THE DEVELOPMENT OF A METHOD FOR THE DETECTION AND ESTIMATION OF CCHF VIRUS RNA IN TICK SPECIES

by

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Hereby I declare that this script submitted towards a M.Med.Sc. degree at the University of the Free State is my original and independent work and has never been submitted to any other university or faculty for degree purposes.

All the sources I have made use of or quoted have been acknowledged by complete references.

P.H. du Preez
May 2000

THIS THESIS IS DEDICATED TO ALL THE PEOPLE WHO DIED OF CCHF VIRUS INFECTION

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To declare that I did it all on my own, would be a lie. To take the glory for all that has been written would also be unjustified. I would like to acknowledge all those many who shared in my progress, occasional frustration and joyous moments. Without their support, the completion of this would not have been possible.

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SUMMARY

Crimean Congo haemorrhagic fever (CCHF), caused by a RNA virus, is a tick-borne viral zoonosis occurring in Europe, Asia and Africa. The fatality rate is ±30%. Rapid and accurate diagnosis is essential. The aim of this study was to develop a reverse transcription-polymerase chain reaction (RT-PCR) with internal control for the detection of CCHF RNA. Primers were selected for a region in the nucleocapsid gene of the S segment. The internal control was constructed by ligating this PCR product into a pGEMEX-I vector. Sequencing of the PCR product (381 bp) revealed two unique restriction sites, *Bln* I and *Bst*E II which were used to delete a fragment of 59 bp. The shortened PCR-product was re-inserted into *E. coli*. T3 RNA polymerase produced plasmid derived RNA (322 bp) was used to spike specimens. Standard RT-PCR was then performed. The minimum concentration of target RNA the RT-PCR can detect was estimated to be 4×10^{-5} pmol RNA, giving more or less the same sensitivity as the PCR alone. The size difference of 59 bp is enough to distinguish between the full-length and the deletion variant inserts when visualised and therefore provides an internal control. RT-PCR on fifty *Hyalomma* ticks was negative. The CCHF virus was probably not present or at concentrations below detection level, as RT-PCR of control CCHF virus RNA confirmed the accuracy of the method. RT-PCR allows rapid detection of CCHF virus RNA. The constructed internal control precludes the use of Dugbe virus, an antigenically related nairovirus.

LIST OF FIGURES

	PAGE
Figure 1: Schematical representation of a Bunyavirus particle	5
Figure 2: Nucleotide sequence of S RNA segment of CCHF C68031 virus	8
Figure 3: CCHF virus maintenance and transmission cycles involving *Hyalomma marginatum marginatum* and associated vertebrate hosts	27
Figure 4: Summary of the development of a method for the detection and estimation of CCHF virus RNA in tick species	68
Figure 5: Agarose gel electrophoresis of the RT-PCR products of the four different primer pair combinations	71
Figure 6: CCHF virus PCR product (381 bp) cloned into the pGEMEX-I plasmid vector at *Eco*R I cloning site to create pHEN I	73
Figure 7: Nucleotide sequence of S RNA segment of CCHF virus (strain SPU 497/89)	74
Figure 8: Agarose gel electrophoresis of the PCR products of the ten-fold dilution series	75
Figure 9: Nucleotide sequence of S RNA segment of CCHF virus (strain SPU497/89) compared with 15 different CCHF variants from Genebank®.	78
Figure 10: Amino acid sequence of the S RNA segment of CCHF virus strain SPU 497/89 compared with strain CCHFSRNA	79
Figure 11: Agarose gel electrophoresis of RT-PCR products	80
Figure 12: Agarose gel electrophoresis of the RT-PCR products of the internal control; RNA dilution series	81

Figure 13: Agarose gel electrophoresis of RT-PCR products 83

LIST OF TABLES

		PAGE
Table 1:	Related antigenical serogroups of Nairoviruses	14
Table 2:	Summary of viraemia and antibody responses of small wild mammals to CCHF virus infection	44
Table 3:	Oligonucleotide primer sequence used for the first round reverse transcription-polymerase chain reactions	52
Table 4:	PCR product lengths of the four different primers	72
Table 5:	Oligonucleotide primer sequences and their positions in the CCHF virus genome	74

