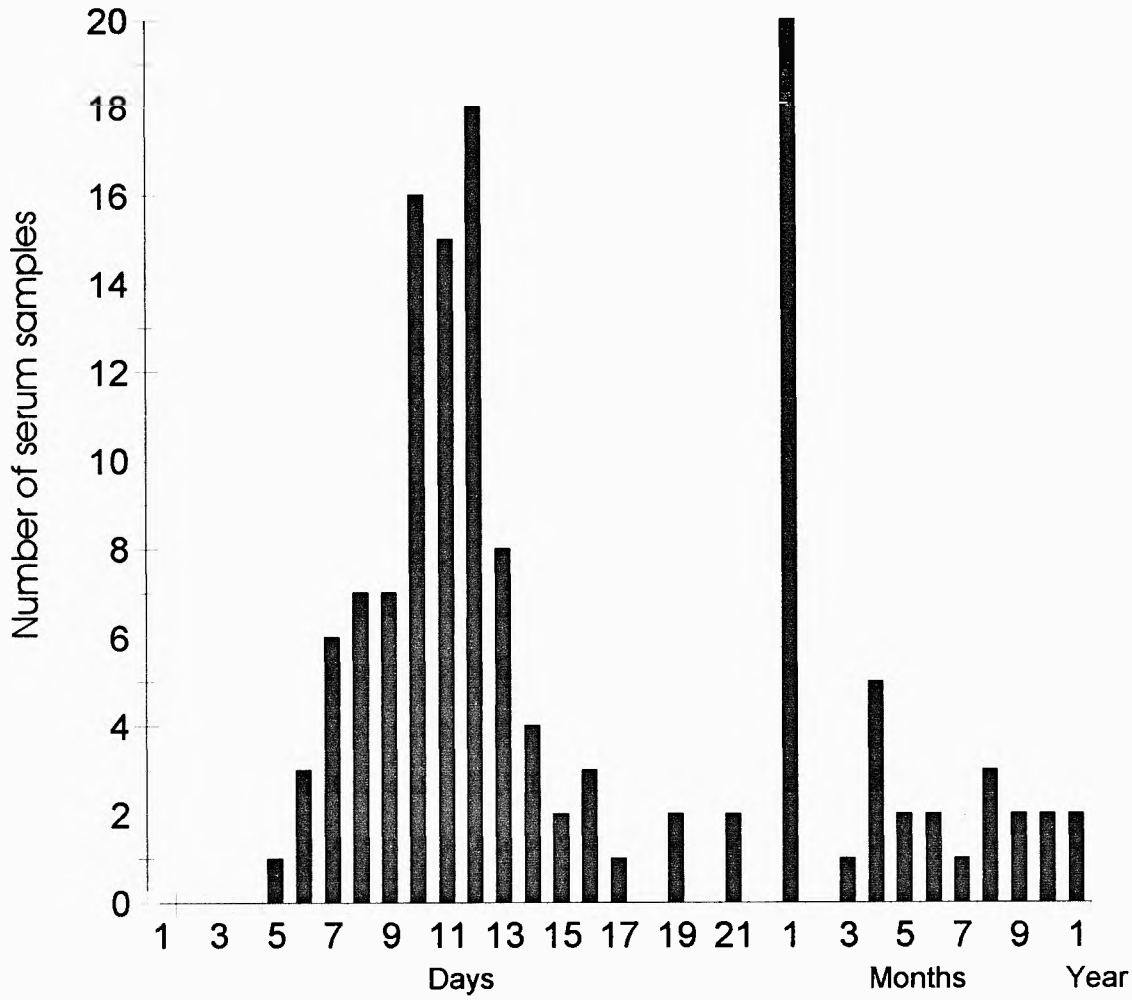


Figure 1. Histogram showing the number of serum samples included in the study which has been collected from CCHF virus infected patients from 1986 to 1999 at the indicated interval after the onset of illness.

sodium hydroxide containing 1% SDS were added to the lysed cells and kept on ice for 5 minutes. A 12 ml aliquot of 3M sodium acetate, pH 4.8, was added and the mixture held for a further 30 minutes on ice. Aliquots of the cell suspension were centrifuged for 10 minutes at 10000x g (Beckman) and nucleic acid was precipitated from supernatant fluid using 2.5 volumes of 96% ethanol and incubated at -70 °C for 1 hour. The precipitate was resuspended in 12 ml of Tris-EDTA (TE) buffer, pH 8.0 (Appendix A) and clarified as previously described. The DNA was precipitated by adding 5 ml of 7.5 M ammonium acetate and holding for 30 minutes on ice. The solution was centrifuged as above at 10000x g for 10 minutes and the DNA precipitated from the supernatant fluid with 2.5 volumes of ethanol at -70°C for 1 hour. The precipitate was washed with 80% ethanol, followed with a wash in absolute alcohol, dried and resuspended in 1 ml of TE buffer. The DNA was quantitated spectrophotometrically assuming that an optical density reading of 1 at a wavelength of 260 nm correspond to approximately 50μg/ml for double stranded DNA. The DNA concentration was then adjusted to contain 1μg DNA in a volume of 20 μl (Maniatis, 1982). A 20 μl aliquot of purified plasmid which contained the CCHF insert was digested with Nhe I (10 units/μl) enzyme (GibcoBRL). The following volumes were added into the microcentrifuge tube for digestion : 20μl of purified plasmid, 3μl of 10X enzyme buffer, 2μl of Nhe I enzyme and 5μl of water. The reaction was incubated for 1 hour at 37°C waterbath. A 70μl aliquot of water was added to make up the volume to 100μl. A further 100μl each of TRIS saturated phenol and chloroform were added and centrifuged at 10000x g for 10 minutes. The aqueous layer was precipitated with 1.5 volumes of isopropanol and incubated at -20°C for 1 hour. Centrifugation was carried out at 10000x g in an Eppendorf Microcentirfuge (Sigma-Aldrich, EschestraBe, Germany) for 15 minutes, the pellet dried and resuspended in 10μl of water. The DNA was then separated by electrophoresis using a 0.8% agarose gel (FMC Bioproducts, Rocklands, ME, USA) prepared in TAE buffer (Appendix A) containing 0.5 μg/ml ethidium bromide. The CCHF segment (1510 bp) was excised from the gel into a microcentrifuge tube and 400μl of TAE buffer added. The tube was heated for 5 minutes at 65°C. A 400μl aliquot of TRIS saturated phenol was added and centrifuged as before in a microfuge (Sigma). A further 400μl aliquot of chloroform and 400μl aliquot of phenol was added to the aqueous layer and centrifuged as above. The aqueous layer was precipitated in 2.5 volumes of ethanol and incubated at -20°C for 1 hour. The pellet was dried and resuspended in 10μl of water.

2.4.2. Cloning of CCHF virus insert into pFASTBAC 1 donor plasmid

The pFASTBAC 1 expression vector (GibcoBRL) was digested with Xba I enzyme (12 units/μl)(GibcoBRL). The following volumes were added into the microcentrifuge tube for digestion: 2μl of purified vector DNA, 1μl of 10X enzyme buffer, 1μl of Xba I enzyme and 6μl of water. The reaction mixture was incubated for 1 hour in 37° C. A 90μl aliquot of water was added to make up the volume to 100μl. A further 100μl of TRIS saturated phenol and chloroform was added to the tube and centrifuged at 10000x g in a microfuge (Sigma) for 10 minutes. The aqueous layer was precipitated with 1.5 volume of isopropanol and incubated at -20° C for 1 hour. Centrifugation was then carried out as above , the pellet was dried and resuspended in 10μl of 10 mM TRIS-HCl, pH 8.0. To dephosphorylate the vector, a 5 μl aliquot of the digested and purified DNA vector was added to 5 μl of 10X calf intestinal alkaline phosphatase (CIP) buffer (Appendix A), 48 μl of water and 2 μl of CIP. After 30 minutes of incubation at 37°C, a further 2 μl of CIP was added and the tube incubated for 30 minutes at 37°C. A 40 μl aliquot of water, 10 μl of STE buffer (Appendix A) and 5 μl of 10% SDS were added to the tube. The tube was then heated to 68^0 C for 15 minutes. The DNA was then extracted twice with phenol/chloroform and twice with chloroform. The DNA was precipitated with ethanol and ready for ligation. To ligate the insert and the vector, a 5μl aliquot of DNA (insert recovered from the gel) and 3μl aliquot of pFASTBAC 1 vector (that was dephosphorylated) were added in a microcentrifuge tube and warmed for 5 minutes at 45^{0}C to melt the cohesive termini that has reannealed. The tube was then chilled on ice. A 1μl aliquot of T4 DNA Ligase (Roche Diagnostics, Manheim, Germany) and 1μl of 10X ligase buffer was added to the microcentrifuge tube. The reaction mixture was incubated for 16 hours at 4°C. The DNA was then ready to be used for transformation.

2.4.3. Transformation of DH5$\propto$ competent cells

The DH5$\propto$ competent cells (GibcoBRL) and pUC19 DNA (GibcoBRL) were stored at -70^{0}C. The competent cells was removed from -70^{0}C and thawed on wet ice. The cells were gently mixed, then 100 μl of competent cells were aliquoted into the chilled microcentrifuge tubes. A 5 μl aliquot of control pUC19 (GibcoBRL) was added to one tube containing 100 μl of competent cells to determine the transformation efficiency. The pipette was moved through the cells while dispensing and the tube

tapped to mix. A 3 μl aliquot of the DNA ligation reaction was added to the second tube containing 100 μl of competent cells, moving the pipette through while dispensing and the tube tapped to mix. The mixture was incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 45 seconds. The cells were then placed on ice for 2 minutes. A 0.95 ml aliquot of S.O.C. medium (Appendix A) was added and incubated for 1 hour at 37°C with shaking for expression. After expression, 0.1 ml of the reaction containing the control pUC19 was diluted with 0.9 ml medium. A 100μl aliquots of the undiluted reaction and the 1/10 dilution were spread on Luria Bertani (LB) plates (Appendix A) with 100 μg/ml ampicillin and 50 μg/ml 5-brom-4-chlor-3-indolyl-β-D-galactopyranosid (X-gal). The experimental reaction was diluted as was done with the control pUC19 cells (outlined above) and 100μl of cells spread on the agar plates as the control. The plates were incubated for 48 hours at 37°C. To confirm that the white colonies were truly white, they were selected and streaked on a plate and incubated as before. The confirmed white colonies (transformed DH5∝ cells) were picked and grown by inoculation of 5 ml LB broth containing 100 μg/ml of ampicillin and DNA was isolated as outlined in section 2.4.1. above. To confirm the presence of transformed DNA in the preparation, the isolated DNA was analysed by electrophoresis using a 0,8% agarose gel as described in Section 2.4.1. and a DNA band of approximately 20 000 base pairs confirms the presence of the transformed DNA in the preparation.

Amplification by polymerase chain reaction (PCR) using primers specific for a region of the CCHF S segment was performed to confirm the presence of the CCHF insert. The primer pair designated F_2(5' ACACCTTCACAAACTC 3') and R_3 (5' GACAAATTCCCGCACCA 3') have previously been described and amplify a 536 base pair region of the nucleocapsid gene (Burt *et al.*, 1995). Briefly a 3 μl aliquot of template was amplified in a tube containing the following reaction mix: 5μl of 10X PCR buffer, 1μl of 10mM dNTP mix (10mM each dATP, dTTP, dCTP, dGTP) (Roche Diagnostics), 1.5μl 50 mM $MgCl_2$, 2.5μl of primer mix (1.25μl each 10μM stock) and 0.5μl Taq Polymerase (2.5 U)(Promega Corporation, Madison, WI, USA). Distilled water was added to final volume of 50μl. The content of the tube was mixed by gentle tapping of the tube. After incubation at 95°C for 3 minutes, 30 cycles of PCR were performed as follows: 94°C for 30 seconds, 47°C for 30 seconds and 68°C for 3 minutes. A final extension of 68°C for 7 minutes. The PCR product was visualised

by electrophoresis using a 0.5% agarose gel (FMC Bioproducts) in TAE (Appendix A) buffer containing 0.5 μg/ml ethidium bromide. A positive was control was prepared using CCHF RNA extracted from infected tissue culture (stored in the Special Pathogens Unit). The RNA was reverse transcribed to cDNA using the CCHF primer F2, M-MLV Reverse Transcriptase, Superscript (Gibco BRL) and the following conditions: 1 pmol F2 primer, 5 μl RNA template made up to 10μl with water and heated at 95 °C for 3 min, cool 42 °C for 3 min. To the tube the following were added, 1 μl RNasin (Roche Diagnostics), 4 μl 5x buffer (Gibco BRL), 2 μl of 10mm dNTP mix (Roche Diagnostics), 2 μl 0.1 M dithiothreital (GibcoBRL) 1 μl M-MLV reverse transcriptase (GibcoBRL). The tube was mixed, incubated for 30 min at 42 °C, heated for 3 min 95 °C and the reaction quenched on ice for 3 min. The cDNA was amplified using PCR as described above, 1 μl of template was used in the PCR reaction.

2.4.4. Transformation of DH10Bac competent cells

The DH10Bac competent cells (GibcoBRL) were stored at -70°C. The cells were removed from the freezer and thawed on wet ice. The cells were gently mixed, then 100μl of competent cells added into the chilled polypropylene tubes. A 5μl aliquot of pUC19 control DNA (GibcoBRL) was added to 100 μl of competent cells to determine the transformation efficiency. To determine the transposition efficiency 5μl of pFASTBAC-gus control DNA (GibcoBRL) was added to 100 μl of competent cells. The cells were incubated on ice for 30 minutes. Without shaking the cells were heat shocked at 42°C for 45 seconds. The cells were then placed on ice for 2 minutes. A 0.9 ml aliquot of S.O.C. medium (Appendix A) was then added. Tubes from the ligation reaction and control were incubated at 37°C with shaking for 4 hours. The reaction containing the pFASTBAC-gus control DNA was diluted at 1/10, 1/100 and 1/1000 with S.O.C. medium and 100μl of each dilution were spread on agar plates that contain 50μg/ml of kanamycin sulphate, 10μg/ml of tetracycline, 7μg/ml of gentamycin, 100μg/ml of X-gal and 40 μg/ml of IPTG. The transformation with the pUC19 control DNA was diluted at 1/10, 1/100 and 1/1000 and plated on agar with 100μg/ml ampicillin. The experimental reactions were diluted as above and 100μl spread on agar that contain 50μg/ml of kanamycin sulphate, 10μg/ml of tetracycline, 7μg/ml of gentamycin, 100μg/ml of X-gal and 40μg/ml of IPTG. The plates were incubated at 37° C for 48 hours.

White colonies were selected from the plate for isolation of recombinant bacmid DNA. Before isolating bacmid DNA, candidate colonies were re-streaked on a new plate to ensure that they are truly white. Using a sterile toothpick, a single isolated bacterial colony was inoculated into 2 ml LB medium supplemented with 50μg/ml kanamycin, 7μg/ml gentamycin and 10μg/ml tetracycline . The cells were grown at 37°C for 24 hours with shaking. A 1.5 ml aliquot of culture was transferred to a tube and centrifuged at 14000x g for 1 minute. The supernatant was removed by aspiration and resuspended by gently pipetting up and down in 0.3 ml of Solution I (Appendix A). A 0.3 ml aliquot of Solution II (Appendix A) was added and gently mixed. The tube was incubated at room temperature for 5 minutes. A 0.3 ml aliquot of 3 M potassium acetate, pH 5.5, was slowly added with gentle mixing during addition. The sample was placed on ice for 10 minutes and centrifuged at 14000x g for 10 minutes. The supernatant was transferred into another microcentrifuge tube containing 0.8 ml of isopropanol. The tube was mixed gently ,placed on ice for 10 minutes, centrifuged at 14000x g in the Beckman high speed centrifuge for 15 minutes and the supernatant discarded. The pellet was washed with 0.5 ml of 70% ethanol. After washing and spinning, the supernatant was discarded and the pellet air dried and resuspended in 40μl of TE. The DNA was stored at -20°C.

2.4.5. Analysis of bacmid DNA

To confirm the presence of bacmid DNA a 5μl aliquot of the Bacmid mini-prep (isolated in section 2.4.4.) was separated by electrophoresis using a 0.5% agarose (FMC Bioproducts) gel in TAE buffer containing 0.5μg/ml ethidium bromide. The size of the bacmid DNA is approximately 23000 bp.

Verification of the insertion of the CCHF gene was done by PCR using pUC/M13 amplification primers. To each tube, the following were added: 5μl of 10X PCR buffer, 1μl of 10mM dNTP mix (10mM each dATP, dTTP, dCTP, dGTP), 1.5μl 50 mM $MgCl_2$, 2.5μl of primer mix (1.25μl each 10μM stock), 1μl template DNA and 0.5μl Taq Polymerase (2.5 U)(Promega Corporation, Madison, WI, USA). Distilled water was added to final volume of 50μl. The content of the tube was mixed by gentle tapping of the tube. After incubation at 95°C for 3 minutes, 35 cycles of PCR were performed as follows: 94°C for 45 seconds, 55°C for 45 seconds and 68°C for 3 minutes. PCR products were

analysed by electrophoresis using a 0.7% agarose (FMC Bioproducts) gel in TAE buffer containing 0.5μg/ml ethidium bromide. The predicted PCR products are as follows: the bacmid only is approximately 300 bp and the transformed bacmid is approximately 2300 bp. Each step of the protocol had been controlled as recommended by the manufacturers of the Bac-to-Bac system, troubleshooting had been performed as suggested, however after numerous attempts the experiment was discontinued at this point as a positive PCR indicating that the bacmid was transformed was not demonstrable.

2.5. Expression and purification of recombinant antigen

2.5.1. Inoculation of cell cultures

The Sf9 cells [(Fall Armyworm Ovary, *Spodoptera frugiperda*) cells obtained from the American Type Culture Collection (ATCC) CRL 1711] were grown in T_{25} tissue culture flasks with 5 ml Grace's medium (Sigma Chemical Company, St.Louis, USA) containing 10% foetal calf serum (FCS), fungizone 20 mg/ml and gentamycin 10 mg/ml, until the cells were confluent. Tissue culture flasks were inoculated with either 1 ml of recombinant baculovirus BacCCHF1 expressing the N-protein of CCHF virus, or with 1 ml of AcNPV as a control. The inoculum was absorbed for 1 hour at 28^0 C. After 1 hour of incubation with regular shaking at room temperature, the inoculum was discarded and 5 ml of Grace's medium without FCS was added to the flasks and incubated at 28 °C for 6 days.

2.5.2. Detection of *in vitro* expression of CCHF nucleoprotein

Two methods were employed to show that the CCHF nucleoprotein was expressed from the baculovirus in cell culture: immunoflourescence was performed on infected cells to show expression of protein and a Western blot was performed to confirm that the protein expressed reacted with mouse anti-CCHF serum and that the size of the protein expressed was that predicted for CCHF nucleoprotein.

2.5.2.1. Immunofluoresence tests

Sf9 cells were inoculated on day 1 with baculovirus BacCCHF-1 as described in section 2.5.1. On

day 2 after inoculation and on each subsequent day, cells were scraped from the tissue culture flask using a pasture pipette and approximately 0.5 ml of medium containing the scraped cells were transferred into a centrifuge tube. Cells were scraped from flasks infected with the wild type AcNPV and used to prepare control slides for immunofluorescence. The tubes was centrifuged for 5 minutes at 3000x g. The medium was discarded and the cells resuspended in 100μl of 2% foetal calf/saline. Ten microlitres of the cell suspension was applied to each well of 8-well multi-test slides (Flow Laboratories, Irvine, UK), dried for 20 minutes at 37^{0}C and fixed in cold acetone for 20 minutes. For the IF test, 10μl of anti-CCHF HMAF diluted 1/10 in PBS was applied to each well of a slide. The slides were incubated in a moist chamber at 37^{0}C for 20 minutes. The slides were then washed for 3 minutes in PBS and for 1 min in water and then dried. Ten microlitres of flourescencein-labelled anti-mouse IgG conjugate diluted 1/40 with PBS containing Evans blue as a counterstain were applied to each well and the slides were incubated at 37^{0}C for 20 min in a moist chamber. The slides were washed as previous, dried, mounted with glycerol mounting fluid and coverslipped. The slides were read using a Nikon ultraviolet light microscope (Nikon Inc. Instrumental Group, Garden City, USA) and photographed. The cells were monitored daily until 80% infectivity was achieved. The cells were then harvested for protein purification.