ABBREVIATIONS

µg	: Microgram
µg/µl	: Microgram/microlitre
µg/ml	: Microgram/millilitre
µl	: Microlitre
ρmol	: Picomol
°C	: Degree Celsius
A	
A	: Adenine
A. albiventris	: *Argas albiventris*
A. p. percicus	: *Argas (Persicargas) persicus*
A. walkerae	: *Argas walkerae*
A. savignyi	: *Argas savignyi*
AC	: Antigen capture
Ag-ELISA	: Antigen-enzyme-linked immunosorbent assay
AGDP	: Agar gel diffusion precipitation
AGPC	: Acid guanidium thiocyanate-phenolchloroform
AMV	: Avian myeloblastosis virus
ATP	: Adenosine 5'-triphosphate
B	
BSL-4	: Biosafety level four
bp	: Base pair
C	: Cytosine
c.	: Circa
$CaCl_2$	: Calcium chloride
Cat. No.	: Catalogue number
CCHF	: Crimean Congo haemorrhagic fever
cDNA	: Complementary deoxyribose nucleic acid
CF	: Complement fixation
CHF	: Congo haemorrhagic fever
CIAP	: Calf intestinal alkaline phosphatase
CTP	: Cytidine 5'-triphosphate
D	
Da	: Dalton
dATP	: Deoxyadenosine 5'-triphosphate
dCTP	: Deoxycytidine 5'-triphosphate
dGTP	: Deoxyguanosine 5'-triphosphate
dH_2O	: Distilled water
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribose nucleic acid
dNTP	: Deoxynucleotide 5'-triphosphate
DTT	: Dithiothreitol
dTTP	: Deoxythymidine 5'-triphosphate

E

E. coli	: *Escherichia coli*
EDTA	: Ethylene diamine tetra-acid
e.g.	: Exempli gratia (for example)
ELISA	: Enzyme-linked immunosorbent assay
et al.	: And others
ETOH	: Ethyl alcohol

F

F2	: Forward primer 2
F3	: Forward primer 3
FAT	: Indirect fluorescent antibody technique

G

g	: Gravitational force
G	: Guanine
G1	: Glycoprotein 1
G2	: Glycoprotein 2
GTP	: Guanosine 5'-triphosphate

H

H. a. anatoliticum	: *Hyalomma anatoliticum anatoliticum*
H. auritus	: *Hemiechinus auritus*
H. m. marginatum	: *Hyalomma marginatum marginatum*
H. m. rufipes	: *Hyalomma marginatum rufipes*
H. truncatum	: *Hyalomma truncatum*
HI	: Haemagglutination-inhibition

I

i.c.	: Intracerebral
i.e.	: Id est (that is)
IF	: Immunofluoresence
IgG	: Immunoglobulin G
IgM	: Immunoglobulin M
IHI	: Indirect haemagglutination-inhibition

K

kDa	: Kilodalton

L

L protein	: large protein
L/K	: Ligase/kinase
LB	: Luria Bertani

M

M	: Molar
mM	: Millimolar
ml	: Millilitre
M protein	: Medium protein
M. coucha	: *Mastomys coucha*
$MgSO_4$	: Magnesium sulphate
M-MuLV	: Moloney Murine Leukaemia Virus
M_r	: Molecular weight
mRNA	: Messenger ribonucleic acid

N

N : Neutralisation
NaAc : Sodium acetate
NaCl : Sodium chloride
NaOH : Sodium hydroxide
ng : Nanogram
NH_4Ac : Ammonium acetate
NIV : National Institute for Virology

M

nm : Nanometer

O

O. sonrai : *Ornithodoros sonrai*

P

PCR : Plymerase chain reaction
PFU : Plague forming units
PNK : *Phosphatase nucleotide kinase*

R

R. d. bursa : *Rhipicephalus (Digineus) bursa*
R. pumilio : *Rhipicephalus pumilio*
R. rossicus : *Rhipicephalus rossicus*
R. sanguineus : *Rhipicephalus sanguineus*
R. turanicus : *Rhipicephalus turanicus*
R2 : Reverse primer 2
R3 : Reverse primer 3
RNA : Ribonucleic acid
RNase : Ribonuclease
RPHA : Reverse passive haemagglutination
RPHI : Reverse passive haemagglutination-inhibition
RT : Reverse transcriptase
RT-PCP : Reverse transcriptase-polymerase chain reaction

S

S protein : Small protein
S RNA : Small ribonucleic acid
SDS : Sodium docecyl sulphate
Sp. : *Species*
Spp. : *Species*