2.5.2.2. Western blot

Sf9 cells infected with AcNPV and BacCCHF-1 were propagated in T_{25} tissue culture flasks for 6 days. Antigen spot slides were performed as described in section 2.5.2.1. to confirm expression of CCHF protein from BacCCHF-1 infected cells. Protein was purified from the Sf9 cells as described in section 2.5.3. below and cell lysates stored at -70° C. Prior to use, 50μl of each sample were boiled in 50μl of 1X SDS electrophoresis loading buffer (Maniatis *et al.*,1982)(Appendix A) for 5 minutes at 100^0 C and proteins resolved using SDS-polyacrylamide gel electrophoresis methods. Polyacrylamide gel electrophoresis was performed in 1.5 mm thick slab gels using a 4% stacking gel and a 10% resolving gel in Tris-glycine buffer system overnight (Appendix A). Protein molecular weight determinations were made by comparison of bands with a known protein molecular weight marker (Roche Diagnostics).

After electrophoresis was completed, the gel was equilibrated for 10 minutes in transfer buffer (Appendix A). A polyvinylidene diflouride (PVDF)(Roche Diagnostics) membrane was soaked in methanol for few seconds and then submerged in transfer buffer (Appendix A) for 3 minutes. Proteins were transferred onto the PVDF membrane using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Biorad, California, USA) for 4 hours at 40 volts. The gels were stained with Coomasie blue (Appendix A) after transfer to confirm that the proteins had been transferred to the membrane. Initially the gels were stained after. To visualise the protein molecular weight marker, the lane on the membrane in which the molecular weight marker was run, was cut off and stained in Ponceau S solution (Appendix A). After visualizing by staining, the protein bands were marked for permanent record. CCHF specific proteins were reacted with anti-CCHF HMAF as the primary antibody and detected using the BM Chromogenic Western Blotting Kit (Roche Diagnostics) according to accompanying instructions. Briefly, the membrane was washed twice with in Tris-buffered saline (TBS)(Appendix A) and non-specific binding of antibody was blocked by incubating the membrane for 30 minutes in 1% blocking solution (Appendix A). The membrane was then incubated with the primary polyclonal antibody, anti-CCHF HMAF fluid, diluted 1/1000 in blocking solution. Following overnight incubation, the membrane was washed 4 times in TBS buffer containing 0.1% Tween 20 for 10 minutes. Antibody was detected using anti-mouse IgG alkaline phosphatase diluted to 800 mU/ml in 1% blocking solution. After 3 hours incubation, the membrane was washed 4 times for 15 minutes as before. Finally, the colour reaction was initiated by the addition of NBT/X phosphate substrate and the membrane incubated at room temperature with no agitation.

2.5.3. Purification of expressed proteins

The infected Sf9 cells grown in T_{25} tissue culture flask were harvested on day7 when the infectivity was approximately 80%, as determined by daily IF tests, centrifuged at 3000x g for 10 minutes and the supernatant stored at -20^0 C for use as inoculum. A crude cell lysate technique is a suitable method for purifying the antigen. The cells were lysed in 2.5 ml of NET/BSA (Appendix A), incubated on ice for 30 minutes and centrifuged at 3000x g for 10 minutes. To the cytosol fraction 0.1% of SDS was added and the mix sonicated for 10 minutes. To the nuclear fraction, 2.5 ml of NET/BSA containing 0.1% of SDS was added and the mix sonicated for 10 minutes. For the

following study the cytosol fraction of the protein was used and the nuclear fraction discarded because it formed insoluble pellets. The recombinant protein was aliquoted and stored at -20°C. For the development of the ELISA the optimal dilution of antigen is determined by checkerboard titrations which must be performed for each batch of antigen prepared.

2.6. Enzyme-linked immunoassay

The ELISA were performed in 96 well immunoassay plates (Maxisorp, Nunc, Roskilde, Denmark), and unless otherwise stated reagent volumes of 100μl were used, the diluent for reagents was PBS containing 2% skimmed milk, incubations were performed for 1 hour at 37°C, wells were post-coated with 200μl PBS containing 10% skimmed milk and plates were washed thrice with PBS containing 0.1% Tween 20. The optimal working dilution of reagents, including the sucrose acetone extracted antigen, were determined by chessboard titration.

2.6.1. Evaluation of recombinant antigens

Recombinant antigens, which included the nucleocapsid protein expressed by the BacCCHF1 and the three recombinant proteins supplied by CDC, were evaluated using a sandwich ELISA. The plates were coated overnight at 4° C with monoclonal antibody 5G2 diluted 1/2000 in PBS. After post-coating, each recombinant antigen was added to duplicate wells in 2-fold dilutions from 1/50, the plates were incubated, washed and reference positive test serum and negative control serum were added to the replicate wells at a dilution of 1/100. The plates were incubated, washed and anti-human IgG HRPO conjugate (Zymed Laboratories Inc., San Franscisco, CA.USA) diluted 1/2000 was added, and the plates incubated. After further washing, the substrate, azino-di-(3-ethyl-benzthiazoline-6-sulfonate) (ABTS) (Kirkgaard and Perry Laboratories, Gaithersburg, Md, USA) was added and the plates incubated at room temperature (22°C) for 30 minutes in the dark. The results were determined by reading the optical density (OD) at 402 nm on a Multiscan spectrophotometer (Flow Laboratories Inc., McLean, VA, USA). A positive result was recorded for OD values greater than twice the OD of the known negative serum at a dilution of 1/100. Optimal dilutions of antigen for use in the routine ELISA were determined in chessboard titrations and recorded as the highest dilutions of antigen which produced a positive result with positive control serum, without a non-specific reaction with

negative control serum (OD <0.200 with negative serum).

2.6.2. IgM-capture ELISA

Plates were coated overnight at 4°C with μ-chain specific anti-human or anti-sheep IgM (Zymed Laboratories) diluted 1/1000 in PBS. After the plates were washed, human or sheep test sera were added to duplicate wells in 4-fold dilutions from 1/100 upwards. The plates were incubated, washed, and test or negative control antigen added at optimal dilution (1/800 for sucrose acetone test and negative control antigens, or 1/50 for recombinant antigen) to the replicate wells. After incubation, the plate was washed, anti-CCHF HMAF diluted 1/1000 added to the wells and the plates were incubated. After incubation and washing, anti-mouse HRPO conjugate (Zymed Laboratories), diluted 1/2000, was added to the wells and the plates were incubated. After further washing, the substrate, ABTS, was added and the plates incubated at room temperature (22°C) for 30 minutes in the dark, and OD recorded as above. Net OD values were calculated by subtracting the OD reading with control antigen for each serum dilution from the OD reading with test antigen. Net OD values of ≥0.400 recorded as positive reactions, and the antibody titre of a serum was recorded as the reciprocal of the highest dilution producing a positive reaction. From statistical analysis of the results of tests on large numbers of sera at CDC and in the SPU it has been established that only sera with titres of ≥400 can be interpreted as positive for antibody to CCHF virus (unpublished laboratory records). Reactions which occur only at the lowest 4-fold dilution of serum tested, 1/100, are rated as equivocal.

2.6.3. IgG sandwich ELISA

The presence of IgG antibody was demonstrated by sandwich ELISA. Plates were coated overnight at 4°C with monoclonal antibody 5G2 diluted 1/2000 in PBS. After post-coating, test or negative control antigen added at optimal dilution (1/1000 for sucrose acetone test and negative control antigens, or 1/100 for recombinant antigen) to replicate wells. The plates were incubated, washed and human or sheep test sera were added in 4-fold dilutions from 1/100 upwards to test and control antigen wells. The plates were incubated, washed and anti-human or anti-sheep IgG HRPO conjugate (Zymed Laboratories) was added at a dilution of 1/2000. After further incubation and washing, ABTS substrate was added, allowed to react and results recorded and interpreted as above.

2.6.4. Competitive ELISA

Total antibody activity in human, ostrich and small mammal sera was determined in a competitive ELISA (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, 5G2, diluted 1/8000. After post-coating, 50μl of test serum was added to duplicate wells in doubling dilutions from 1/10 upwards, along with 50μl of test or negative control antigen added at optimal dilution (1/400 for sucrose acetone test and negative control antigens, or 1/50 for recombinant antigen). The plates were incubated for 3 hours at 37°C with mixing at 30 minutes intervals. After washing, anti-CCHF HRPO conjugate (prepared as described in section 2.2) was added to the wells at a dilution of 1/1000, the plates were incubated, washed, and reactions detected with ABTS substrate as described above. The CELISA produces much lower titres than the IgM-capture or the IgG sandwich ELISA, and the sera were recorded as positive if the optical density reading was ≤50% of that produced by negative control serum at the lowest dilution tested, 1/10.

2.6.5 Calculation of sensitivity and specificity of serological tests with recombinant antigen

For purposes of calculating the sensitivity and specificity of tests for antibody to CCHF virus using recombinant antigen, the tests using sucrose acetone antigen were regarded as reference techniques which produced true positive and true negative results. By comparison, the results produced for the same samples in tests with recombinant antigen were rated as true or false positive results, and true or false negative results. The sensitivity of the tests with recombinant antigen were then calculated as: True positives/(True positives + False negatives) x 100 (Galen and Gambino, 1975).

The specificity of the tests with recombinant antigen were calculated as:

True negatives/(True negatives + False positives) x 100 (Galen and Gambino, 1975).

2.7. Antigenic specificity testing of CCHF recombinant antigen

To determine the antigenic specificity of the recombinant CCHF nucleocapsid protein in ELISA, the antigen was reacted with sera from guinea pigs hyperimmunised against three other nairoviruses: Dugbe, Hazara and Nairobi sheep disease. An IgG sandwich ELISA was performed as described in

section 2.6.3. using anti-guinea pig HRPO (Zymed) diluted 1/2000.

2.8. Stability testing of recombinant antigen

Aliquots of recombinant antigen were incubated at different temperatures to determine the stability of the protein. The aliquots were left at room temperature (22°C), 37°C, 4°C, and their potency tested at two week intervals for 5 months by ELISA as described in 2.6.1 above. To determine the effect of freeze drying on the protein, 0.5ml aliquots were freeze dried and tested after reconstitution.

3.0 Results

3.1. Preparation of CCHF baculovirus

The pBluebac baculovirus transfer plasmid containing the 1510 base pairs CCHF virus insert was isolated and digested with Nhe I restriction enzyme (GibcoBRL). The products were separated by electrophoresis using a 0.8% agarose gel and the 1510 base pairs CCHF virus insert was excised from the gel (Figure 2) and subjected to purification and ligation with the pFastBac1 plasmid vector. Figure 2 shows undigested pBluebac transfer vector plasmid which is approximately 9000 base pairs and after digestion with Nhe I, the CCHF insert of approximately 1510 base pairs. The insert was then cloned into the pFastBac 1 plasmid. The ligated DNA was transformed into the DH5∝ competent cells. After 24 hours, the confirmed white colonies were picked, grown and the DNA isolated from the cells after 24 hours. The transformed DNA was isolated and analysed by electrophoresis. Figure 3 shows a DNA fragment of approximately 20 000 base pairs which confirms the presence of the transformed DNA in the preparation. Figure 4 shows the PCR product obtained after amplification of the DNA with CCHF specific primers F2 and R3. The primers amplify a 536 base pair region of the nucleocasid gene confirming the insertion of the CCHF S segment. Since the transformed DNA from the DH5∝ competent cells was shown by PCR to contain the CCHF insert, the DNA was transformed into the DH10Bac competent cells. The white colonies obtained from the transformation into the DH10Bac competent cells were picked and grown in LB broth and the bacmid DNA isolated after 24 hours. To confirm the presence of Bacmid DNA an aliquot was visualised by electrophoresis and a DNA band of approximately 23 000 base pairs was identified as illustrated in Figure 5. However repeated attempts to show that transformation had occurred using pUC/M13 primers and PCR as described in Section 2.4.5. were unsuccessful and the preparation was abandoned.

3.2. *In vitro* expression of CCHF recombinant protein

The expression of the CCHF recombinant protein in cell culture was detected by IF test and a Western blot was performed to confirm that the specific CCHF protein of the predicted size was expressed. Cells harvested from flasks infected with BacCCHF1 on day six after inoculation were positive for CCHF antigen using IF as shown in Figure 6. Negative control slides prepared using cells infected with wild type showed no fluorescence (not shown). The Western blot (Figure 7)

showed an expressed protein of approximately 50 kDa that reacted with mouse anti-CCHF HMAF fluid. Only recombinant protein expressed by cells infected with BacCCHF1 and sucrose acetone antigen reacted with anti-CCHF antibody in Western blots. A larger band of approximately 97 000 base pairs present in lane 2 is probably CCHF glycoprotein. The nucleocapsid protein in lane 2 is very faint but was present.

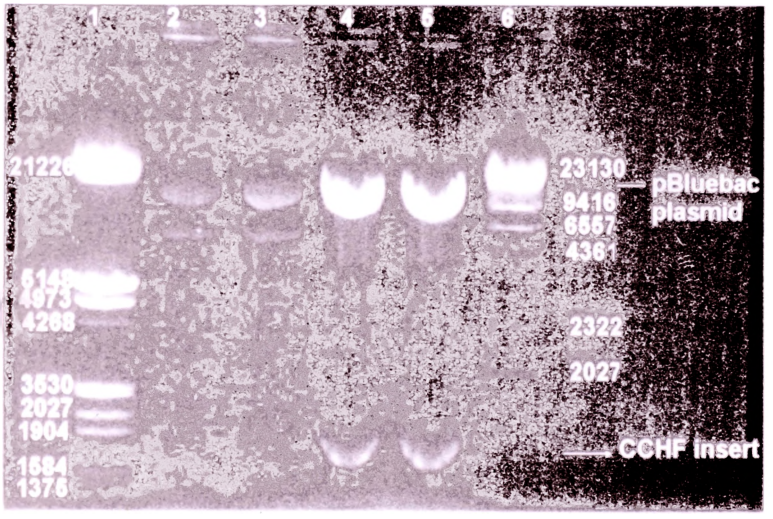


Figure 2. Agarose gel analysis of pBluebac DNA. Lane 1: DNA Marker III fragments. Lane 2 and 3: Undigested pBluebac DNA. Lane 4 and 5: Digested pBluebac DNA showing the 1510 bp CCHF insert. Lane 6: DNA Marker II fragments. Fragment sizes for the molecular weight markers are shown on the left and right of the figure.

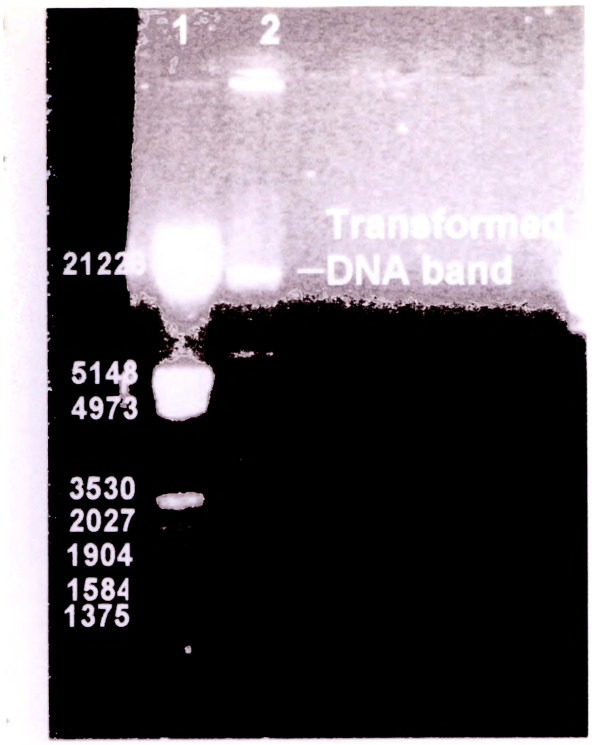


Figure 3. Agarose gel analysis of transformed DNA isolated from the DH5∝ cells. Lane 1: DNA Marker III fragment. Lane 2: Transformed DNA. Fragments sizes for the molecular weight marker are shown on the left of the figure.

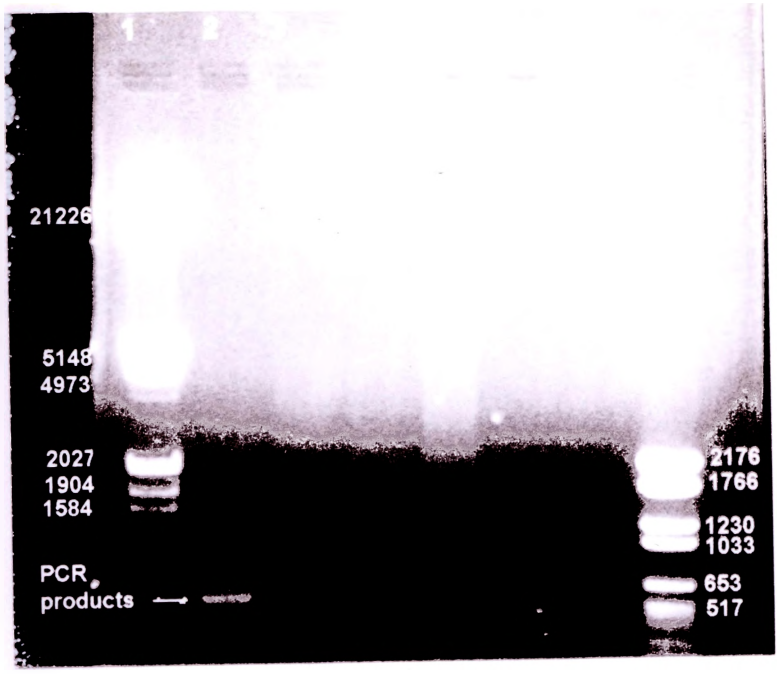


Figure 4. Agarose gel analysis of PCR products from transformed DNA isolated from DH5∝ cells and controls. Lane 1: DNA Marker III fragments. Lane 2: 536 base pair amplicon, CCHF positive control. Lane 3, 4, 5: 536 base pair product amplified from transformed DNA. Lane 6 and 7:negative controls. Lane 8: DNA Marker VI fragments. Fragments sizes for the molecular weight markers are shown on the left and right of the figure.



Figure 5. Agarose gel analysis of Bacmid DNA isolated from DH10 cells. Lane 1: DNA marker II fragments. Lane 2: DNA marker III fragments. Lanes 3, 4, 5: Bacmid DNA. Fragment sizes for molecular weight marker II shown on left of figure.

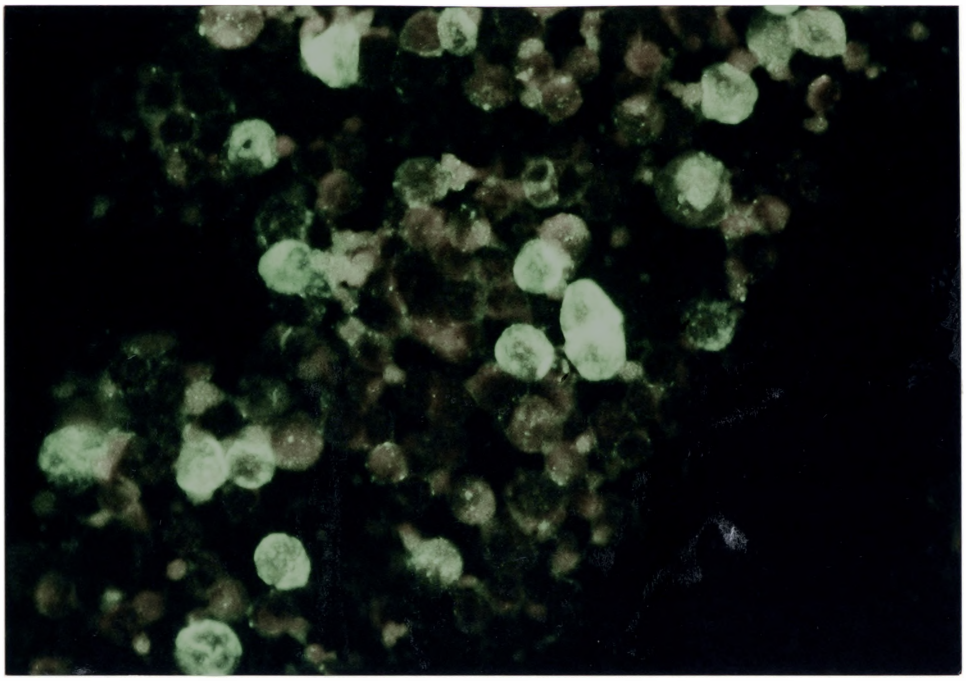


Figure 6. Detection of expressed CCHF virus nucleoprotein from CCHF infected Sf9 cells using immunoflourescence.