T

T : Thymidine
Taq : *Thermus aquaticus*
TB : Terrific broth
TBE : Tris-borate with EDTA
TE : Tris-ethylene diamine tetra-acid
Tris : 2-Amino-2(hydroxymethyl)-1,3-propandiol
TRIZOL : Total RNA isolation reagent

U

USSR : Union of Socialistic Soviet Republics
UTP : Uridine 5'-triphosphate
U : Enzyme unit
UV : Ultra violet

Q

Q-fever : Query-fever

TABLE OF CONTENTS

	PAGE
DEDICATION	**i**
ACKNOWLEDGEMENTS	**ii**
SUMMARY	**iii**
LIST OF FIGURES	**iv**
LIST OF TABLES	**vi**
LIST OF ABBREVIATIONS	**vii**
TABLE OF CONTENTS	**x**
1. GENERAL INTRODUCTION	1
1.1 INTRODUCTION	1
1.1.1 *HISTORICAL BACKGROUND OF CCHF IN CENTRAL ASIA AND EUROPEAN RUSSIA*	2
1.1.2 DISCOVERY OF THE VIRUS	3
1.2 CCHF VIRUS	4
1.2.1 STRUCTURAL CHARACTERISTICS	4
1.2.1.1 CCHF virion structure	4
1.2.1.2 Genetic organisation of the CCHF virus genome	5
1.2.2 HOST RANGE	9
1.2.2.1 Vertebrates	9