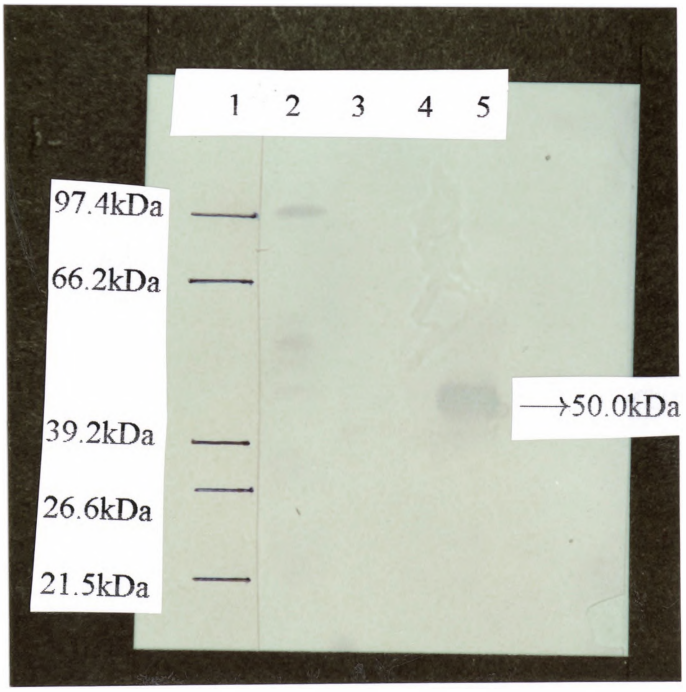


Figure 7. Western blot to demonstrate expressed CCHF nucleocapsid protein by recombinant baculovirus. Lane 1: molecular weight marker. Lane 2: recombinant antigen. Lane 3: sucrose acetone extracted antigen. Lane 4: cell lysate. Lane 5: control AcNPV

3.3. Antigenic reactivity of recombinant proteins

Only the recombinant protein expressed by the baculovirus BacCCHF1 supplied by USAMRIID was found to react satisfactorily with reference CCHF antibody in ELISA titrations. The three recombinant proteins supplied by CDC showed weak activity in the ELISA, lower dilutions of these antigens gave non-specific reactions and hence the proteins were excluded from the study at this point as being unsuitable for use as diagnostic antigens.

The optimal dilution of sucrose acetone extracted antigen as determined in chessboard titration was 1/800 for use in the IgM-capture ELISA, 1/1000 for use in the IgG sandwich ELISA, and 1/400 for use in the CELISA, while the optimal dilution of the BacCCHF1 recombinant antigen was 1/50 for use in the IgM-capture ELISA, 1/100 for the IgG sandwich ELISA, and 1/50 for the CELISA

Although only sera which react positively in IgM and IgG ELISA at dilutions $\geq$ 1/400 are interpreted as being positive for antibody to CCHF virus (See Materials and methods section 2.6.2), all serum reactions detected in the present study, including equivocal reactions (which are positive only at the lowest 4-fold dilution of serum tested, 1/100), are reported in sections 3.4 1-3.4.2 and 3.5.1-3.5.2 below, and in Figures 8,9, and 11-16, plus Appendices B-E, and H-M. However, only IgM and IgG ELISA reactions interpreted as positive (with titres $\geq$400) are used in calculating the sensitivity and specificity of recombinant antigen in relation to sucrose acetone extracted antigen (Section 3.8 below). In contrast, antibody titres detected in the CELISA are lower than those detected in the IgM and IgG ELISA, and positive reactions recorded at the lowest dilution of serum tested, 1/10, are interpreted as positive for antibody to CCHF virus (See Materials and methods section 2.6.4). Published literature showed higher titres than some recorded in this study. One explanation could be that the sera have been frozen and thawed for a number of related studies which could be responsible for the drop in antibody titre (Burt *et al.*, 1994).

3.4. Detection of antibody response to CCHF virus infection in human sera

3.4.1. IgM detection by ELISA using sucrose acetone extracted antigen

The 135 serum samples of confirmed CCHF patients included in the study were collected from day 5 to one year after the onset of illness as shown in Figure 1. The results of IgM-capture ELISA for detection of antibody to CCHF virus are summarised in Figure 8A, and the titres recorded for

individual serum samples are shown in Appendix B.

No IgM antibody was detected in the earliest serum sample available for testing which was collected on day 5 after the onset of illness. An IgM antibody response was detected in only 2/3 serum samples collected on day 6, while 5/6 sera were positive on day 7, and 5/ 7 were positive on day 8. By day 9 after the onset of illness all of the serum samples were positive for IgM antibody, although only 15/16 serum samples were positive on day 10. All serum samples were positive for IgM antibody from day 11 to day 21. One month after the onset of illness there were still 15/19 positive serum samples, while the only serum analysed on month 2 was also positive. Thereafter the proportion of positive sera declined steadily and all of the samples analysed 8 months after the onset of illness were negative for IgM antibody, and only isolated positive reactions were recorded after that (Figure 8A). The maximum titre was 6400, and titres started to decline from the first month after the onset of illness.

3.4.2. IgM detection by ELISA using recombinant antigen

The results obtained in the IgM-capture ELISA using recombinant antigen on the 135 CCHF patient sera were similar to those reported above for sucrose acetone extracted antigen, and are summarised in Figure 8B, while the titres recorded for individual sera are shown in Appendix C.

The serum sample taken on day 5 after the onset of illness was negative for IgM antibody. On day 6 there were 2/3 sera IgM antibody positive, on day 7 there were 4/6 positive sera, 4/7 were positive on day 8, and by day 10 after the onset of illness 15/16 serum samples were positive for IgM antibody to CCHF virus. From day 11-21 all of the serum samples had demonstrable IgM antibody. One month after the onset of illness 14/19 serum samples were still positive and then the proportion of positive sera declined steadily until all samples analysed at 8 and 9 months were negative, and only isolated positive reactions were recorded after that (Figure 8B). The maximum titre attained was 6400 and levels started to decline steadily after the first month.

3.4.3. IgG detection by ELISA using sucrose acetone extracted antigen

The results obtained for IgG antibody detection by ELISA using sucrose acetone antigen on the 135 sera from CCHF patients are summarised in Figure 9A, and the titres are recorded in Appendix D.

No antibody was detected in the earliest serum samples available for testing which were collected from day 5 and 6 after the onset of illness. Antibody was detected in 3/6 sera on day 7, and 2/7 were positive on day 8, while on day 8 after the onset of illness 6/7 were positive. By day 10 15/16 serum sample were positive, and from day 11 to first the year after the onset of illness, all serum samples showed demonstrable IgG antibody response except for the seventh month when there was one negative serum. The maximum titre attained was 6400.

3.4.4. IgG detection by ELISA using recombinant antigen

Serological results obtained from the IgG sandwich ELISA using recombinant antigen on 135 CCHF patient sera were similar to those described using sucrose acetone extracted antigen, and are summarised in Figure 9B, while the titres are recorded in Appendix E.

The serum samples collected on day 5 and 6 were negative for IgG antibody. Antibody was demonstrable in 3/6 sera on day 7, in 1/7 sera on day 8, in 6/7 sera on day 9, and by day 10 there were 15/16 sera positive. All serum samples analysed from day 11 to the end of the first year were positive except one from month 7. This serum was negative by ELISA using both sucrose acetone antigen and recombinant antigen, and was negative for antibody to CCHF using IF (laboratory results) and this was probably due to an individual response. The maximum titre attained was 6400.

3.4.5. CELISA using the sucrose acetone extracted antigen

The results obtained for total antibody detection by CELISA using sucrose acetone antigen on the 135 CCHF patient sera are summarised in Figure 10A, and the titres are recorded in Appendix F. The total antibody response detected using sucrose acetone extracted antigen was demonstrable at the earliest in 4/6 serum samples collected on day 7 after the onset of illness. On day 8 there were 4/7 rising to 12/18 on day twelve. From day 15-23 all serum samples were positive except the only available serum sample from the seventh month.

3.4.6. CELISA using the recombinant antigen

The results obtained in CELISA using recombinant antigen for the detection of total antibody activity in CCHF patient sera are summarised in Figure 10B, and the titres are recorded in Appendix G. Antibody was detected in 1/3 sera on day 6. On day 7, 4/6 serum samples were positive, 4/7 on day

8, 6/7 on day 9, 14/16 on day 10, rising to 16/18 on day 12. From day 15 all samples were positive until the end of the first year except for the single sample available for the seventh month.

3.4.7. Control panel of negative sera

A total of 160 sera from known non-CCHF patients were tested for IgG, IgM and total antibody to CCHF using ELISA with both antigens and CELISA. All the sera were screened at a dilution of 1:100 and were antibody negative. The results for the optical density values obtained are presented in Appendix H.

3.5. Detection of antibody response to CCHF virus in experimentally infected sheep

3.5.1. IgM ELISA using sucrose acetone extracted and recombinant antigens

The antibody responses to CCHF virus detected by IgM-capture ELISA using sucrose acetone and recombinant antigens on 63 serum samples collected from day 0 to month 8 post-infection from three experimentally inoculated sheep designated 92, 48 and 37, are presented graphically in Figures 11-13, and the titres are recorded in Appendices I-K.

There was no detectable IgM antibody to CCHF virus in sera collected from sheep 92 from day 0-6 post-inoculation when using the sucrose acetone extracted antigen as illustrated in Figure 11A. Antibody was detected in sera collected on days 7-28, but from month 5-8 IgM antibody tests were again negative with sucrose acetone extracted antigen. Similar results were obtained with recombinant antigen (Figure 11B).

The serum samples collected from sheep 48, showed no demonstrable IgM antibody from day 0-6 post-inoculation in tests using the sucrose acetone antigen, as illustrated in Figure 12A. IgM antibody was demonstrable from day 7-21, and not after that. Similar esults were obtained with recombinant antigen (Figure 12B).

Antibody was detected from day 4-14 post-inoculation in sheep 37 using sucrose acetone antigen, as illustrated in Figure 13A, and after that no IgM antibody was detectable The results of tests with recombinant antigen for the detection of IgM antibody were negative on days 0-3 post-inoculation, and positive from day 4-14. After that there was no demonstrable IgM antibody activity (Figure 13B).

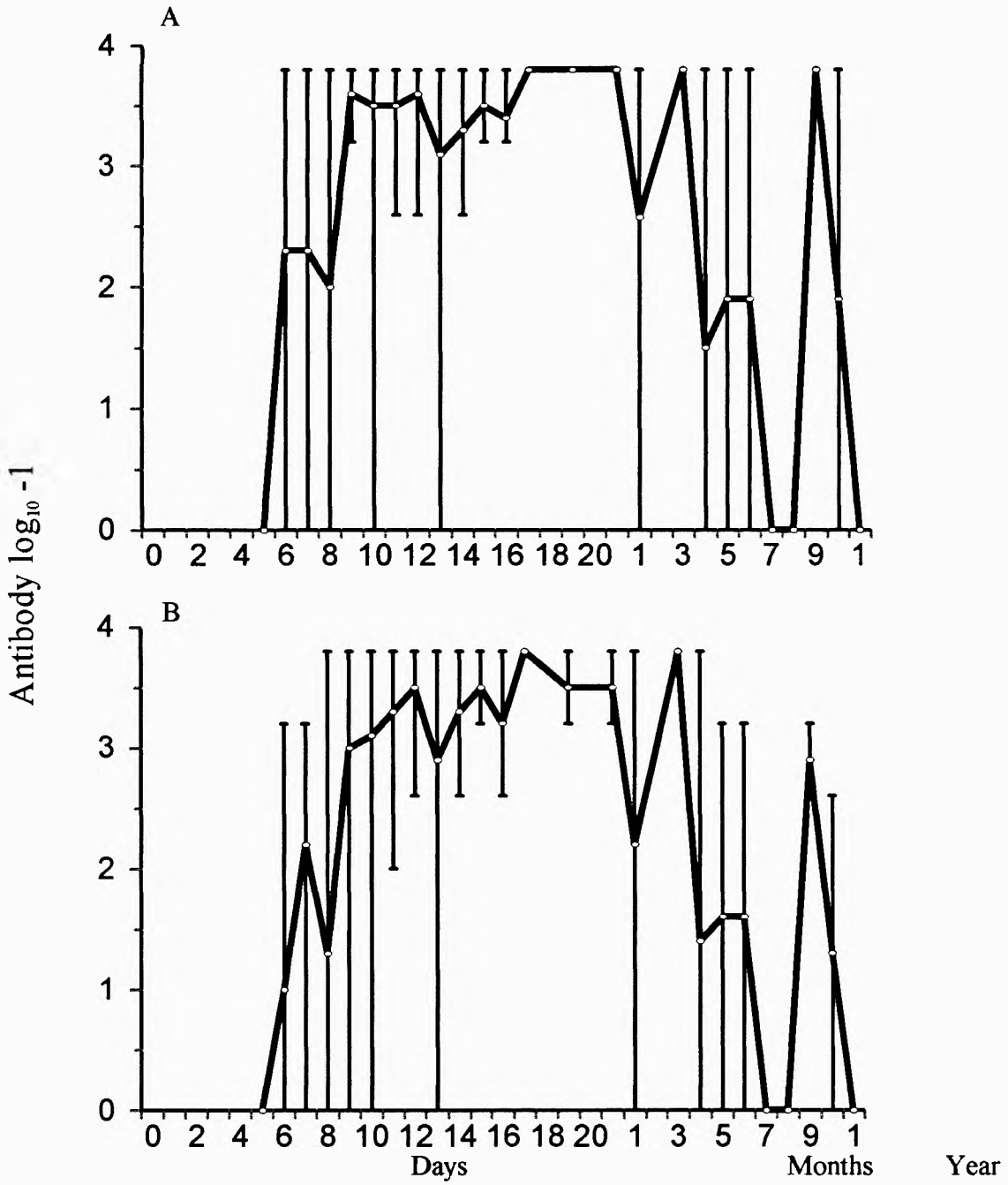


Figure 8. IgM antibody level to CCHF in human serum samples collected on different days after the onset of illness, detected by IgM-capture ELISA using sucrose acetone-extracted antigen (A) and recombinant antigen (B).

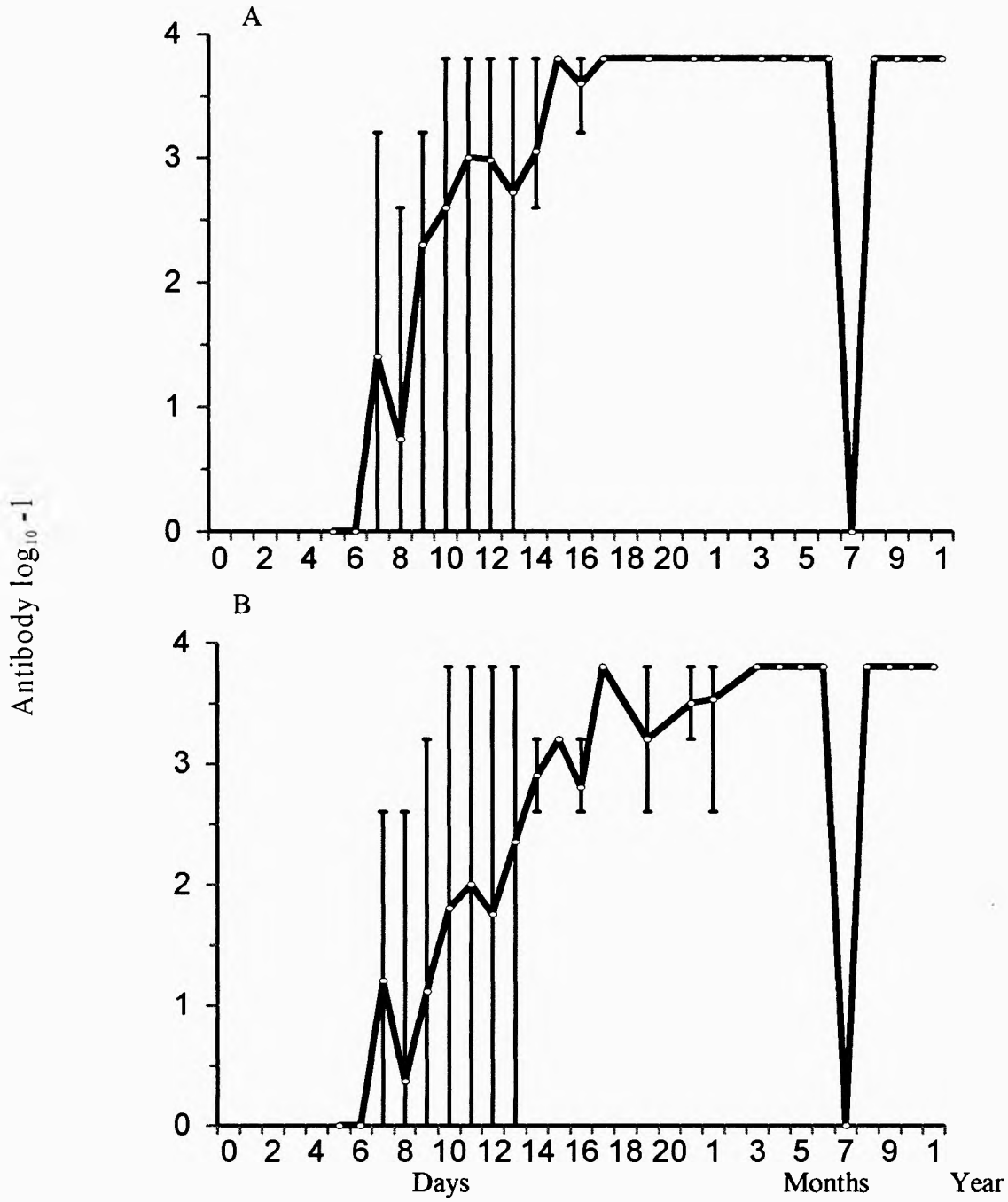


Figure 9. IgG antibody level to CCHF in human serum samples collected on different days after the onset of illness, detected by IgG sandwich ELISA using sucrose acetone extracted antigen (A) and recombinant antigen (B).