1.2.3.2 Invertebrates 9

1.2.3 VARIOUS DETECTION METHODS 9

1.2.4 DIAGNOSTIC PROCEDURES 11

1.2.4.1 Clinical diagnosis 11

1.2.4.2 Serological and virological diagnosis 12

1.2.5 ANTIGENIC RELATIONSHIPS 13

1.2.6 STRAIN VARIATION AMONG CCHF VIRUSES 15

1.2.7 *STABILITY* 16

1.3 EPIDEMIOLOGY 16

1.3.1 GEOGRAPHICAL LOCATION 16

1.3.2 OCCURRENCE OF CCHF VIRUS INFECTION 17

1.3.3 SEASONAL ACTIVITY AND DISTRIBUTION 18

1.3.4 RISK FACTORS 20

1.4 TRANSMISSION CYCLES 21

1.4.1 VECTORS 21

1.4.2 VERTEBRATE HOSTS 22

1.4.3 EXPERIMENTAL INFECTION 24

1.4.4 HIBERNATION 25

1.4.5 TRANSSTADIAL SURVIVAL AND TRANSOVARIAL TRANSMISSION 26

1.5 TICK ECOLOGICAL DYNAMICS 27

1.5.1 *BITING ACTIVITY AND HOST PREFERENCE OF*

VECTOR TICKS 29

1.5.1.1 One-host ticks 30

1.5.1.2 Two-host ticks 30

1.5.1.3 Three-host ticks 31

1.5.1.4 Multi-host ticks 31

1.5.2 MACRO- AND MICROENVIRONMENT 32

1.5.3 VECTOR OVIPOSITION 32

1.5.4 *DENSITY, FERTILITY AND LONGEVITY OF VECTOR TICKS* 33

1.5.5 *VERTEBRATE HOSTS AND SEROLOGICAL BACKGROUND OF CCHF VIRUS* 34

1.5.5.1 Horizontal transmission 34

1.5.5.2 Vertical transmission 35

1.5.6 VECTOR CAPABILITY 38

1.5.7 MOVEMENTS AND MIGRATIONS OF VECTORS AND HOSTS 39

1.5.8 HUMANS IN DISEASE ECOLOGY 39

1.5.9 ASSOCIATION OF CCHF WITH CERTAIN TICK SPECIES 40

1.6 CCHF VIRUS DISEASE ASSOCIATIONS 41

1.6.1 HUMANS 41

1.6.2 DOMESTIC ANIMALS 42

1.6.3 LABORATORY ANIMALS 43

1.6.4 SMALL AND MEDIUM-SIZED WILD MAMMALS 43

1.6.5 BIRDS 45

2. MATERIALS AND METHODS 47

2.1 SOURCE OF BIOLOGICAL MATERIALS 47

2.1.1 CCHF VIRUS 47

2.1.2 TICKS 47

2.1.3 ESCHERICHIA COLI TRANSFORMATION 47

2.1.4 REAGENTS 48

2.2 EXTRACTION OF NUCLEIC ACIDS 48

2.2.1 RIBONUCLEIC ACID (RNA) EXTRACTION 48

2.2.1.1 Preparation of CCHF viral nucleic acid 48

2.2.1.2 Total RNA isolation from ticks 49

2.2.1.2.1 RNeasy™ Total RNA kit 49

2.2.1.2.2 Trizol® 50

2.2.2 *DEOXYRIBONUCLEIC ACID (DNA)* 50

2.2.2.1 The pGEMEX-I plasmid vector 50

2.3 CONSTRUCTION OF THE INTERNAL CONTROL 51

2.3.1 *GENERAL RECOMBINANT DNA METHODS* 51

2.3.2 *REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)* 51

2.3.2.1 cDNA synthesis from the CCHF virus 51

2.3.2.2 PCR amplification 51

2.3.2.3 Analysis of PCR-amplification products on agarose gels 52

2.3.2.4 Purification of PCR products 53

2.3.2.5 Labelling of cDNA with [γ-^{32}P]ATP 53

2.3.2.6 Ligation 54

2.3.2.7 *Kinase of labelled cDNA* 55

2.3.3 *PREPARATION OF THE pGEMEX-I PLASMID VECTOR* 56

2.3.3.1 *Dephosphorylation reaction of pGEMEX-I* 56

2.3.4 *CLONING OF PCR PRODUCT INTO THE pGEMEX-I PLASMID* 57

2.3.4.1 *Ligation of pGEMEX-I vector and insert cDNA* 57

2.3.5 *E. COLI TRANSFORMATION AND COLONY HYBRIDISATION* 57

2.3.5.1 *Labelling of probe with [α-^{32}P]dATP* 58

2.3.5.2 *Plasmid extraction and purification* 59

2.3.5.3 *Determination of optical density of pHEN I DNA* 60

2.3.5.4 *Ten-fold dilution series of pHEN I DNA* 60

2.3.6 *SEQUENCING OF THE CLONED CCHF VIRUS RT-PCR PRODUCT* 61

2.3.7 *CONSTRUCTION OF DELETION VARIANT* 62

2.3.7.1 *Plasmid isolation* 62

2.3.7.2 *Transcription of the DNA insert and pGEMEX-I positive control* 64

2.3.7.3 *Removal of DNA template following transcription* 65

2.3.7.4 *Isopropyl alcohol precipitation of the transcribed internal control RNA* 65

2.3.7.5 *Determination of the optical density of the internal control RNA* 65

2.3.7.6 *Ten-fold dilution series of the internal control RNA* 66

2.3.7.7 Determination of the optical density of tick RNA 66

2.4 INTERNAL CONTROL USED TO SPIKE THE RT-PCR 66

2.5 SUMMARY OF THE DEVELOPMENT OF THE METHOD FOR THE DETECTION AND ESTIMATION OF CCHF VIRUS RNA IN TICK SPECIES 67

3. **RESULTS & DICUSSION** 69

3.1 INTRODUCTION 69

3.2 RT-PCR 70

3.3 CLONING AND CARACTERIZATION OF PCR PRODUCTS 72

3.4 ESTIMATION OF THE SENSITIVITY OF THE PCR STEP 75

3.5 CONSTRUCTION OF THE DELETION VARIANT 80

3.6 IN VITRO TRANSCRIPTION OF INTERNAL CONTROL RNA 80

3.7 ESTIMATION OF THE CONCENTRATION OF INTERNAL CONTROL RNA REQUIRED FOR RT-PCR 81

3.8 IMPLEMENTATION OF THE INTERNAL CONTROL WITH ISOLATED TICK RNA 82

3.9 CONCLUSION AND FUTURE APPLICATIONS 84

4. REFERENCES 86

5. APPENDIX A 97

6. APPENDIX B 99

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Crimean-Congo haemorrhagic fever (CCHF) is a good example of an emerging infectious disease, which has caused several epidemics in South Africa and other parts of the world. Currently the diagnosis of acute CCHF virus infection relies on the isolation of the virus in cell culture and suckling mouse brain, passive haemagglutination, indirect immunofluorescence for the detection of IgG and IgM antibodies to the CCHF virus and antigen capture enzyme-linked immunosorbent assay (ELISA) (Joubert *et al.*, 1985; Shepherd *et al.*, 1986; Logan *et al.*, 1993). A reverse transcriptase polymerase chain reaction (RT-PCR) has also been widely used for the detection of several members of the Bunyaviridae of which CCHF virus is a member (Horling *et al.*,1995).