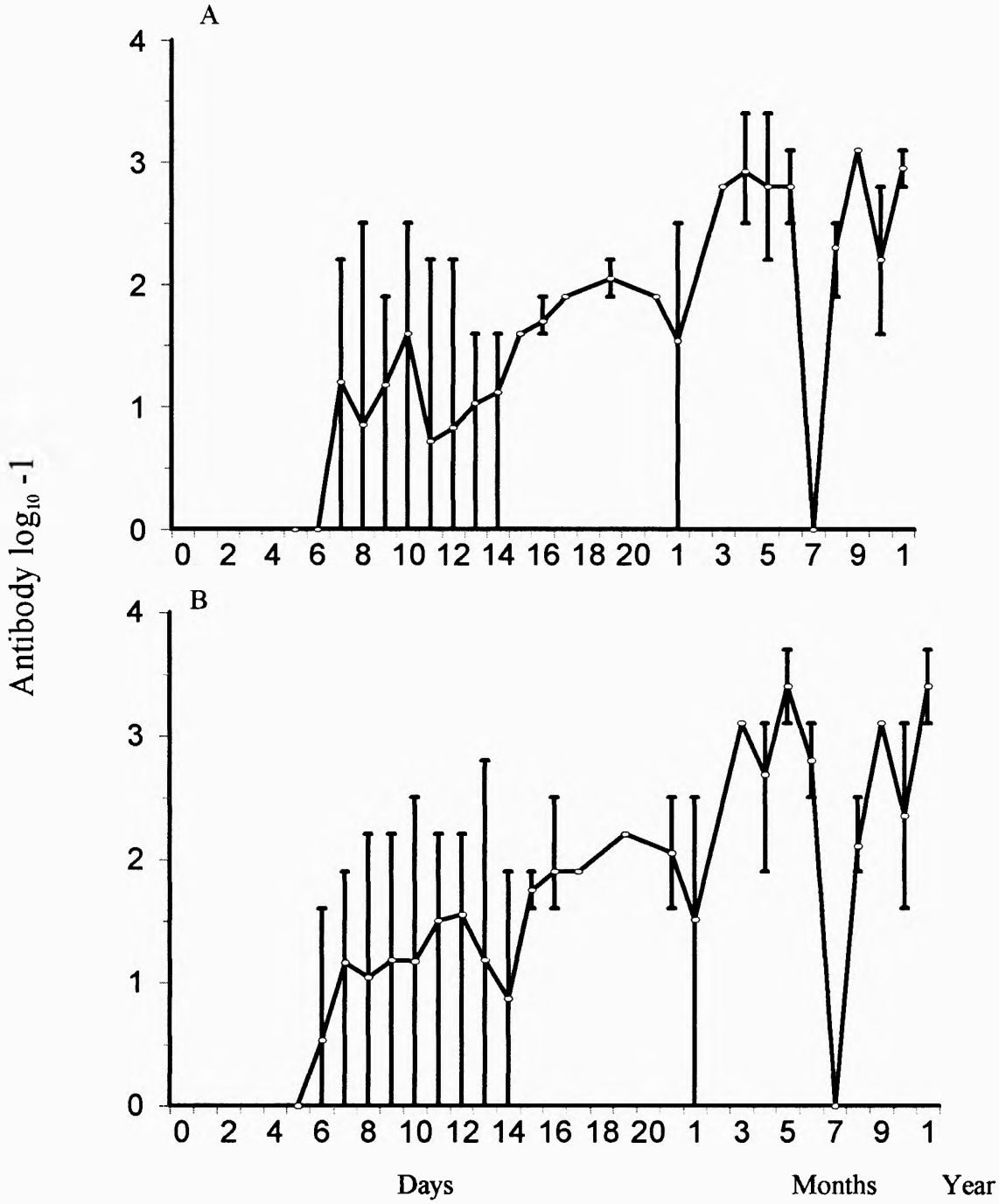


Figure 10. Total antibody level to CCHF virus in human serum samples collected on different days after the onset of illness, detected by CELISA using sucrose acetone extracted antigen (A) and recombinant antigen (B).

3.5.2. IgG ELISA using sucrose acetone extracted and recombinant antigens

The results of IgG antibody tests on the three experimentally infected sheep using sucrose acetone extracted and recombinant antigens are presented graphically in Figures 14-16, and the titres are recorded in Appendices L-N.

For sheep 92, IgG antibody response was detected from day 7 post-inoculation up until month 8, the last serum available, when using the sucrose acetone extracted antigen (Figure 14A) and similar results were obtained with recombinant antigen (Figure 14B).

In tests with sucrose acetone extracted antigen the serum samples of sheep 48 showed IgG antibody response from day 7 post-inoculation up to month 8, except for a negative result on the month 7 sample (Figure 15A). In tests with the recombinant antigen similar results were obtained (Figure 15B).

Serum samples collected from sheep 37 showed demonstrable IgG antibody from day 5 until month 8 using sucrose acetone extracted antigen (Figure 16A). Similar results were obtained with recombinant antigen (Figure 16B).

3.6. Detection of CCHF antibody in ostrich sera

A total of 271 ostrich sera submitted to SPU for screening for antibody to CCHF virus as a prerequisite to export were tested by CELISA using sucrose acetone extracted antigen, and with recombinant antigen for comparison. The sera were screened at a single dilution of 1/10 to produce a positive or negative result, and were not titrated to end-point. The results are shown in Table 1.

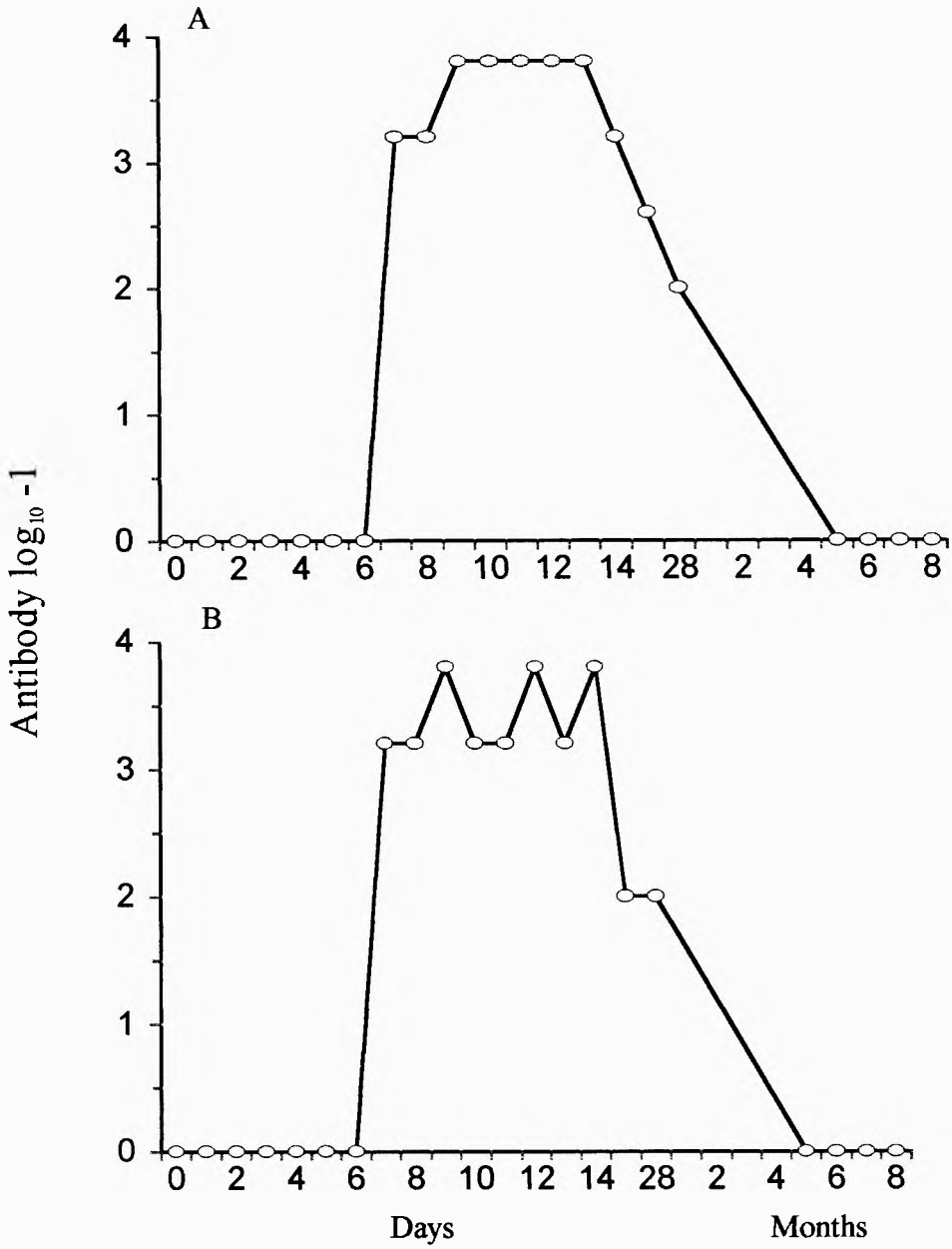


Figure 11. IgM antibody log titre to CCHF virus in relation to day after infection as demonstrated by IgM capture ELISA using sucrose acetone antigen (A) and recombinant antigen (B), in serum samples from experimentally infected sheep number 92.

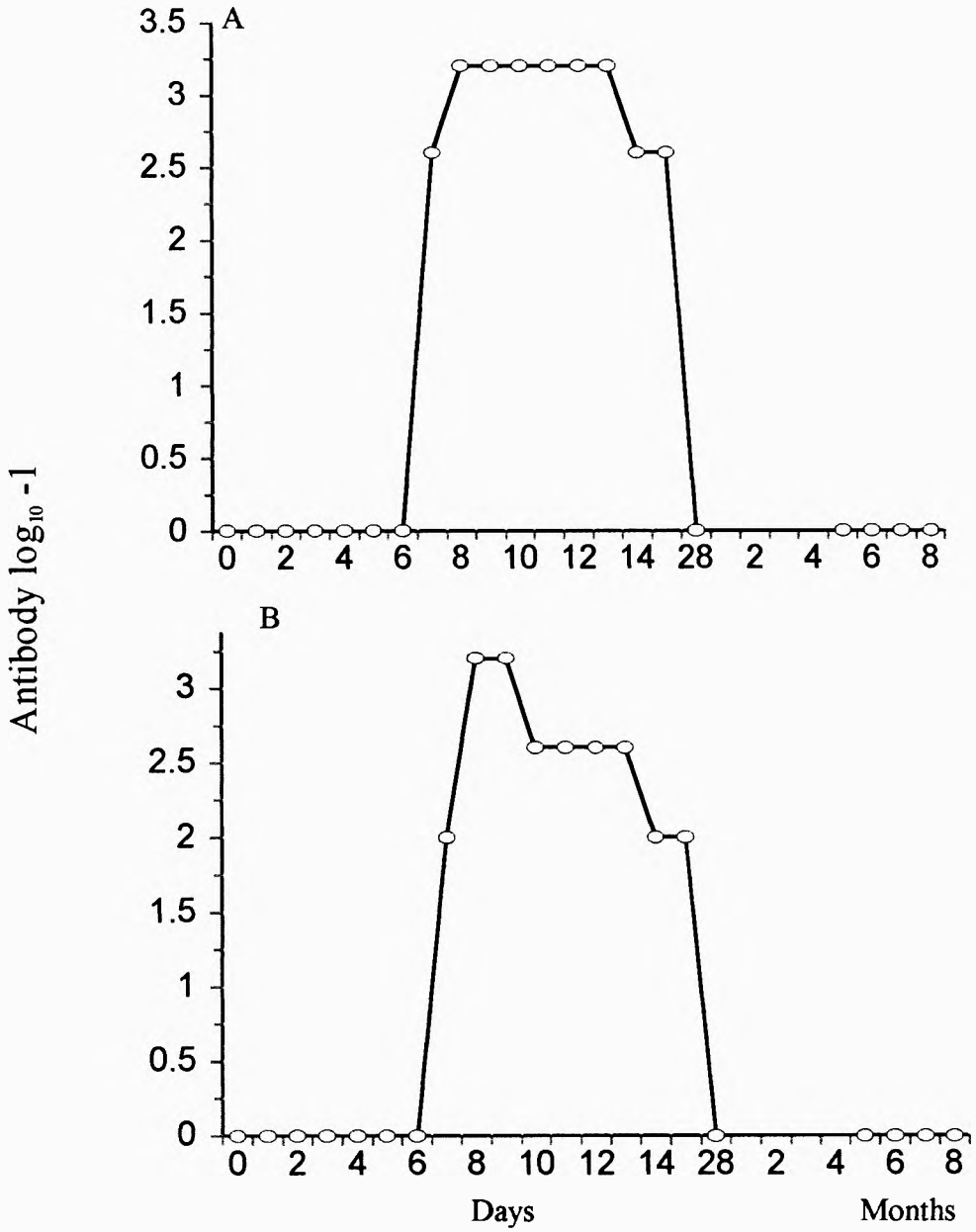


Figure 12. IgM antibody log titre to CCHF virus in relation to day after infection as detected by IgM capture ELISA using sucrose acetone antigen (A) and recombinant antigen (B), in serum samples from experimentally infected sheep number 48.

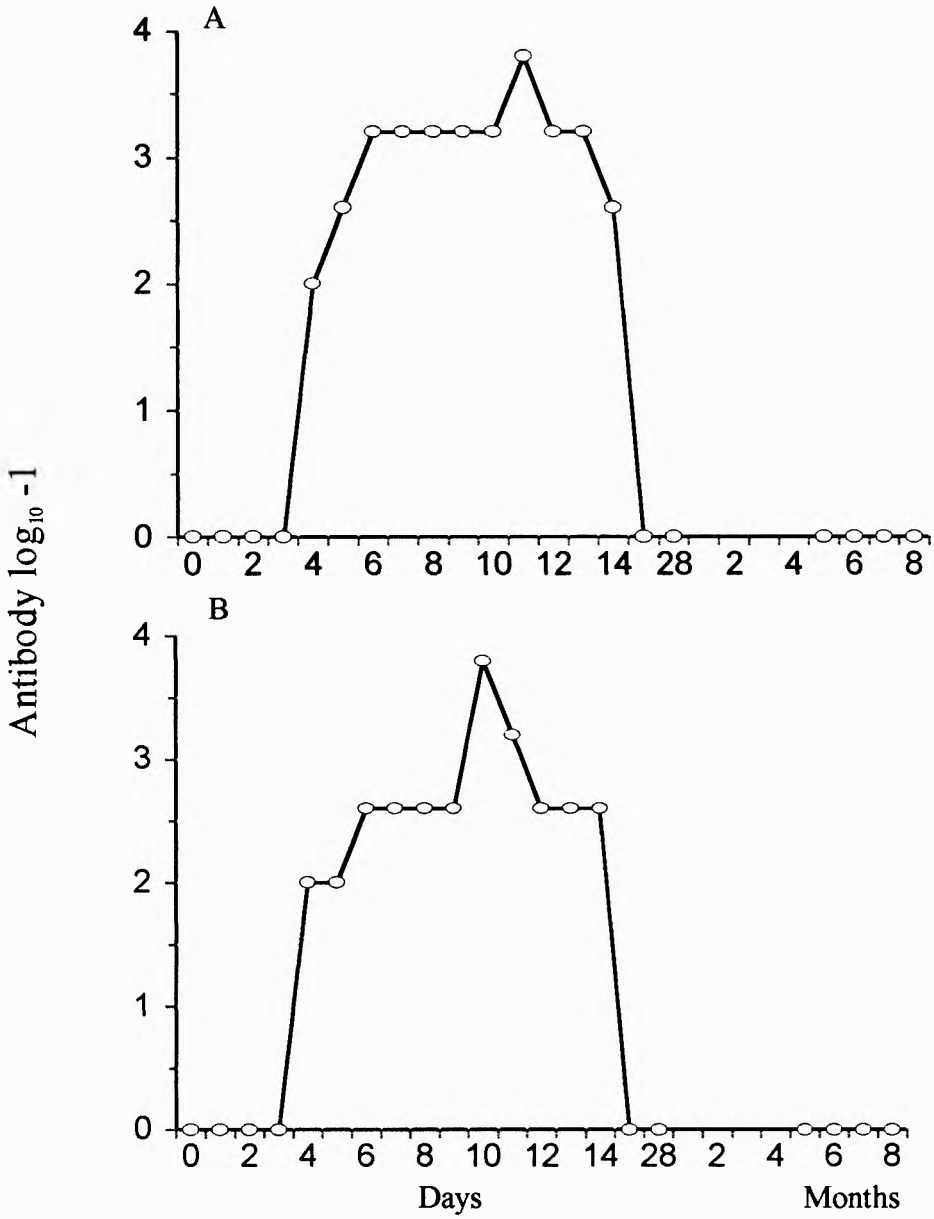


Figure 13. IgM antibody log titre to CCHF virus in relation to day after infection as detected by IgM capture ELISA using sucrose-acetone extracted antigen (A) and recombinant antigen (B), in serum samples from experimentally infected sheep number 37.

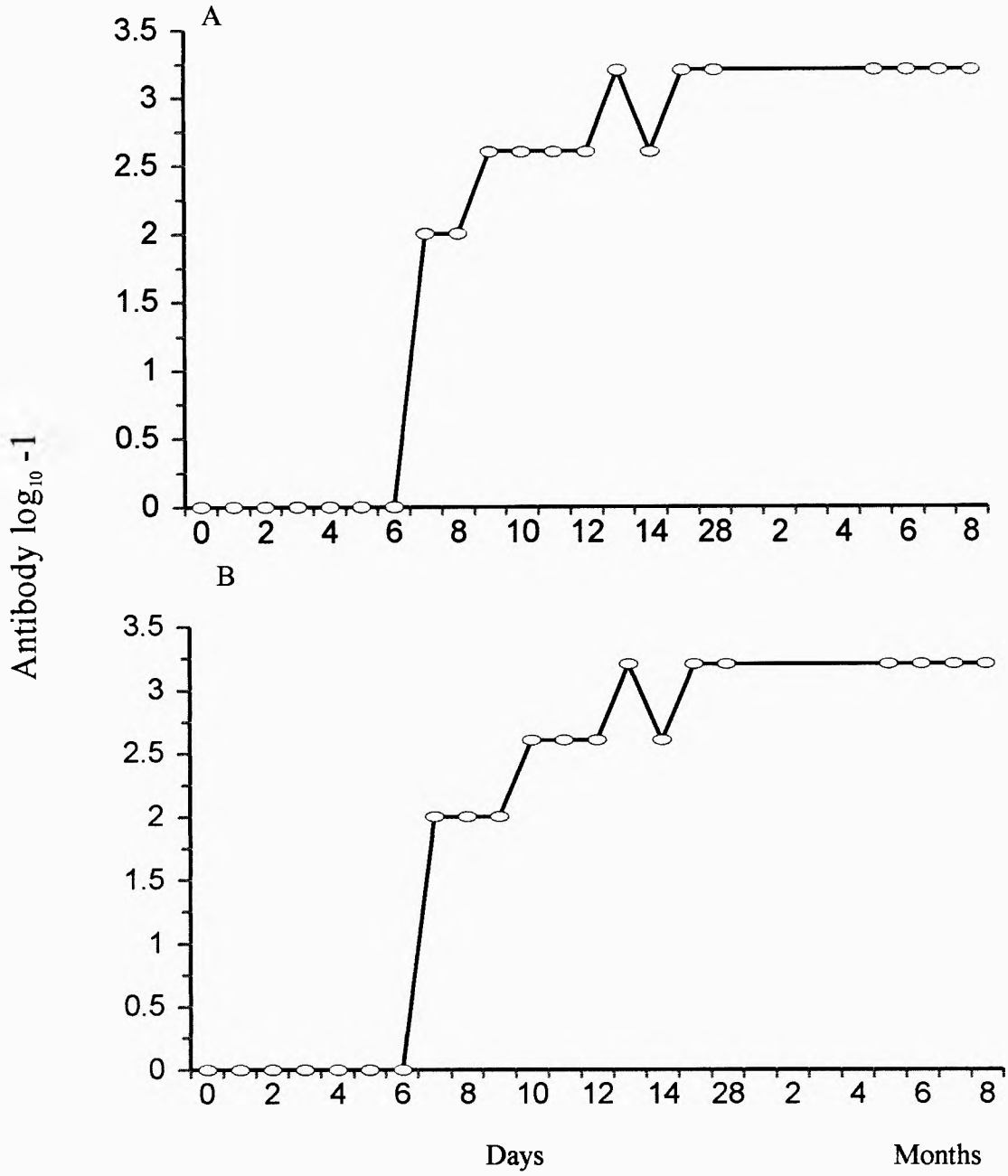


Figure 14. IgG antibody log titre to CCHF virus in relation to day after infection as detected by IgG sandwich ELISA using sucrose acetone extracted antigen (A) and recombinant antigen (B), in serum samples from experimentally infected sheep number 92.