Human infections with Bunyaviridae have become increasingly important. Virus isolation often requires special containment laboratories that may not be available in some regions where infections are endemic. Instead of virus isolation, RT-PCR can be used to detect genomic bunyavirus RNA. In a study by Schwartz in the United Arab Emirates, CCHF virus RNA was detected in 25% of the sera of patients with suspected haemorrhagic fever, despite inadequate storage of the sera over several months. It may thus be worthwhile to perform RT-PCR on the sera of patients with suspected CCHF virus infection (Schwartz *et al.*, 1996).

CCHF virus has a propensity to cause nosocomial infections, hence a rapid diagnosis is important for the treatment of the patient and to install control measures to protect medical staff. However, several factors hamper attempts to achieve a specific diagnosis rapidly by conventional techniques (Schwartz *et al.*, 1996) and the RT-PCR technique may prove to be more appropriate. Furthermore, this technique may also prove more suitable to detect the presence of CCHF virus in viremic livestock as well as *Hyalomma* tick species, the vectors of this virus. Finally, economic losses may result from restricted exportation of livestock and animal products to non-enzootic CCHF virus countries and limited attempts to control CCHF have been an unaffordable expense (Hoogstraal, 1979).

Taking previous research and documentation into account it is obvious that a more appropriate technique should be found for the detection of the CCHF virus. This study was initiated to determine whether RT-PCR could be applied for the detection of CCHF virus in *Hyalomma* tick species.

1.1.1 HISTORICAL BACKGROUND OF CCHF IN CENTRAL ASIA AND EUROPEAN RUSSIA

In The Thesaurus of the Shah of Khwarazm (Dzhurzhoni, c. 1110), written in Persian, the physician Zayn ad-Din abu Ibrahim Ismacil ibn Muhamad al-Husayini al-Jurjani described a haemorrhagic disease that is now considered to have been CCHF (from the area that is presently Tadzhikistan). Symptoms of this disease included the presence of blood in the urine, rectum, gums, vomit, sputum and abdominal cavity. The arthropod implicated in causing the disease was said to be tough, small, related to a louse or tick and normally parasitic in a black bird. Treatment, which was sometimes ineffectual, included the application of *bodzkhar* - an essence of red sandalwood - at the site of the bite, fresh goat milk together with

butter, *khot'ma* flowers, leaves or essence of *khovre,* essence of flax seed, chicory and ground to eat. CCHF was also recognised for centuries under at least 3 different names by indigenous people of southern Uzbekistan. The first detailed clinical accounts date from the World War II epidemic in the Crimea during 1944 to 1945 (Hoogstraal, 1979).

1.1.2 DISCOVERY OF CCHF VIRUS

According to Chumakov CCHF virus was first described as a clinical entity in 1944 and 1945 during an epidemic in the western steppe region of the Crimean. Subsequent attempts to determine the aetiology of clinically diagnosed CHF during epidemics in Eurasia led to the discovery in 1967 of the agent replicated in new-born white mice. Intracerebral (i.c.) inoculation of mice with blood from clinically diagnosed Congo haemorrhagic fever (CHF) patients and corpses led to the isolation of the virus, subsequently designated CHF virus (Watts *et al.*, 1988). CHF virus was shown to be antigenically indistinguishable from Congo virus (Casals, 1969) originally isolated in 1956 from a febrile patient in the Belgian Congo (Zaire) (Simpson *et al.*, 1967). In addition, an antigenic relationship was demonstrated between Eurasian strains of CHF virus and several strains of Congo virus (Casals, 1969) isolated from the initial Zaire patient's physician and additional febrile patients, including laboratory workers in Uganda (Woodall *et al.*, 1965). From wild and domestic animals, ticks and biting gnats in Nigeria, (Causey *et al.*, 1970; Kemp *et al.*, 1974) and *Hyalomma* ticks in Pakistan (Begum *et al.*, 1970). Observations that CHF and Congo virus were antigenically indistinguishable gave rise to the new name Crimean-Congo haemorrhagic fever virus (CCHF) (Hoogstraal, 1979).