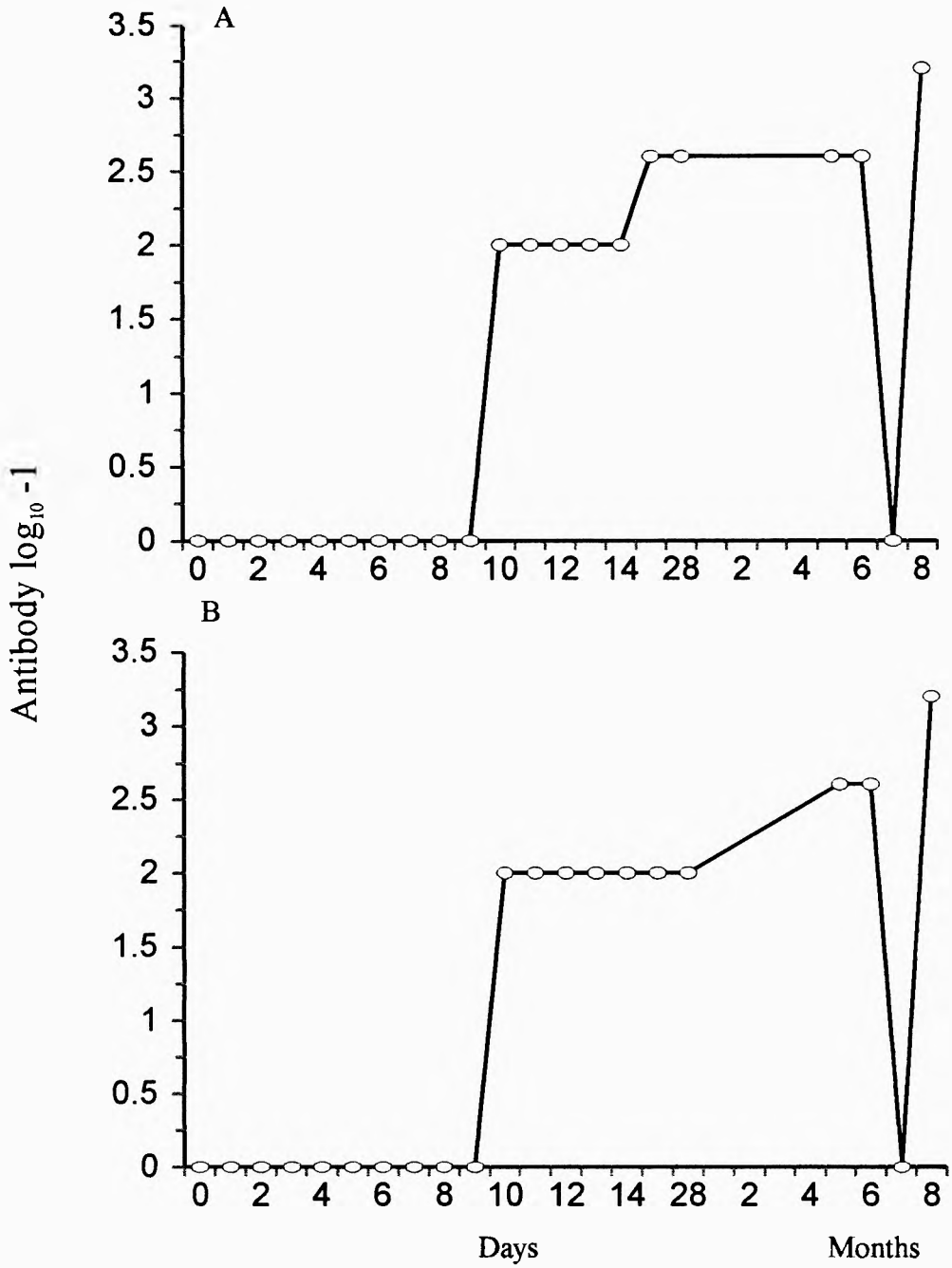


Figure 15. IgG antibody log titre in relation to day after infection as detected by IgG sandwich ELISA using sucrose acetone extracted antigen (A) and recombinant antigen (B), in serum samples from experimentally infected sheep number 48.

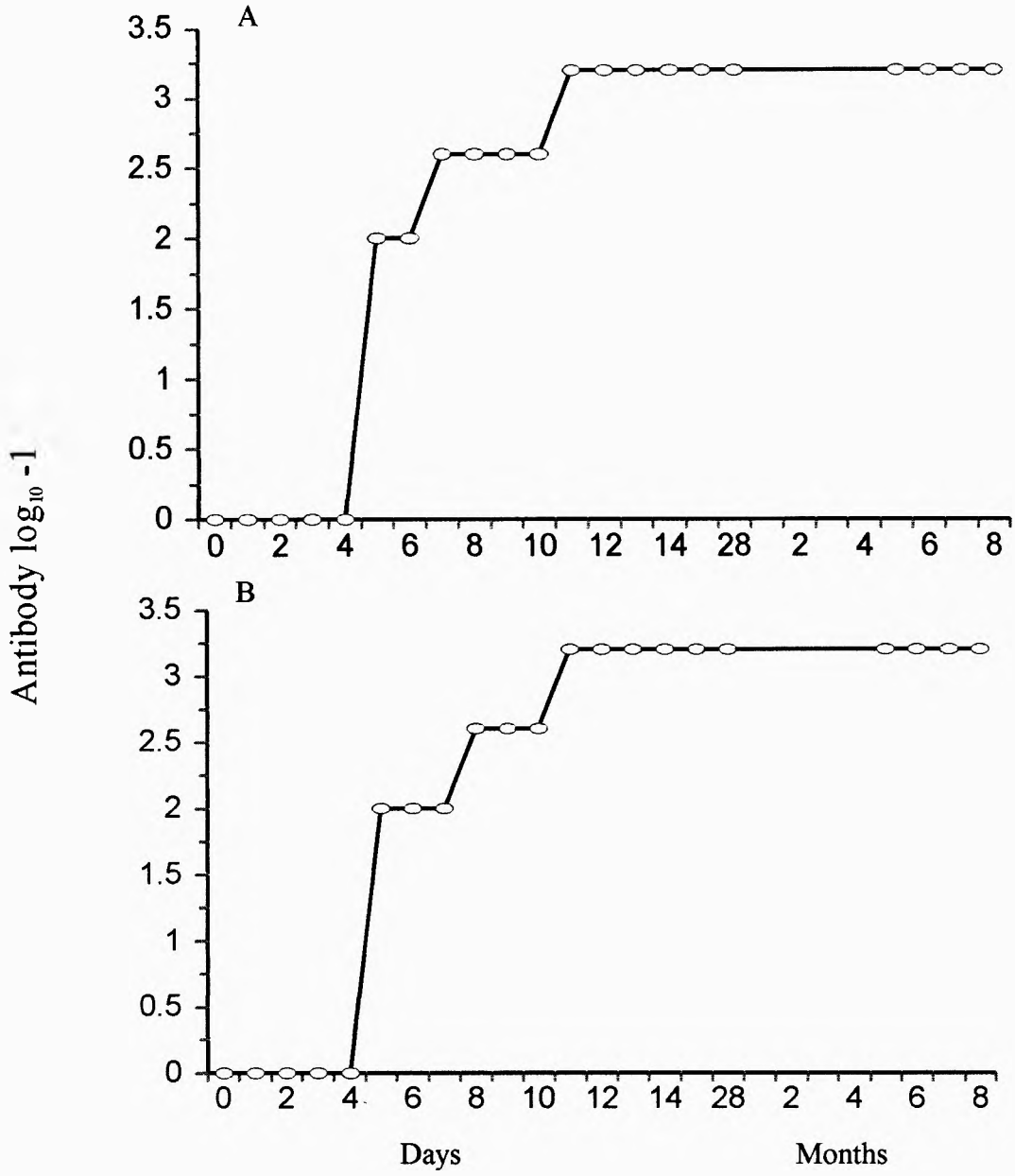


Figure 16. IgG antibody log titre to CCHF virus in relation to day after infection as detected by IgG sandwich ELISA using sucrose acetone extracted antigen (A) and recombinant antigen (B), in serum samples from experimentally infected sheep number 37.

Table 1. Results of screening tests for antibody activity to CCHF virus performed on 271 ostrich serum samples by CELISA using sucrose acetone extracted antigen in comparison with recombinant antigen.

	Recombinant antigen	
Sucrose acetone extracted antigen	**Positive**	**Negative**
Positive	14	2
Negative	0	255

3.7. Detection of antibody by CELISA in experimentally infected small mammals

The antibody response to CCHF virus in 35 serum samples collected from small mammals at the indicated intervals after experimental infection, was detected by CELISA using sucrose acetone extracted antigen in comparison with recombinant antigen (Table 2).

Table 2. Total antibody titres in the sera of small mammals experimentally infected with CCHF virus as detected by CELISA using sucrose acetone extracted and recombinant antigens, shown in relation to day after inoculation.

Day	Species	Animal number	sucrose acetone extracted antigen Antibody titre	Recombinant antigen Antibody titre
	Mystromys albicaudatus			
1		1	0	0
1		2	0	0
1		3	0	0
1		4	0	0
14		1	20	20
14		2	40	20
21		1	160	160
21		2	80	40
21		3	20	20
28		1	0	80
28		2	160	80
28		3	40	80
Unrecorded		2	0	160
Unrecorded		3	160	160
Unrecorded		4	160	160
	Tatera brantsii			
1		1	0	0
1		2	0	0
1		3	0	0
21		1	0	0
21		2	160	80
35		1	20	20
35		2	20	20
35		3	80	160
	Tatera leucogaster			
1		2	0	0
1		4	0	0
2		4	0	0
3		1	0	0
28		1	80	20
28		2	160	80
28		3	80	80
	Aethomys namaquensis			
1		1	0	0
1		2	0	0
14		1	20	20
14		2	0	0
14		3	0	0

3.8 Sensitivity and specificity of serological tests with recombinant antigen

3.8.1 IgM-capture ELISA on CCHF patient sera

The results obtained on 295 serum samples from confirmed CCHF patients in IgM capture ELISA with sucrose acetone extracted and recombinant antigens (Appendices B, C and H) were interpreted as positive (titres ≥400) or negative (titres <400), and the totals are shown in Table 3.

In addition to 160 sera from non-CCHF patients there were 135 sera from confirmed patients, of which not all would show a demonstrable antibody response. One would expect a negative result from sera collected early after onset of illness and in the case of IgM antibody, sera collected a month after onset as IgM is no longer detected after a few weeks. Hence the number of true positives indicated in the following calculations for each ELISA reflect number of antibody positive specimens using the reference ELISA, in this case the ELISA using sucrose acetone antigen. The true negatives include the sera from non-CCHF patients and samples from CCHF patients that were negative using the reference ELISA.

Table 3. Summary of the results obtained on 295 serum samples from confirmed CCHF patients in IgM capture ELISA with sucrose acetone extracted and recombinant antigens (antibody titres ≥400 interpreted as positive results).

	Recombinant antigen	
Sucrose acetone extracted antigen	**Positive**	**Negative**
Positive	104	6
Negative	0	185

As described in Materials and methods (Section 2..6.5), tests using sucrose acetone antigen are regarded as reference techniques which produce true positive and true negative results. By comparison, the results produced in tests with recombinant antigen are rated as true or false positive results, and true or false negative results. The sensitivity of the test with recombinant antigen is then calculated as:

True positives/(True positives + False negatives) x 100

In the present instance = 104/110 x 100 = 94.5%

The specificity of the tests with recombinant antigen were calculated as:

True negatives/(True negatives + False positives) x 100 (Galen and Gambino, 1975).

In the present instance =185/185 x 100 = 100%

3.8.2 IgG sandwich ELISA on CCHF patient sera

The results obtained on 295 serum samples from confirmed CCHF patients in IgG sandwich ELISA with sucrose acetone extracted and recombinant antigens (Appendices D, E and H) were interpreted as positive (titres ≥400) or negative (titres <400), and the totals are shown in Table 4.

Table 4. Summary of the results obtained on 295 serum samples from confirmed CCHF patients in IgGsandwich ELISA with sucrose acetone extracted and recombinant antigens (antibody titres ≥400 interpreted as positive results).

	Recombinant antigen	
Sucrose acetone extracted antigen	**Positive**	**Negative**
Positive	115	5
Negative	3	172

The sensitivity of the test with recombinant antigen is: 115/120 x 100 = 95.8%

The specificity of the tests with recombinant antigen is: 172/175 x 100 = 98.3%

3.8.3 CELISA on CCHF patient sera

The results obtained on 295 serum samples from confirmed CCHF patients in CELISA with sucrose acetone extracted and recombinant antigens (Appendices F, G and H) are summarised in Table 5

(titres ≥10 are recorded as positive).

Table 5. Summary of the results obtained on 295 serum samples from confirmed CCHF patients in CELISA with sucrose acetone extracted and recombinant antigens (titres ≥10 are recorded as positive).

	Recombinant antigen	
Sucrose acetone extracted antigen	**Positive**	**Negative**
Positive	97	8
Negative	14	176

The sensitivity of the test with recombinant antigen is: 97/105 x 100 = 92.4%

The specificity of the tests with recombinant antigen is: 176/190 x 100 = 92.6%

3.8.4 IgM-capture ELISA on experimentally infected sheep sera

The results obtained on 63 serum samples from 3 sheep experimentally infected with CCHF virus in IgM capture ELISA with sucrose acetone extracted and recombinant antigens (Appendices I, J and K) were interpreted as positive (titres ≥400) or negative (titres <400), and the totals are shown in Table 6.

Table 6. Summary of the results obtained on 63 serum samples from 3 sheep infected with CCHF virus in IgM capture ELISA with sucrose acetone extracted and recombinant antigens (antibody titres ≥400 interpreted as positive results).

	Recombinant antigen	
Sucrose acetone extracted antigen	**Positive**	**Negative**
Positive	23	5
Negative	0	35

The sensitivity of the test with recombinant antigen is: 23/28 x 100 = 82.1%

The specificity of the tests with recombinant antigen is: 35/35 x 100 = 100%

3.8.5.IgG sandwich ELISA on experimentally infected sheep sera

The results obtained on 63 serum samples from 3 sheep experimentally infected with CCHF virus in IgG sandwich ELISA with sucrose acetone extracted and recombinant antigens (Appendices L, M and N) were interpreted as positive (titres ≥400) or negative (titres <400), and the totals are shown in Table 7.

Table 7. Summary of the results obtained on 63 serum samples from 3 sheep infected with CCHF virus in IgG sandwich ELISA with sucrose acetone extracted and recombinant antigens (antibody titres ≥400 interpreted as positive results).

	Recombinant antigen	
Sucrose acetone extracted antigen	**Positive**	**Negative**
Positive	26	4
Negative	1	31

The sensitivity of the test with recombinant antigen is: 26/30 x 100 = 86.7%

The specificity of the tests with recombinant antigen is: 31/32 x 100 = 96.9%

3.8.6 CELISA on ostrich sera

The results obtained on 271 ostrich serum samples in CELISA screening tests with sucrose acetone extracted and recombinant antigens are summarised in Table 1 above (Section 3.6) (titres ≥10 are recorded as positive).

The sensitivity of the test with recombinant antigen is: 14/16 x 100 = 87.5%

The specificity of the tests with recombinant antigen is: 255/255 x 100 = 100%

3.8.7 CELISA on experimentally infected small mammal sera

The results obtained on 35 serum samples from small mammals experimentally infected with CCHF virus in CELISA with sucrose acetone extracted and recombinant antigens (Table 2) are summarised in Table 8 (titres ≥10 are recorded as positive).

Table 8. Summary of the results obtained on 35 serum samples from small mammals experimentally infected with CCHF virus in CELISA with sucrose acetone extracted and recombinant antigens (titres ≥10 are recorded as positive). (The full results are presented in Table 2.)

	Recombinant antigen	
Sucrose acetone extracted antigen	**Positive**	**Negative**
Positive	17	0
Negative	2	16

The sensitivity of the test with recombinant antigen is: 17/17 x 100 = 100%

The specificity of the tests with recombinant antigen is: 16/18 x 100 = 88.8%

3.9. Antigenic specificity of CCHF recombinant antigen

The antigenic specificity of the recombinant CCHF nucleocapsid protein purified protein was determined in an IgG sandwich ELISA using guinea pig sera raised against other nairoviruses antigenically related to CCHF virus, namely, Dugbe, Hazara and Nairobi sheep disease (NSD) viruses. Guinea pig sera against Dugbe and Hazara viruses showed no reaction with the recombinant antigen. The serum against NSD virus reacted positively at a dilution of 1/100 and was negative at 1/400. Since specimens are routinely recorded as positive only if reaction is present at 1/400, the weak reaction with the recombinant antigen would be recorded as equivocal or non-specific.

3.10. Stability testing of CCHF recombinant antigen

Aliquots of recombinant antigen left at room temperature and at 37°C lost activity after two weeks; the antigen left at room temperature had lost about 25% of activity and the one left at 37°C lost about 45%. By the end of the first month the aliquots left at room temperature had already lost about 75% of activity whilst antigen left at 37°C had almost completely lost activity. The aliquots left at 4°C were stable up to the fifth month of testing with <1% of activity lost. Freeze drying did not affect the activity of the recombinant antigen.

4.0 Discussion

As mentioned in the introduction, CCHF is being recognized with increasing frequency as a cause of illness in Africa, probably as a result of increased awareness of the disease and the availability of a specific diagnostic service in South Africa (Swanepoel, 1998). The virus has the propensity to cause nosocomial infection, and hence rapid and accurate laboratory confirmation of the diagnosis is important to allow for timely isolation of the patient and the institution of barrier-nursing procedures for the protection of health care personnel and the community at large, and also to allow for the institution of appropriate therapy for the patient. However, it has been the experience of the SPU over the past 21 years that the vast majority of suspected cases of haemorrhagic fever, approximately 95%, prove to be severe cases of more common diseases, such as bacterial septicaemias, viral hepatitis or even malaria (unpublished laboratory records). Nevertheless, these false alarms can be extremely inconvenient and costly for hospitals since barrier-nursing is an expensive procedure to implement which frequently requires the emptying of wards in order to place patients in isolation, sometimes with resultant overloading of neighbouring medical facilities. In particular, the dedication of a modern multi-bed intensive care ward, with highly trained staff and expensive equipment to the care of a single patient, can be extremely costly. Thus, it is almost equally important to eliminate false suspected cases of haemorrhagic fever rapidly as it is to confirm genuine cases.

Laboratory investigation of CCHF is undertaken only in BSL4 laboratories, such as that operated by the SPU for South Africa. However, the virus is widely distributed in Africa, Eastern Europe and Asia, and in recent years the SPU has diagnosed cases of the disease or found serological evidence of the presence of the virus in several countries in Africa and in the Middle East, including Iraq, Iran, Afghanistan, and Pakistan (unpublished laboratory records). As mentioned in the Introduction many of these countries do not have the finances to build and maintain BSL4 laboratories of their own, and the SPU has received several requests from health authorities in these countries, and even from Australia and the World Health Organization in Geneva, to be of assistance in developing safe diagnostic procedures which can be used in less established laboratories.

A diagnosis of CCHF is generally confirmed in the laboratory by detection of viral RNA amplified by RT-PCR, isolation of virus, demonstration of seroconversion or a >4-fold increase in antibody

titre, or detection of specific IgM antibody activity (Shepherd *et al.*,1986; Burt, *et al.*, 1994; 1998) Of these techniques, the RT-PCR is more amenable to being performed safely than is the isolation of live virus, and this would probably suffice as a diagnostic technique if it could be supported by an antibody test using safe antigen, in particular an IgM test. In other words the RT-PCR can be performed safely outside a P4 laboratory on acute stage specimens in which antibody is not yet demonstrable. However after the acute stage, when viral nucleic acid is no longer present then an antibody test is required for convalescent phase sera in which an antibody response is demonstrable. In the instance of CCHF, these procedures could further be rendered safe through inactivating patients' blood or serum samples at 60°C/30 min before testing since the infectivity of the virus is thermolabile. An early publication on a limited number of confirmed CCHF patients showed that ELISA was a suitable method for detection of IgG amd IgM antibody to CCHF virus (Shepherd *et al.*, 1989c). The use of ELISA was further investigated using a larger number of samples which showed that the ELISA yielded results in close agreement with the IF test for detection of an antibody response and provided evidence that the ELISA using a sucrose acetone antigen was a suitable gold standard or reference test for serodiagnosis (Burt *et al.*, 1994). This study has now compared the ELISA using sucrose acetone antigen a potentially biohazardous antigen, with ELISA using a recombinant antigen based on the nucleocapsid protein of CCHF virus which is a non-infectious antigen.

A further demand for safe reagents for performing antibody tests has arisen from the requirement imposed by certain countries which are free of CCHF, notably countries in the European Union, that live animals of susceptible species from countries where the virus is present should be free of antibody as a pre-requisite to importation. The requirement applies particularly to ostriches, and was imposed following an outbreak of CCHF among workers in an ostrich abattoir in Oudsthoorn, South Africa, in 1996 (Swanepoel *et al.,* 1998). Since then the SPU has tested serum from large numbers of exported ostriches on behalf of farmers in South Africa and neighbouring states. Moreover, the birds are re-bled on arrival in Europe and serum sent back to SPU for re-testing. Similar restrictions are imposed on the international movement of certain zoo animals. It would be most convenient if importing and exporting countries could perform their own tests. Furthermore, many countries where the virus is known or suspected to be present wish to conduct serosurveys on livestock and wild animals in order to determine the prevalence of CCHF virus. For most domestic animal species

anti-immunoglobulin peroxidase conjugates which permit ELISA to be performed with CCHF antigen are commercially available, and for the testing of wild animal and ostrich sera for which no conjugates are available, the SPU developed the CELISA (Burt *et al.,* 1993). Thus it is clear that safe antigen is needed for veterinary use in addition to the requirement for diagnosing human infections.

Recombinant antigens are being used more frequently as diagnostic antigens, and apart from safety they have the advantage that they reduce the use of laboratory animals since sucrose acetone extracted mouse brain is currently the most effective CCHF antigen. Monoclonal antibody studies have shown that the nucleocapsid is the most highly conserved viral protein (Blackburn *et al.,* 1987), and it is therefore most useful for diagnostic assays. Recombinant antigens have previously been investigated as diagnostic tools for CCHF (Marriott *et al.,* 1994). The investigators produced a full length nucleocapsid protein as well as three nucleoprotein-derived peptides from the cDNA of CCHF virus. Two of the peptides had poor antigenic reactivity, but the full length nucleoprotein and one of the peptides had some potential for use as diagnostic tools since each of these two reagents reacted with 6/8 known positive human sera. Full evaluation would require a larger collection of positive sera, and there does not appear to have been further development of these antigens.

The failure of the recombinant baculovirus produced in the present project to express antigenically active nucleoprotein is not surprising since this has seldom been achieved successfully, and indeed the inadequately antigenic recombinant nucleoprotein and peptides received from CDC were produced in an M.Sc. project in Atlanta. The 3 peptides showed even poorer reactivity than the baculovirus-expressed full length nucleoprotein, and this could be due to their method of expression as bacterial products, with incorrect folding or fusion of parts of the structure that affect antigenicity (Morii *et al.*,1998). However, baculovirus BacCCHF1 received from USAMRIID was found to express adequate quantities of nucleoprotein of suitable antigenic reactivity, which could be purified and evaluated against the comparatively large collection of sera available for the present study, including sera from confirmed human cases of CCHF, sera from experimentally infected sheep and small mammals, plus ostrich sera.

In the event, the nucleoprotein antigen prepared from BacCCHF1 produced extremely promising results in comparison with the reference sucrose acetone extracted antigen, although the congruity

of the results produced by the two antigens varied with the type of test (IgG ELISA, IgM ELISA and CELISA), and with the species from which the sera were derived (human patients, sheep, small mammals and ostriches) (Appendices B-N and Tables 1-8). For example, the results obtained for human IgM antibody had a 94.5 % sensitivity and 100% specificity in comparison with the results obtained with sucrose acetone antigen, whereas the sensitivity was only 82.1%, and specificity 100%, in the sheep IgM test (Sections 3.8.1 and 3.8.4). Similarly, sensitivity was relatively low in the ostrich CELISA (87.5%), and specificity in the small mammal CELISA (88.8%) (Sections 3.8.6 and 3.8.7). Nevertheless, all of the results obtained with the recombinant antigen lie within a range that should be amenable to relatively simple adjustment.

The sensitivity and specificity of serological tests are adjusted by varying the concentrations of reagents or the test conditions, but primarily by adjusting the cut off values for positivity. In this regard it is notable that by simply dropping the cut off level for positivity from a titre of 400 to 100, the sensitivity and specificity of ELISA for IgM and IgG in sheep sera with recombinant antigen both become 100%, as can be seen readily from inspection of Appendices I-N. In other words, there is complete congruity between results obtained in IgM and IgG tests on sheep sera with the two antigens if the cut off titre for positivity is adjusted to 100. However, the cut off titre of 400 used for IgM and IgG ELISA with sucrose acetone antigen is based on the analysis of results obtained on sera from hundreds of suspected and confirmed human cases of CCHF, and thousands of sera from ostriches and sheep, including experimentally infected animals, tested by the SPU over a period of years, and the value is calculated to retain adequate sensitivity while increasing specificity to the point where non-specific reactors in test populations are not recorded as false positives. It can be argued that for the purposes of the present study it would have been satisfactory to obtain large numbers of randomly bled samples from human or livestock subjects, such as human blood donors, in order to perform the necessary tests to adjust cut off values. However, it is a common misconception that tests with known positive sera can be evaluated in comparison with random samples from general populations; in reality tests should be evaluated in the context of the syndrome under investigation (Galen and Gambino, 1975). This means that the definitive evaluation of the recombinant antigen for use in the diagnosis of the human disease will have to be achieved by prospectively using it on suspected cases of CCHF, the vast majority of which can be expected to be false alarms on the basis of past experience. The present results serve to indicate that there is a sound basis for proceeding with such

prospective evaluation of the recombinant antigen.

In the broad sense discussed above, specificity refers to the ability of a test or antigen to avoid producing false positive reactions when applied to samples from a particular syndrome. Antigenic specificity forms a component of this broader specificity; it is a measure of whether or not the test or antigen is likely to produce false positive results when applied to serum from individuals infected with known antigenic relatives of the pathogen under investigation. The antigenic specificity of the recombinant CCHF nucleocapsid antigen was assessed by performing ELISA with guinea pig sera raised against the closest known antigenic relatives of CCHF virus, namely, the nairoviruses Hazara, Dugbe, and Nairobi sheep disease (NSD). Weak cross-reactivity was found only with with the NSD antiserum. However, extensive tests on human patient and livestock sera have previously indicated that the only nairovirus other than CCHF likely to be encountered in southern Africa is Dugbe virus, and this is likely to be restricted to locations where ticks of the genus *Amblyomma* occur (Burt *et al.*, 1996).

The remarkable stability of the recombinant antigen observed at 4°C bodes well for its use as a diagnostic antigen (Section 3.10). The antigen retained virtually all of its potency for 5 months at 4°C, and this greatly exceeds the norm for commercially available ELISA reagents. Moreover, the stability of the antigen when subjected to freeze drying should facilitate distribution of the reagent to other laboratories.

It can be concluded that there are good grounds for proceeding with further evaluation of the recombinant nucleoprotein antigen expressed by the baculovirus BacCCHF1, through using the antigen prospectively in parallel with sucrose acetone and/or cell culture derived antigens (which lack potency and stability) in ELISA on human and livestock specimens routinely received by the SPU and other institutions engaged in similar work on CCHF.

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Appendix A: Buffers, reagents, pbluebac restriction map

A. Preparation of Baculovirus

1. Isolation of pBluebac plasmid from *E. coli*

Lauria Bertani (LB) Broth. pH 7.5: 1% Bacto-tryptone
0.5% Bacto-Yeast extract
1% NaCl

Lysosome solution: 2μg/ml lysosome
50mM glucose
10mM EDTA
25mM Tris-Cl, pH 8.0

Tris-EDTA (TE) buffer, pH 8.0: 10mM Tris
1mM EDTA

2. Restriction enzyme digest and recovery of DNA from the gel

Tris-acetate EDTA (TAE) buffer, pH 8.0: 0.04 M Tris-acetate
0.001 M EDTA

3. Dephosphorylation of the pFASTBac 1 vector

10X calf intestinal alkaline phophatase (CIP) buffer: 0.5M Tris -HCl
10mM $MgCl_2$
1mM $ZnCl_2$
10mM spermidine, pH 9.0

10X Sodium-Tris-EDTA (STE) buffer, pH 8.0: 100mM Tris-Cl
1M NaCl
10mM EDTA

4. Transformation of DH5α competent cells

Lauria Bertani (LB) agar: 10g Bactotryptone
5g Yeast extract
10g NaCl
12g Bacteriological agar

Stir to dissolve and autoclave. Cool to 55^0 C and add 50μg/ml of kanamycin sulphate, 10μg/ml of tetracycline, 7μg/ml of gentamycin, 100μg/ml of Bluo-gal and 40 μg/ml of IPTG. Stir and pour to a plate.

S.O.C. medium: 2g Bactotryptone
0.55g Yeast extract
1ml 1M NaCl
1ml 1M KCl, pH 7.0

Autoclave and cool to 55^0 C, add 1 ml 2 M Mg^{++} (1 M $MgCl_2$, 1 M $MgSO_4$) and add 1 ml of glucose. Filter through a 0.2μm filter unit.

5. Isolation of recombinant bacmid DNA

Solution I: 15 mM Tris-Cl
10 mM EDTA
100μg/ml Rnase A, pH 8.0

Solution II: 0.2 N NaoH
1% SDS

B. Purification of protein

NET/BSA: 150mM NaCl
5mM EDTA
50mM Tris-HCl
0.5% NP40
1mg/ml BSA

0.1% sodium dodecyl sulphate (SDS)

C. Western blot

Acrylamide-bisacrylamide: Prepared in ratio 30:0.8 and stored in dark bottle

10X Electrophoresis bath buffer: 0.25M Tris-HCl, pH 8.3
1.92M glycine
1% SDS

10% resolving gel: 1M Tris-HCl, pH 8.8

26.7 ml	Acrylamide/bisacrylamide (30:0.8)
30 ml	1M Tris-HCl, pH 8.8
0.8 ml	10% SDS
18.5 ml	distilled water
4 ml	1.% ammonium persulphate
20 μl	TEMED

4% stacking gel: 1M Tris-HCl, pH 6.8

2 ml	Acrylamide/bisacrylamide (30:0.8)
1.9 ml	1M Tris-HCl, pH 6.8
0.15 ml	10% SDS
1 ml	80% glycerol
9.25 ml	distilled water
0.7 ml	1.5% ammonium persulphate
20 μl	TEMED

2X SDS loading buffer:

0.06M Tris-HCl, pH 6.8

3% dithiothreiltol

4% SDS

40% glycerol

0.005% bromophenol blue

Transfer buffer: 2.9g glycine

5.8g Tris-HCl

0.37g SDS

200ml methanol, pH 8.3 per litre

Coomasie blue : O.1% Coomasie blue in water:methanol:acetic acid (5:5:2 by volume)

Tris-buffered saline (TBS): 6.05g Tris base

8.76g sodium chloride

800 ml distilled. water, adjust pH to 7.5 with hydrochloric acid

Tris buffered saline tween (TBST): 0.1% Tween 20/TBS

1% blocking solution: 1% skimmed milk in phosphae buffered saline pH 7.4

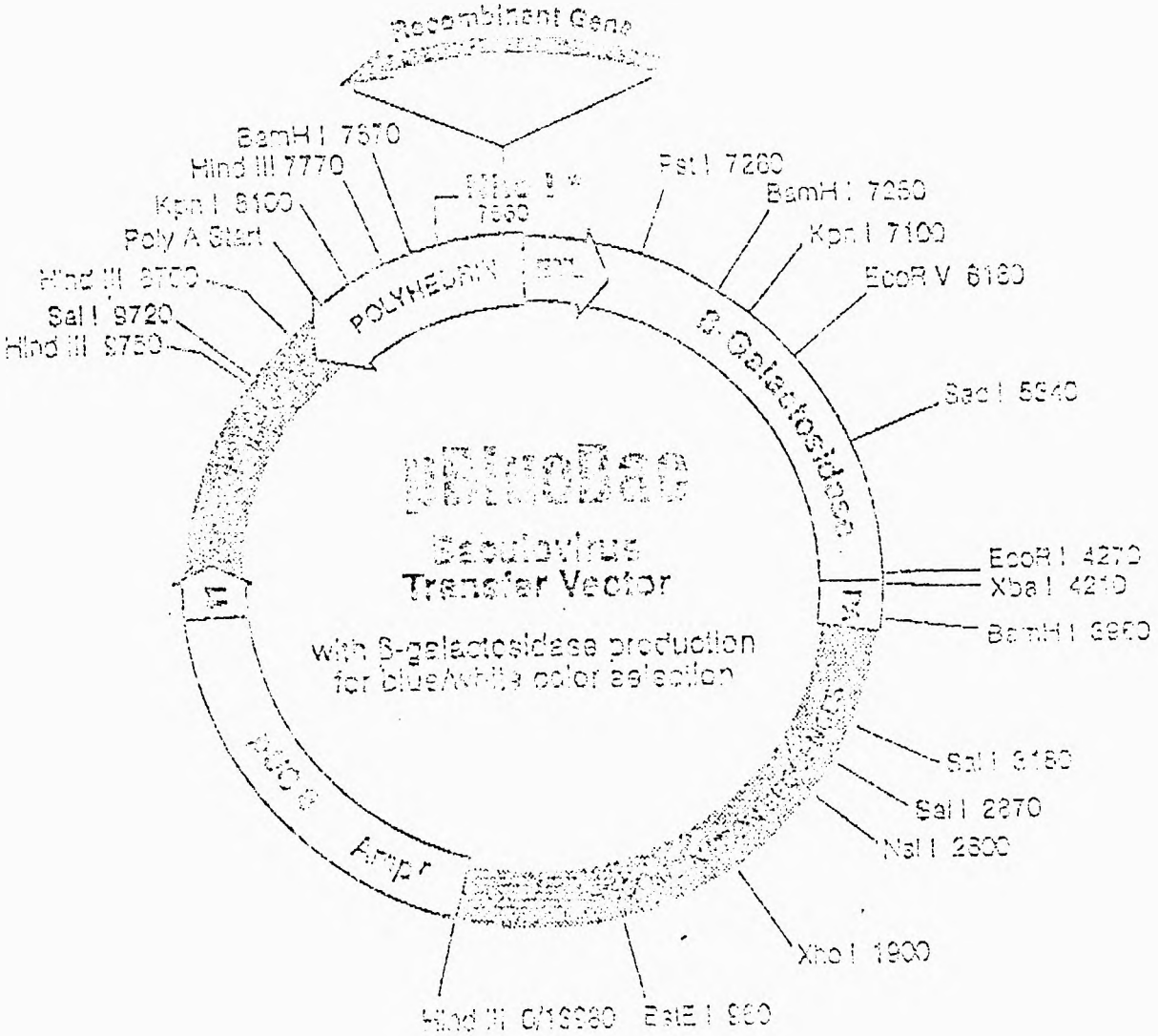
Ponceau S solution: 2g Ponceau S

30g trichloroacetic acid

30g sulfosalcylic acid

Add water to 100 ml

D: pBlueBac transfer plasmid map, Vialard *et al.*, (1990).



Nhe I out vector accepts cDNA with
Nhe I, Xba I and Spe I overhangs.

Appendix B: IgM antibody titre to CCHF virus in relation to day after the onset of illness as detected by IgM capture ELISA using sucrose acetone extracted antigen, in serum samples collected from CCHF virus infected patients from 1986 to 1999.

Specimen number	Day of illness	IgM titre	Interpretation
70/98	D5	0	neg
198/96	D6	0	neg
198/98	D6	6400	pos
50/95	D6	1600	pos
47/97/10	D7	0	neg
214/96	D7	6400	pos
51/95	D7	1600	pos
344/98	D7	1600	pos
184/96	D7	6400	pos
123/95	D7	100	neg
95/95	D8	100	neg
279/98	D8	6400	pos
74/96	D8	400	pos
48/97/10	D8	0	neg
181/96	D8	0	neg
182/96	D8	6400	pos
55/95	D8	6400	pos
77/96	D9	1600	pos
58/95	D9	6400	pos
365/95	D9	1600	pos
193/96	D9	6400	pos
190/96	D9	6400	pos
201/96	D9	6400	pos
183/96	D9	6400	pos
288/98	D10	6400	pos
47/97/5	D10	6400	pos
197/96	D10	6400	pos
207/96	D10	0	neg
191/96	D10	6400	pos
47/97/4	D10	6400	pos
199/96	D10	6400	pos
200/96	D10	6400	pos
208/96	D10	6400	pos
280/98	D10	6400	pos
209/96	D10	6400	pos
226/96	D10	6400	pos
78/96	D10	6400	pos
59/95	D10	6400	pos
363/95	D10	6400	pos
103/95	D10	6400	pos
47/97/12	D11	6400	pos

Appendix B (contd)

Specimen number	Day of illness	IgM titre	Interpetation
104/95	D11	6400	pos
81/96	D11	400	pos
47/92/1	D11	400	pos
62/95	D11	6400	pos
47/97/2	D11	6400	pos
192/96	D11	6400	pos
47/97/9	D11	400	pos
48/97/5	D11	6400	pos
46/97/13	D11	6400	pos
196/96	D11	6400	pos
47/97/11	D11	6400	pos
48/97/4	D11	6400	pos
47/97/14	D11	6400	pos
47/97/15	D11	6400	pos
47/97/12	D12	6400	pos
81/98	D12	1600	pos
194/96	D12	1600	pos
47/97/2	D12	6400	pos
48/97/3	D12	6400	pos
48/97/15	D12	6400	pos
48/97/9	D12	6400	pos
210/96	D12	6400	pos
47/97/8	D12	6400	pos
49/97/2	D12	6400	pos
48/97/11	D12	6400	pos
82/96	D12	400	pos
63/95	D12	6400	pos
370/95	D12	6400	pos
105/95	D12	6400	pos
48/97/1	D12	1600	pos
48/97/12	D12	6400	pos
48/97/2	D12	6400	pos
83/96	D13	100	neg
371/95	D13	6400	pos
48/97/15	D13	6400	pos
47/97/6	D13	1600	pos
225/96	D13	1600	pos
48/97/8	D13	6400	pos
48/97/13	D13	6400	pos
47/97/16	D13	6400	pos
48/97/16	D14	6400	pos
372/95	D14	6400	pos
48/97/6	D14	1600	pos
84/96	D14	400	pos

Appendix B (contd)

Specimen number	Day of illness	IgM titre	Interpretation
373/95	D15	6400	pos
85/96	D15	1600	pos
49/97/1	D16	1600	pos
86/96	D16	1600	pos
19/96	D16	6400	pos
90/96	D17	6400	pos
92/96	D19	6400	pos
74/95	D19	6400	pos
97/96	D21	6400	pos
374/95	D21	6400	pos
99/96	D23	6400	pos
50/97/5	WK4	6400	pos
50/97/4	WK4	1600	pos
94/95	WK4	6400	pos
50/97/2	WK4	1600	pos
50/97/1	WK4	6400	pos
50/97/6	WK4	6400	pos
51/97/6	WK5	0	neg
51/97/7	WK5	0	neg
51/97/9	WK5	1600	pos
51/97/1	WK5	1600	pos
51/97/5	WK5	0	neg
51/97/4	WK5	1600	pos
51/97/10	WK5	6400	pos
51/97/8	WK5	0	neg
51/97/1	WK5	1600	pos
51/97/2	WK5	1600	pos
51/97/11	WK5	400	pos
51/97/12	WK5	100	pos
40/99	WK6	6400	pos
121/99	WK14	6400	pos
43/89	WK17	0	neg
525/99	WK17	6400	pos
309/87	WK19	0	neg
219/89	WK19	6400	pos
62/89	WK19	0	neg
573/99	WK20	6400	pos
131/87	WK21	0	neg
281/88	WK26	6400	pos
140/96	WK28	0	neg
350/89	WK32	0	neg
298/88	WK33	0	neg
507/87	WK33	0	neg
496/87	WK33	0	neg
247/88	WK38	6400	pos
414/88	WK40	6400	pos
73/89	WK44	0	neg
413/88	WK45	6400	pos
508/87	M13	0	neg
85/88	M16	0	neg

Appendix C: IgM antibody titre to CCHF virus in relation to day after the onset of illness as detected by IgM capture ELISA using recombinant antigen, in serum samples collected from CCHF virus infected patients from 1986 to 1999.