1.2 CCHF VIRUS

1.2.1 STRUCTURAL CHARACTERISTICS

1.2.1.1 CCHF virion structure

With the electron microscope, negatively stained Bunyaviridae particles appear spherical and have a bilipid-layer envelope from which a fringe of glycoprotein spikes project (Pettersson & Kääriäinew, 1973; Murphy *et al.*, 1973; Smith & Pifat, 1982; Hung *et al.*, 1983; Pettersson & von Bonsdorff, 1987). Partikels of the CCHF virus were approximately 90 nm in diameter and have very small morphologic surface units (Martin *et al.*, 1985). The CCHF virus has a single-strand, negative-sense, three-segment RNA genome. The three RNA segments are designated according to their size: large (L), medium (M) and small (S) and is contained in a separate nucleocapsid within the virion. This circular, helical viral nucleocapsids can be obtained from virus preparations. Each is composed of a nucleocapsid protein (N), which separates the RNA segments (L, M or S). The L RNA segment (molecular weight (4.1 to 4.9 x 10^6 Da) codes for the viral transcriptase component. The M RNA segment (1.5 to 2.3 x 10^6 Da) codes for the external glycoproteins, termed G1 and G2, which are inserted in the viral membrane. The glycoproteins are located on the outer surface of the virus particle. By convention the larger M_r glycoproteins are designated G1. The S RNA segment (0.6 to 0.7 x 10^6 Da) codes for the N protein (Bishop *et al.,* 1980, 1986; Swanepoel, 1998a). The virion contains three major structural proteins: two enveloped glycoproteins, G1 and G2, with molecular weights 72 to 84 and 30 to 40 kDa; and minor quantities of large protein, L (>200 kDa), the viral transcriptase (Swanepoel, 1998a).

The RNA segments in the nucleocapsid have unique complementary 5'- and 3'-end sequences that may be hydrogen bonded to allow circular conformation and can be extracted from nucleocapsids as non-covalent closed circles. The genus Nairovirus

is named after Nairobi sheep disease. All Nairoviruses are tick borne. Dugbe and Ganjam viruses have been isolated from culicine mosquitoes and CCHF virus is the medically most important member of this genus (Elliott, 1990).

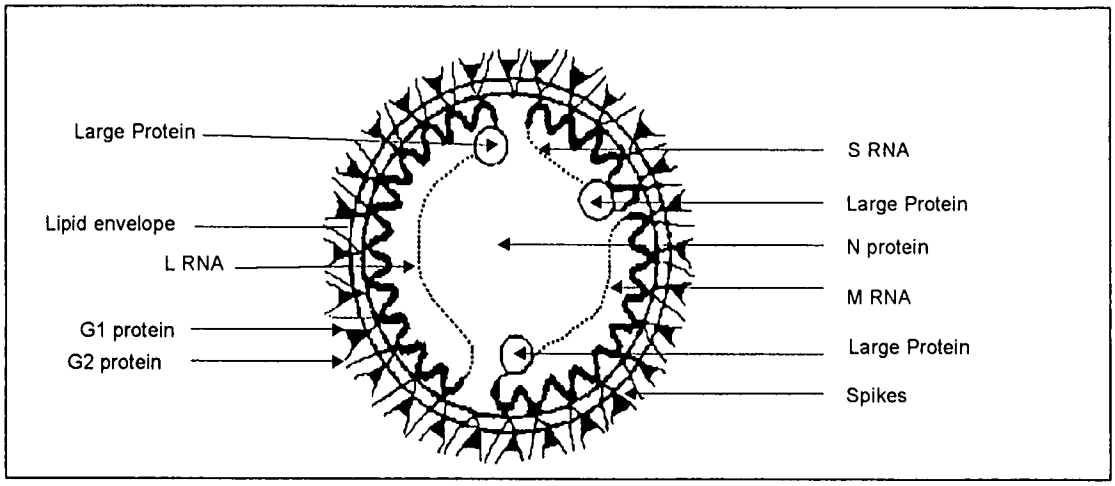

Figure 1: Schematic representation of a Bunyavirus particle (Bishop *et al.*, 1980, 1986)

Some nairoviruses are exceptions to the above mentioned pattern, as Foulke *et al.* (1981) detected three glycoprotein species in Hazara virus. The Bunyaviridae do not encode an internal matrix protein and therefore the virion structure may be stabilised by direct interaction of the internal nucleocapsids with the membrane or with the cytoplasmic domain of the inserted glycoprotein (Pettersson & von Bonsdorff, 1987; Talmon *et al.*, 1987). Virus replication occurs in the cytoplasm of infected cells. Virus particles form by budding into the Golgi cisternae. Virions are released either from infected cells by fusion of the intracellular vacuoles with the cellular plasma membrane and subsequent virus budding, or by cell membrane disruption and discharge of the cell contents (Bishop *et al.*, 1980).