Specimen number	Day of illness	IgM titre	Interpretation
70/98	D5	0	neg
198/96	D6	0	neg
198/98	D6	1600	pos
50/95	D6	1600	pos
47/97/10	D7	0	neg
214/96	D7	6400	pos
51/95	D7	1600	pos
344/98	D7	400	pos
184/96	D7	6400	pos
123/95	D7	0	neg
95/95	D8	100	neg
279/98	D8	1600	pos
74/96	D8	0	neg
48/97/10	D8	0	neg
181/96	D8	0	neg
182/96	D8	1600	pos
55/95	D8	1600	pos
77/96	D9	0	neg
58/95	D9	6400	pos
365/95	D9	1600	pos
193/96	D9	6400	pos
190/96	D9	6400	pos
201/96	D9	6400	pos
183/96	D9	1600	pos
288/98	D10	6400	pos
47/97/5	D10	6400	pos
197/96	D10	1600	pos
207/96	D10	0	neg
191/96	D10	6400	pos
47/97/4	D10	1600	pos
199/96	D10	6400	pos
200/96	D10	6400	pos
208/96	D10	6400	pos
280/98	D10	6400	pos
209/96	D10	6400	pos
226/96	D10	1600	pos
78/96	D10	100	neg
59/95	D10	6400	pos
363/95	D10	1600	pos
103/95	D10	1600	pos
47/97/12	D11	6400	pos
104/95	D11	1600	pos
81/96	D11	400	pos

Appendix C (contd)

Specimen number	Day of illness	IgM titre	Interpretation
47/92/1	D11	100	neg
62/95	D11	1600	pos
47/97/2	D11	6400	pos
192/96	D11	6400	pos
47/97/9	D11	6400	pos
48/97/5	D11	6400	pos
46/97/13	D11	1600	pos
196/96	D11	6400	pos
47/97/11	D11	6400	pos
48/97/4	D11	1600	pos
47/97/14	D11	1600	pos
47/97/15	D11	1600	pos
47/97/12	D12	1600	pos
81/98	D12	6400	pos
194/96	D12	1600	pos
47/97/2	D12	6400	pos
48/97/3	D12	6400	pos
48/97/15	D12	6400	pos
48/97/9	D12	1600	pos
210/96	D12	6400	pos
47/97/8	D12	6400	pos
49/97/2	D12	1600	pos
48/97/11	D12	6400	pos
82/96	D12	400	pos
63/95	D12	6400	pos
370/95	D12	6400	pos
105/95	D12	1600	pos
48/97/1	D12	1600	pos
48/97/12	D12	6400	pos
48/97/2	D12	6400	pos
83/96	D13	100	neg
371/95	D13	6400	pos
48/97/15	D13	1600	pos
47/97/6	D13	1600	pos
225/96	D13	1600	pos
48/97/8	D13	1600	pos
48/97/13	D13	1600	pos
47/97/16	D13	6400	pos
48/97/16	D14	6400	pos
372/95	D14	6400	pos
48/97/6	D14	1600	pos
84/96	D14	400	pos
373/95	D15	6400	pos
85/96	D15	1600	pos
49/97/1	D16	1600	pos
86/96	D16	400	pos
19/96	D16	6400	pos
90/96	D17	1600	pos
92/96	D19	1600	pos

Appendix C (contd)

Specimen number	Day of illness	IgM titre	Interpretation
74/95	D19	6400	pos
97/96	D21	1600	pos
374/95	D21	6400	pos
99/96	D23	1600	pos
50/97/4	WK4	1600	pos
50/97/5	WK4	1600	pos
94/95	WK4	6400	pos
50/97/2	WK4	0	neg
50/97/1	WK4	1600	pos
50/97/6	WK4	1600	pos
51/97/6	WK5	0	neg
51/97/7	WK5	0	neg
51/97/9	WK5	1600	pos
51/97/1	WK5	400	pos
51/97/5	WK5	0	neg
51/97/4	WK5	0	neg
51/97/10	WK5	1600	pos
51/97/8	WK5	0	neg
51/97/1	WK5	6400	pos
51/97/2	WK5	1600	pos
51/97/11	WK5	400	pos
51/97/12	WK5	400	pos
40/99	WK6	6400	pos
121/99	WK14	1600	pos
43/89	WK17	0	neg
525/99	WK17	1600	pos
309/87	WK19	0	neg
219/89	WK19	6400	pos
62/89	WK19	0	neg
573/99	WK20	1600	pos
131/87	WK21	0	neg
281/88	WK26	1600	pos
140/96	WK28	0	neg
350/89	WK32	0	neg
298/88	WK33	0	neg
507/87	WK33	0	neg
496/87	WK33	0	neg
247/88	WK38	1600	pos
414/88	WK40	400	pos
73/89	WK44	0	neg
413/88	WK45	400	pos
508/87	M13	0	neg
85/88	M16	0	neg

Appendix D: IgG antibody titre to CCHF virus in relation to day after the onset of illness as detected by IgG sandwich ELISA using sucrose acetone extracted antigen, in serum samples collected from CCHF virus infected patients from 1986 to 1999.

Specimen number	Day of illness	IgG titre	Interpretation
70/98	D5	0	neg
198/96	D6	0	neg
50/95	D6	0	neg
198/98	D6	0	neg
214/96	D7	1600	pos
123/95	D7	0	neg
47/97/10	D7	0	neg
51/95	D7	0	neg
184/96	D7	400	pos
344/98	D7	400	pos
55/95	D8	400	pos
95/95	D8	0	neg
182/96	D8	400	pos
48/97/10	D8	0	neg
279/98	D8	0	neg
74/96	D8	0	neg
181/96	D8	0	neg
58/95	D9	1600	pos
201/96	D9	400	pos
183/96	D9	400	pos
190/96	D9	1600	pos
193/96	D9	400	pos
77/96	D9	100	pos
365/95	D9	0	neg
197/96	D10	1600	pos
47/97/5	D10	400	pos
288/98	D10	400	pos
363/95	D10	100	pos
47/97/4	D10	1600	pos
191/96	D10	1600	pos
78/96	D10	100	pos
207/96	D10	0	neg
199/96	D10	6400	pos
226/96	D10	400	pos
208/96	D10	6400	pos
200/96	D10	1600	pos
103/95	D10	100	pos
280/98	D10	400	pos
209/96	D10	6400	pos
59/95	D10	1600	pos
47/97/14	D11	1600	pos
47/97/15	D11	1600	pos
46/97/13	D11	100	pos
81/96	D11	400	pos
104/95	D11	6400	pos

Appendix D(contd)

Specimen number	Day of illness	IgG titre	Interpretation
62/95	D11	6400	pos
192/96	D11	6400	pos
47/97/2	D11	1600	pos
47/92/1	D11	1600	pos
47/97/9	D11	400	pos
47/97/11	D11	6400	pos
47/97/12	D11	400	pos
48/97/4	D11	1600	pos
48/97/5	D11	1600	pos
196/96	D11	1600	pos
48/97/1	D12	400	pos
48/97/11	D12	6400	pos
49/97/2	D12	400	pos
47/97/2	D12	6400	pos
48/97/15	D12	1600	pos
210/96	D12	400	pos
48/97/12	D12	100	pos
47/97/12	D12	400	pos
48/97/9	D12	400	pos
105/95	D12	6400	pos
63/95	D12	6400	pos
82/96	D12	1600	pos
370/95	D12	6400	pos
48/97/2	D12	6400	pos
47/97/8	D12	1600	pos
48/97/3	D12	6400	pos
194/96	D12	400	pos
81/98	D12	100	pos
371/95	D13	6400	pos
48/97/13	D13	400	pos
83/96	D13	1600	pos
225/96	D13	1600	pos
47/97/6	D13	100	pos
48/97/8	D13	1600	pos
48/97/15	D13	1600	pos
47/97/16	D13	400	pos
48/97/6	D14	400	pos
48/97/16	D14	400	pos
372/95	D14	6400	pos
84/96	D14	1600	pos
373/95	D15	6400	pos
85/96	D15	6400	pos
86/96	D16	6400	pos
19/96	D16	6400	pos
49/97/1	D16	1600	pos
90/96	D17	6400	pos
92/96	D19	6400	pos
74/95	D19	6400	pos
97/96	D21	6400	pos

Appendix D(contd)

Specimen number	Day of illness	IgG titre	Interpretation
374/95	D21	6400	pos
99/96	D23	6400	pos
50/97/6	WK4	6400	pos
50/97/5	WK4	6400	pos
50/97/2	WK4	6400	pos
50/97/1	WK4	6400	pos
94/95	WK4	6400	pos
50/97/4	WK4	6400	pos
51/97/7	WK5	6400	pos
51/97/6	WK5	6400	pos
51/97/1	WK5	6400	pos
51/97/5	WK5	6400	pos
51/97/4	WK5	6400	pos
51/97/2	WK5	6400	pos
51/97/8	WK5	6400	pos
51/97/1	WK5	6400	pos
51/97/12	WK5	6400	pos
51/97/10	WK5	6400	pos
51/97/9	WK5	6400	pos
51/97/11	WK5	6400	pos
40/99	WK6	6400	pos
121/99	WK14	6400	pos
525/99	WK17	6400	pos
43/89	WK17	6400	pos
309/87	WK19	6400	pos
219/89	WK19	6400	pos
62/89	WK19	6400	pos
573/99	WK20	6400	pos
131/87	WK21	6400	pos
281/88	WK26	6400	pos
140/96	WK28	6400	pos
350/89	WK32	0	neg
507/87	WK33	6400	pos
496/87	WK33	6400	pos
298/88	WK33	6400	pos
247/88	WK38	6400	pos
414/88	WK40	6400	pos
73/89	WK44	6400	pos
413/88	WK45	6400	pos
508/87	M13	6400	pos
85/88	M16	6400	pos

Appendix E: IgG antibody titre to CCHF virus in relation to day after the onset of illness as detected by IgG sandwich ELISA using recombinant antigen, in serum samples collected from CCHF virus infected patients from 1986 to 1999.

Specimen number	Day of illness	IgG titre	Interpretation
70/98	D5	0	neg
198/96	D6	0	neg
198/98	D6	0	neg
50/95	D6	0	neg
184/96	D7	400	pos
51/95	D7	0	neg
214/96	D7	100	pos
123/95	D7	0	neg
344/98	D7	400	pos
47/97/10	D7	0	neg
55/95	D8	400	pos
182/96	D8	0	neg
279/98	D8	0	neg
48/97/10	D8	0	neg
95/95	D8	0	neg
74/96	D8	0	neg
181/96	D8	0	neg
190/96	D9	0	neg
77/96	D9	100	pos
183/96	D9	400	pos
365/95	D9	0	neg
201/96	D9	100	pos
193/96	D9	100	pos
58/95	D9	1600	pos
47/97/5	D10	400	pos
197/96	D10	400	pos
288/98	D10	400	pos
207/96	D10	0	neg
191/96	D10	400	pos
47/97/4	D10	400	pos
199/96	D10	6400	pos
200/96	D10	100	pos
208/96	D10	1600	pos
280/98	D10	100	pos
209/96	D10	400	pos
226/96	D10	400	pos
78/96	D10	100	pos
59/95	D10	100	pos
363/95	D10	400	pos
103/95	D10	100	pos
192/96	D11	400	pos
47/97/2	D11	1600	pos
81/96	D11	400	pos
47/92/1	D11	100	pos
62/95	D11	100	pos
104/95	D11	100	pos

Appendix E(contd)

Specimen number	Day of illness	IgG titre	Interpretation
48/97/4	D11	400	pos
47/97/14	D11	1600	pos
196/96	D11	400	pos
47/97/9	D11	400	pos
46/97/13	D11	100	pos
47/97/12	D11	100	pos
48/97/5	D11	400	pos
47/97/15	D11	1600	pos
47/97/11	D11	6400	pos
82/96	D12	100	pos
47/97/8	D12	400	pos
194/96	D12	400	pos
210/96	D12	400	pos
48/97/11	D12	6400	pos
81/98	D12	100	pos
49/97/2	D12	100	pos
63/95	D12	400	pos
48/97/9	D12	400	pos
48/97/12	D12	400	pos
47/97/12	D12	100	pos
48/97/2	D12	1600	pos
48/97/15	D12	1600	pos
370/95	D12	100	pos
105/95	D12	100	pos
47/97/2	D12	1600	pos
48/97/3	D12	1600	pos
48/97/1	D12	100	pos
48/97/15	D13	1600	pos
83/96	D13	400	pos
225/96	D13	400	pos
371/95	D13	100	pos
47/97/6	D13	400	pos
48/97/8	D13	400	pos
48/97/13	D13	400	pos
47/97/16	D13	400	pos
372/95	D14	1600	pos
84/96	D14	1600	pos
48/97/6	D14	400	pos
48/97/16	D14	400	pos
373/95	D15	1600	pos
85/96	D15	1600	pos
86/96	D16	400	pos
19/96	D16	1600	pos
49/97/1	D16	400	pos
90/96	D17	6400	pos
92/96	D19	6400	pos
74/95	D19	1600	pos
97/96	D21	1600	pos

Appendix E(contd)

Specimen number	Day of illness	IgG titre	Interpretation
374/95	D21	6400	pos
99/96	D23	6400	pos
50/97/5	WK4	6400	pos
50/97/4	WK4	1600	pos
94/95	WK4	6400	pos
50/97/2	WK4	1600	pos
50/97/6	WK4	400	pos
50/97/1	WK4	1600	pos
51/97/6	WK5	6400	pos
51/97/7	WK5	6400	pos
51/97/9	WK5	6400	pos
51/97/1	WK5	6400	pos
51/97/5	WK5	6400	pos
51/97/4	WK5	1600	pos
51/97/10	WK5	6400	pos
51/97/8	WK5	6400	pos
51/97/1	WK5	1600	pos
51/97/2	WK5	6400	pos
51/97/11	WK5	1600	pos
51/97/12	WK5	1600	pos
40/99	WK6	6400	pos
121/99	WK14	6400	pos
43/89	WK17	6400	pos
525/99	WK17	6400	pos
219/89	WK19	6400	pos
309/87	WK19	6400	pos
62/89	WK19	6400	pos
573/99	WK20	6400	pos
131/87	WK21	6400	pos
281/88	WK26	6400	pos
140/96	WK28	6400	pos
350/89	WK32	0	neg
298/88	WK33	6400	pos
507/87	WK33	6400	pos
496/87	WK33	6400	pos
247/88	WK38	6400	pos
414/88	WK40	6400	pos
73/89	WK44	6400	pos
413/88	WK45	6400	pos
508/87	M13	6400	pos
85/88	M16	6400	pos

Appendix F: Total antibody titre to CCHF virus in relation to day after the onset of illness as detected by CELISA using sucrose acetone extracted antigen, in serum samples from CCHF virus infected patients collected from 1986 to 1999

Specimen number	Day of illness	Total antibody titre	Interpretation
70/98	D5	0	neg
198/96	D6	0	neg
198/98	D6	0	neg
50/95	D6	0	neg
47/97/10	D7	40	pos
214/96	D7	40	pos
51/95	D7	80	pos
344/98	D7	0	neg
184/96	D7	160	pos
123/95	D7	0	neg
95/95	D8	80	pos
279/98	D8	20	pos
74/96	D8	0	neg
48/97/10	D8	320	pos
181/96	D8	0	neg
182/96	D8	40	pos
55/95	D8	0	neg
77/96	D9	80	pos
58/95	D9	0	neg
365/95	D9	40	pos
193/96	D9	0	neg
190/96	D9	40	pos
201/96	D9	40.	pos
183/96	D9	40	pos
288/98	D10	80	pos
47/97/5	D10	40	pos
197/96	D10	20	pos
207/96	D10	40	pos
191/96	D10	0	neg
47/97/4	D10	320	pos
199/96	D10	0	neg
200/96	D10	40	pos
208/96	D10	320	pos
280/98	D10	80	pos
209/96	D10	160	pos
226/96	D10	160	pos
78/96	D10	160	pos
59/95	D10	160	pos
363/95	D10	40	pos
103/95	D10	40	pos
47/97/12	D11	40	pos
104/95	D11	20	pos
81/96	D11	0	neg
47/92/1	D11	0	neg
62/95	D11	40	pos

Appendix F(contd)

Specimen number	Day of illness	Total antibody titre	Interpretation
47/97/2	D11	40	pos
192/96	D11	20	pos
47/97/9	D11	0	neg
48/97/5	D11	160	pos
46/97/13	D11	40	pos
196/96	D11	160	pos
47/97/11	D11	0	neg
48/97/4	D11	0	neg
47/97/14	D11	20	pos
47/97/15	D11	20	pos
47/97/12	D12	40	pos
81/98	D12	0	neg
194/96	D12	40	pos
47/97/2	D12	0	neg
48/97/3	D12	0	neg
48/97/15	D12	40	pos
48/97/9	D12	40	pos
210/96	D12	40	pos
47/97/8	D12	0	neg
49/97/2	D12	160	pos
48/97/11	D12	160	pos
82/96	D12	40	pos
63/95	D12	0	neg
370/95	D12	20	pos
105/95	D12	20	pos
48/97/1	D12	40	pos
48/97/12	D12	20	pos
48/97/2	D12	0	neg
83/96	D13	40	pos
371/95	D13	0	neg
48/97/15	D13	40	pos
47/97/6	D13	40	pos
225/96	D13	80	pos
48/97/8	D13	0	neg
48/97/13	D13	0	neg
47/97/16	D13	80	pos
48/97/16	D14	80	pos
372/95	D14	0	neg
48/97/6	D14	40	pos
84/96	D14	40	pos
373/95	D15	40	pos
85/96	D15	40	pos
49/97/1	D16	80	pos
86/96	D16	40	pos
19/96	D16	40	pos
90/96	D17	80	pos
92/96	D19	160	pos
74/95	D19	80	pos
97/96	D21	80	pos

Appendix F(contd)

Specimen number	Day of illness	Total antibody titre	Interpretation
374/95	D21	80	pos
99/96	D23	80	pos
50/97/4	WK4	80	pos
50/97/5	WK4	40	pos
94/95	WK4	40	pos
50/97/2	WK4	40	pos
50/97/1	WK4	0	neg
50/97/6	WK4	20	pos
51/97/6	WK5	80	pos
51/97/7	WK5	80	pos
51/97/9	WK5	40	pos
51/97/1	WK5	40	pos
51/97/5	WK5	40	pos
51/97/4	WK5	40	pos
51/97/10	WK5	40	pos
51/97/8	WK5	40	pos
51/97/1	WK5	20	pos
51/97/2	WK5	80	pos
51/97/11	WK5	40	pos
51/97/12	WK5	40	pos
40/99	WK6	320	pos
121/99	WK14	640	pos
43/89	WK17	2560	pos
525/99	WK17	2560	pos
309/87	WK19	320	pos
219/89	WK19	640	pos
62/89	WK19	320	pos
573/99	WK20	160	pos
131/87	WK21	2560	pos
281/88	WK26	1280	pos
140/96	WK28	320	pos
350/89	WK32	0	neg
298/88	WK33	320	pos
507/87	WK33	80	pos
496/87	WK33	320	pos
247/88	WK38	1280	pos
414/88	WK40	1280	pos
73/89	WK44	40	pos
413/88	WK45	640	pos
508/87	M13	640	pos
85/88	M16	1280	pos

Appendix G: Total antibody titre to CCHF virus in relation to day after the onset of illness detected by CELISA using recombinant antigen, in serum samples from CCHF virus infected patients collected from 1986 to 1999.

Specimen number	Day of illness	Total antibody titre	Interpretation
70/98	D5	0	neg
198/96	D6	40	pos
198/98	D6	0	neg
50/95	D6	0	neg
47/97/10	D7	80	pos
214/96	D7	40	pos
51/95	D7	40	pos
344/98	D7	0	neg
184/96	D7	80	pos
123/95	D7	0	neg
95/95	D8	160	pos
279/98	D8	80	pos
74/96	D8	0	neg
48/97/10	D8	0	neg
181/96	D8	80	pos
182/96	D8	0	neg
55/95	D8	20	pos
77/96	D9	160	pos
58/95	D9	160	pos
365/95	D9	0	neg
193/96	D9	40	pos
190/96	D9	40	pos
201/96	D9	40	pos
183/96	D9	20	pos
288/98	D10	160	pos
47/97/5	D10	160	pos
197/96	D10	160	pos
207/96	D10	160	pos
191/96	D10	0	neg
47/97/4	D10	160	pos
199/96	D10	0	neg
200/96	D10	40	pos
208/96	D10	80	pos
280/98	D10	320	pos
209/96	D10	160	pos
226/96	D10	40	pos
78/96	D10	80	pos
59/95	D10	80	pos
363/95	D10	80	pos
103/95	D10	40	pos
47/97/12	D11	160	pos
104/95	D11	80	pos
81/96	D11	20	pos
47/92/1	D11	0	neg
62/95	D11	80	pos
47/97/2	D11	80	pos

Appendix G(contd)

Specimen number	Day of illness	Total antibody titre	Interpretation
192/96	D11	80	pos
47/97/9	D11	40	pos
48/97/5	D11	80	pos
46/97/13	D11	40	pos
196/96	D11	40	pos
47/97/11	D11	40	pos
48/97/4	D11	0	neg
47/97/14	D11	40	pos
47/97/15	D11	40	pos
47/97/12	D12	80	pos
81/98	D12	40	pos
194/96	D12	80	pos
47/97/2	D12	0	neg
48/97/3	D12	40	pos
48/97/15	D12	80	pos
48/97/9	D12	80	pos
210/96	D12	80	pos
47/97/8	D12	160	pos
49/97/2	D12	40	pos
48/97/11	D12	80	pos
82/96	D12	40	pos
63/95	D12	40	pos
370/95	D12	40	pos
105/95	D12	40	pos
48/97/1	D12	80	pos
48/97/12	D12	0	neg
48/97/2	D12	20	pos
83/96	D13	640	pos
371/95	D13	0	neg
48/97/15	D13	40	pos
47/97/6	D13	0	neg
225/96	D13	40	pos
48/97/8	D13	0	neg
48/97/13	D13	40	pos
47/97/16	D13	80	pos
48/97/16	D14	80	pos
372/95	D14	0	neg
48/97/6	D14	0	neg
84/96	D14	40	pos
373/95	D15	80	pos
85/96	D15	80	pos
49/97/1	D16	320	pos
86/96	D16	40	pos
19/96	D16	40	pos
90/96	D17	80	pos
92/96	D19	160	pos
74/95	D19	160	pos
97/96	D21	320	pos
374/95	D21	40	pos

Appendix G(contd)

Specimen number	Day of illness	Total antibody titre	Interpretation
99/96	D23	80	pos
50/97/4	WK4	320	pos
50/97/5	WK4	0	neg
94/95	WK4	20	pos
50/97/2	WK4	40	pos
50/97/1	WK4	0	neg
50/97/6	WK4	40	pos
51/97/6	WK5	40	pos
51/97/7	WK5	40	pos
51/97/9	WK5	80	pos
51/97/1	WK5	80	pos
51/97/5	WK5	40	pos
51/97/4	WK5	0	neg
51/97/10	WK5	40	pos
51/97/8	WK5	40	pos
51/97/1	WK5	40	pos
51/97/2	WK5	80	pos
51/97/11	WK5	80	pos
51/97/12	WK5	20	pos
40/99	WK6	320	pos
121/99	WK14	1280	pos
43/89	WK17	1280	pos
525/99	WK17	640	pos
309/87	WK19	640	pos
219/89	WK19	80	pos
62/89	WK19	640	pos
573/99	WK20	1280	pos
131/87	WK21	2560	pos
281/88	WK26	1280	pos
140/96	WK28	640	pos
350/89	WK32	0	neg
298/88	WK33	320	pos
507/87	WK33	1280	pos
496/87	WK33	80	pos
247/88	WK38	1280	pos
414/88	WK40	1280	pos
73/89	WK44	40	pos
413/88	WK45	1280	pos
508/87	M13	1280	pos
85/88	M16	1280	pos

AppendixH: Optical density (OD) values obtained from screening non-CCHF patients by ELISA. Serum samples were tested by ELISA for detection of IgG, IgM and total antibody using sucrose acetone extracted (sa) and recombinant antigen (rec).

SPU no.	IgG sa[1]	IgG rec[2]	IgM sa[1]	IgM rec[2]	CELISA sa[1]	CELISA rec[2]
147/00	0.185	0.155	0.123	0.141	1.228	1.279
150/00	0.239	0.193	0.141	0.159	1.178	1.399
152/00	0.211	0.174	0.224	0.222	1.082	1.136
153/00	0.166	0.152	0.122	0.129	1.153	1.171
155/00	0.205	0.188	0.132	0.161	1.075	0.835
156/00	0.251	0.207	0.131	0.152	1.148	1.213
157/00	0.146	0.154	0.126	0.13	1.039	1.165
159/00	0.187	0.17	0.127	0.147	0.991	1.22
160/00	0.128	0.117	0.138	0.152	1.09	1.226
165/00	0.201	0.144	0.126	0.134	0.987	1.118
166/00	0.176	0.15	0.143	0.158	1.006	1.23
168/00	0.247	0.172	0.167	0.196	1.074	1.032
178/00	0.381	0.232	0.127	0.141	1.057	1.164
180.1/00	0.192	0.159	0.242	0.243	1.054	1.237
180.2/00	0.36	0.265	0.18	0.212	1.033	1.309
180.3/00	0.199	0.18	0.116	0.143	1.056	1.231
180.4/00	0.167	0.081	0.155	0.182	0.999	0.854
180.5/00	0.185	0.161	0.134	0.155	1.126	1.065
180.6/00	0.22	0.206	0.139	0.148	1.094	1.219
180.7/00	0.153	0.136	0.121	0.139	1.165	1.264
180.8/00	0.172	0.142	0.131	0.149	1.148	1.173
182/00	0.155	0.143	0.117	0.131	1.058	1.148
183/00	0.363	0.311	0.132	0.147	0.949	1.115
184/00	0.219	0.175	0.123	0.137	1.057	1.221
186/00	0.297	0.239	0.11	0.119	1.086	1.128
188/00	0.225	0.201	0.116	0.128	1.031	1.138
189/00	0.269	0.199	0.159	0.181	0.993	1.202
191/00	0.2	0.164	0.219	0.258	1.026	1.156
193/00	0.228	0.172	0.121	0.134	1.102	0.936
196/00	0.093	0.091	0.209	0.257	1.152	1.188
197/00	0.23	0.247	0.194	0.235	1.196	1.227
201/00	0.193	0.156	0.125	0.141	1.016	1.099
202/00	0.255	0.214	0.104	0.114	0.869	1.026
205/00	0.15	0.129	0.103	0.117	0.813	1.177
206/00	0.165	0.147	0.106	0.12	0.859	0.997
207/00	0.195	0.231	0.114	0.127	0.972	1.138
210/00	0.203	0.168	0.119	0.123	1.101	1.129
211/00	0.16	0.163	0.117	0.122	1.193	1.057
212/00	0.362	0.287	0.139	0.137	1.019	1.179
214/00	0.236	0.182	0.111	0.108	1.026	1.152
215/00	0.237	0.194	0.112	0.108	0.997	1.003
216/00	0.091	0.192	0.155	0.14	1.002	0.939
217/00	0.095	0.179	0.228	0.217	1.237	1.051
219/00	0.236	0.167	0.116	0.107	1.145	1.041
223/00	0.291	0.24	0.129	0.115	1.257	0.972

Appendix H (contd)

SPU no.	IgG sa	IgG rec	IgM sa	IgM rec	CELISA sa	CELISA rec
225/00	0.188	0.178	0.132	0.122	1.097	1.005
228/00	0.105	0.193	0.117	0.111	1.143	1.005
231/00	0.299	0.248	0.143	0.134	1.174	1.11
233/00	0.39	0.318	0.124	0.12	1.061	1.17
234/00	0.286	0.189	0.115	0.113	1.035	1.162
237/00	0.2	0.18	0.115	0.111	1.041	1.149
238/00	0.381	0.328	0.118	0.186	0.987	1.051
239/00	0.087	0.227	0.102	0.102	0.988	0.989
240/00	0.352	0.291	0.118	0.116	1	1.069
242/00	0.361	0.299	0.228	0.208	1.005	0.948
248/00	0.294	0.207	0.181	0.198	1.3	0.995
249/00	0.12	0.111	0.113	0.122	1.149	1.018
250/00	0.265	0.194	0.121	0.125	1.113	1.073
251/00	0.102	0.092	0.17	0.168	1.005	1.174
256/00	0.21	0.188	0.128	0.132	1.215	1.206
259/00	0.176	0.151	0.114	0.117	1.107	1.206
263/00	0.225	0.197	0.117	0.119	0.903	1.23
269/00	0.212	0.16	0.121	0.12	1.021	1.085
270/00	0.207	0.151	0.106	0.126	1.057	1.118
271/00	0.247	0.192	0.127	0.126	1.186	1.065
273/00	0.219	0.2	0.115	0.118	1.118	1.063
277/00	0.209	0.187	0.139	0.144	1.111	1.0011
279/00	0.259	0.232	0.115	0.122	1.197	1.082
280/00	0.106	0.092	0.188	0.194	1.08	1.533
282/00	0.221	0.201	0.13	0.134	1.095	1.246
283/00	0.224	0.207	0.123	0.125	1.195	1.287
284/00	0.164	0.138	0.132	0.135	1.056	1.169
285/00	0.2	0.159	0.154	0.154	1.128	1.196
287/00	0.193	0.163	0.123	0.122	1.065	1.182
288/00	0.217	0.186	0.128	0.133	1.119	1.172
289/00	0.31	0.308	0.124	0.125	1.044	1.071
290/00	0.271	0.224	0.119	0.116	1.314	1.108
296/00	0.216	0.196	0.116	0.116	1.083	1.145
297/00	0.252	2.116	0.154	0.144	1.106	1.097
300/00	0.221	0.185	0.136	0.122	1.151	1.158
303/00	0.129	0.163	0.154	0.16	1.102	1.205
15/01	0.255	0.256	0.136	0.142	1.323	1.153
18/01	0.176	0.179	0.146	0.156	1.04	1.157
26/01	0.204	0.183	0.133	0.144	1.194	1.21
27/01	0.322	0.296	0.183	0.196	1.141	1.262
29/01	0.281	0.313	0.156	0.16	1.09	1.173
31/01	0.343	0.225	0.168	0.174	1.02	1.1
33/01	0.105	0.228	0.162	0.174	1.041	1.26
35/01	0.098	0.091	0.174	0.177	1.131	1.17
39/01	0.213	0.201	0.13	0.137	1.163	1.154
40/01	0.249	0.231	0.114	0.122	1.123	1.121
45/01	0.351	0.283	0.125	0.131	1.336	1.124
48/01	0.105	0.19	0.143	0.143	1.132	1.249
55/01	0.248	0.227	0.134	0.14	1.153	1.098
58/01	0.095	0.088	0.195	0.159	1.174	1.204

Appendix H (contd)

SPU no.	IgG sa	IgG rec	IgM sa	IgM rec	CELISA sa	CELISA rec
59/01	0.338	0.256	0.132	0.153	1.09	1.234
60/01	0.301	0.265	0.261	0.262	1.105	1.26
61/01	0.304	0.285	0.12	0.123	1.158	1.238
62/01	0.095	0.286	0.145	0.185	0.96	1.219
71/01	0.113	0.109	0.136	0.136	1.033	1.127
73/01	0.22	0.193	0.165	0.174	1.079	1.003
75/01	0.268	0.257	0.136	0.147	1.193	1.088
78/01	0.196	0.161	0.121	0.132	1.053	1.109
79/01	0.239	0.225	0.183	0.19	1.006	1.094
80/01	0.228	0.196	0.136	0.15	1.172	1.158
82/01	0.36	0.312	0.171	0.177	1.121	1.128
84/01	0.296	0.167	0.126	0.128	1.028	1.191
86/01	0.241	0.082	0.131	0.134	1.077	1.215
88/01	0.316	0.221	0.134	0.143	1.004	1.153
96/01	0.196	0.155	0.164	0.176	1.162	1.141
101/01	0.299	0.231	0.126	0.131	1.222	1.127
107/01	0.297	0.241	0.149	0.144	1.194	1.045
110/01	0.246	0.222	0.118	0.12	0.992	1.101
117/01	0.25	0.197	0.134	0.14	1.323	1.268
121/01	0.133	0.085	0.12	0.123	1.078	1.205
123/01	0.369	0.297	0.312	0.336	0.95	1.12
129/01	0.187	0.17	0.125	0.131	0.952	0.752
130/01	0.169	0.158	0.111	0.111	0.924	1.094
132/01	0.259	0.239	0.117	0.117	0.875	1.091
133/01	0.299	0.314	0.151	0.116	0.928	1.143
134/01	0.202	0.185	0.147	0.159	0.885	1.102
135/01	0.246	0.219	0.133	0.14	1.032	1.252
136/01	0.157	0.164	0.128	0.141	0.985	1.135
142/01	0.133	0.129	0.115	0.163	1.046	0.988
144/01	0.299	0.284	0.112	0.13	0.982	1.072
157/01	0.277	0.249	0.147	0.159	1.102	0.955
158/01	0.28	0.24	0.117	0.123	0.973	0.768
160/01	0.266	0.236	0.13	0.14	0.915	1.059
162/01	0.209	0.181	0.111	0.117	0.916	1.055
166/01	0.169	0.169	0.105	0.107	0.959	1.045
167/01	0.204	0.195	0.106	0.108	0.956	1.033
169/01	0.204	0.16	0.099	0.103	0.87	1.004
172/01	0.146	0.167	0.093	0.097	0.923	0.949
177/01	0.142	0.133	0.106	0.117	0.904	0.966
181/01	0.127	0.122	0.105	0.115	0.928	0.949
182/01	0.126	0.124	0.111	0.129	0.991	1.043
184/01	0.126	0.137	0.108	0.124	1.073	1.014
185/01	0.382	0.368	0.196	0.222	1.074	1.115
186/01	0.276	0.212	0.103	0.112	0.952	0.973
187/01	0.152	0.144	0.119	0.129	0.937	1.181
188/01	0.177	0.154	0.18	0.207	0.833	1.174
193/01	0.182	0.146	0.098	0.105	0.912	0.953
197/01	0.184	0.166	0.142	0.157	0.823	0.994
200/01	0.23	0.205	0.123	0.138	0.869	1.02
201/01	0.1	0.116	0.121	0.14	1.03	0.981

Appendix H (contd)

SPU no.	IgG sa	IgG rec	IgM sa	IgM rec	CELISA sa	CELISA rec
202/01	0.266	0.217	0.124	0.146	1.05	0.961
203/01	0.22	0.193	0.108	0.124	0.989	0.993
204/01	0.195	0.177	0.106	0.13	1.122	1.156
205/01	0.268	0.216	0.118	0.13	1.038	1.192
210/01	0.13	0.111	0.0109	0.115	1.038	0.953
212/01	0.172	0.145	0.107	0.117	1.125	1.282
215/01	0.078	0.077	0.159	0.174	0.963	1.184
218/01	0.239	0.178	0.116	0.126	1.004	1.13
221/01	0.199	0.17	0.107	0.115	0.909	1.103
223/01	0.155	0.118	0.105	0.111	1.003	1.096
228/01	0.264	0.25	0.111	0.124	1.004	1.059
231/01	0.21	0.281	0.157	0.193	1.069	1.112
233/01	0.083	0.091	0.195	0.207	1.185	1.183
239/01	0.165	0.143	0.133	0.155	1.144	1.246
242/01	0.164	0.136	0.135	0.149	0.96	0.812

sa[1]: sucrose acetone extracted antigen

rec[2]: recombinant antigen.

Appendix I: IgM antibody titre to CCHF virus in relation to day post infection as detected by IgM capture ELISA using sucrose acetone extracted antigen and recombinant antigen in serum samples collected from an experimentally infected sheep (number 92).

	sucrose acetone antigen		Recombinant antigen	
Day	IgM titre	Interpretation	IgM titre	Interpretation
0	0	neg	0	neg
1	0	neg	0	neg
2	0	neg	0	neg
3	0	neg	0	neg
4	0	neg	0	neg
5	0	neg	0	neg
6	0	neg	0	neg
7	1600	pos	1600	pos
8	1600	pos	1600	pos
9	6400	pos	6400	pos
10	6400	pos	1600	pos
11	6400	pos	1600	pos
12	6400	pos	6400	pos
13	6400	pos	1600	pos
14	1600	pos	6400	pos
21	400	pos	100	neg
28	100	neg	100	neg
Months				
5	0	neg	0	neg
6	0	neg	0	neg
7	0	neg	0	neg
8	0	neg	0	neg

Appendix J: IgM antibody titre to CCHF virus in relation to day post infection as detected by IgM capture ELISA using sucrose acetone extracted and recombinant antigen in serum sample collected from an sheep (number 48).

	sucrose acetone antigen		Recombinant antigen	
Day	IgM titre	Interpretation	IgM titre	Interpretation
0	0	neg	0	neg
1	0	neg	0	neg
2	0	neg	0	neg
3	0	neg	0	neg
4	0	neg	0	neg
5	0	neg	0	neg
6	0	neg	0	neg
7	400	pos	100	neg
8	1600	pos	1600	pos
9	1600	pos	400	pos
10	1600	pos	400	pos
11	1600	pos	400	pos
12	1600	pos	400	pos
13	1600	pos	400	pos
14	400	pos	100	neg
21	400	pos	100	neg
28	0	neg	0	neg
Months				
5	0	neg	0	neg
6	0	neg	0	neg
7	0	neg	0	neg
8	0	neg	0	neg

Appendix K: IgM antibody titre to CCHF virus in relation to day post infection as detected by IgM capture ELISA using sucrose acetone extracted and recombinant antigen in serum samples collected from an experimentally infected sheep (number 37).

	sucrose acetone antigen		Recombinant antigen	
Day	IgM titre	Interpretation	IgM titre	Interpretation
0	0	neg	0	neg
1	0	neg	0	neg
2	0	neg	0	neg
3	0	neg	0	neg
4	100	neg	100	neg
5	400	pos	100	neg
6	1600	pos	400	pos
7	1600	pos	400	pos
8	1600	pos	400	pos
9	1600	pos	400	pos
10	1600	pos	6400	pos
11	6400	pos	1600	pos
12	1600	pos	400	pos
13	1600	pos	400	pos
14	400	pos	400	pos
21	0	neg	0	neg
28	0	neg	0	neg
Months				
5	0	neg	0	neg
6	0	neg	0	neg
7	0	neg	0	neg
8	0	neg	0	neg

Appendix L: IgG antibody titre to CCHF virus in relation to day post infection as detected by IgG sandwich ELISA using sucrose acetone extracted and recombinant antigen in serum samples collected from an experimentally infected sheep (number 92).

	sucrose acetone antigen		Recombinant antigen	
Day	IgG titre	Interpretation	IgG titre	Interpretation
0	0	neg	0	neg
1	0	neg	0	neg
2	0	neg	0	neg
3	0	neg	0	neg
4	0	neg	0	neg
5	0	neg	0	neg
6	0	neg	0	neg
7	100	neg	100	neg
8	100	neg	100	neg
9	400	pos	100	neg
10	400	pos	400	pos
11	400	pos	400	pos
12	400	pos	400	pos
13	1600	pos	1600	pos
14	400	pos	400	pos
21	1600	pos	1600	pos
28	1600	pos	1600	pos
Months				
5	1600	pos	1600	pos
6	1600	pos	1600	pos
7	1600	pos	1600	pos
8	1600	pos	1600	pos

Appendix M: IgG antibody titre to CCHF virus in relation to day post infection as detected by IgG sandwich ELISA using sucrose acetone extracted and recombinant antigen in serum samples collected from an experimentally infected sheep (number 48).

	sucrose acetone antigen		Recombinant antigen	
Day	IgG titre	Interpretation	IgG titre	Interpretation
0	0	neg	0	neg
1	0	neg	0	neg
2	0	neg	0	neg
3	0	neg	0	neg
4	0	neg	0	neg
5	0	neg	0	neg
6	0	neg	0	neg
7	0	neg	0	neg
8	0	neg	0	neg
9	0	neg	0	neg
10	100	neg	100	neg
11	100	neg	100	neg
12	100	neg	100	neg
13	100	neg	100	neg
14	100	neg	100	neg
21	400	pos	100	neg
28	400	pos	100	neg
Months				
5	400	pos	400	pos
6	400	pos	400	pos
7	0	neg	0	neg
8	1600	pos	1600	pos

Appendix N: IgG antibody titre to CCHF virus in relation to day post infection as detected by IgG sandwich ELISA using sucrose acetone extracted and recombinant antigen in serum samples collected from an experimentally infected sheep (number 37).

	sucrose acetone antigen		Recombinant antigen	
Day	IgG titre	Interpretation	IgG titre	Interpretation
0	0	neg	0	neg
1	0	neg	0	neg
2	0	neg	0	neg
3	0	neg	0	neg
4	0	neg	0	neg
5	100	neg	100	neg
6	400	pos	100	neg
7	400	pos	100	neg
8	400	pos	400	pos
9	400	pos	400	pos
10	1600	pos	400	pos
11	1600	pos	1600	pos
12	1600	pos	1600	pos
13	1600	pos	1600	pos
14	1600	pos	1600	pos
21	400	pos	1600	pos
28	1600	pos	1600	pos
Months				
5	1600	pos	1600	pos
6	1600	pos	1600	pos
7	1600	neg	1600	pos
8	1600	pos	1600	pos