1.2.1.2 Genetic organisation of the CCHF virus genome

The CCHF virus has a single-stranded, negative-sense (complementary to mRNA), three-segmented RNA genome (Swanepoel, 1998a). Studies done with monoclonal

antibodies have shown that the CCHF nucleocapsid protein is the most type-specific polypeptide (Smith *et al.*, 1991) and that certain bunyaviruses are capable of genetic reassortment (Bishop *et al.*, 1980).

Preliminary data indicate that the bunyavirus L RNA codes for the viral protein in a viral-complementary sequence (Clerx-van Haaster *et al*, 1982).

Until recently, little was known of the coding strategy of the nairovirus M RNA segment, although it was assumed to encode the surface glycoproteins by analogy with other members of the family Bunyaviridae. The M RNA consists of 4888 nucleotides and encodes a long open reading frame in the viral-complementary strand with a capacity for a 173.3 kDa protein. The ends of the M RNA show conserved sequences, a general feature of this virus family, 9 nucleotides of which are identical between both ends of the M and S segments of Dugbe virus. These 9 nucleotides are also conserved in the S segments of CCHF and Hazara nairoviruses (Marriott & Nuttall, 1992).

The bunyavirus M RNA in its viral-complementary sequence code for a precursor to both the viral glycoproteins and second non-structural proteins, NS_M (Fuller & Bishop, 1982).

The S genome segment of CCHF virus consists of 1672 nucleotides with a single open reading frame in the viral-complementary strand which encodes a protein of 482 amino acids with a predicted molecular weight of 53 966 Da. Hazara virus S RNA comprises of 1677 nucleotides and also has a single open reading frame in the viral-complementary strand which encodes for a protein of 485 amino acids with a predicted molecular weight of 54 186 Da. The S RNA of both these nairoviruses shows a similar coding strategy to that of Dugbe virus (Ward *et al.*, 1990a). The S RNA of the Dugbe virus is 1712 nucleotides long. The lengths of the 5' and 3' untranslated regions vary between the three viruses. The nucleotide homologies

between the sequences are 49.7% for CCHF and Hazara, 48.2% for CCHF and Dugbe and 45.6% for Hazara and Dugbe virus. These figures explain the weak hybridisation detected between Dugbe riboprobe and the S RNAs of CCHF and Hazara viruses (Marriott *et al.*, 1990).

Alignment of the nucleoprotein sequences of CCHF, Hazara and Dugbe viruses shows that the CCHF and Hazara sequences are somewhat more closely related to each other than either is to the Dugbe sequences (Marriott & Nuttall, 1992).

Genetic and molecular studies, including sequence analyses of DNA copies of the individual RNA species, have shown that the S RNA segment of bunyaviruses codes for two proteins that are read in overlapping reading frames from a single viral-complementary mRNA species (Bishop *et al.*, 1982; Fuller *et al.*, 1983). These proteins are the N protein and a non-structural protein, designated NS_S (Fuller & Bishop, 1982, Marriott & Nuttall, 1992). The function of the NS_S protein is not known, but presumably it is not involved in RNA transcription or RNA replication. Since the NS_S and N proteins are similar in size, the location of NS_S in infected cells has been difficult to determine. However, NS_S does not appear to be a structural protein (Bishop, 1986).

Both CCHF and Hazara virus N proteins are larger than that of Dugbe virus, having an extra sequence at the carboxyl terminus (Marriott & Nuttall, 1992). The comparison of the nairovirus N protein sequences with N and NS_S sequences of other Bunyaviridae, especially the similar-sized N protein of Hantaan virus (Schmaljohn *et al.*, 1986). The N and NS_S proteins of the tick-transmitted Uukuniemi virus (Simons *et al.*, 1990) showed no homology nor common amino acid motifs.

The coding strategy of the bunyaviruses appears to be similar to that of three other groups of negative-stranded RNA viruses (i.e. rhabdoviruses, paramyxoviruses and orthomyxoviruses) and involves proteins coded in the viral complementary RNA

sequences. Unlike rhabdoviruses and paramyxoviruses, the initiation of mRNA transcription for bunyaviruses involves the use of host cell-derived primers, in a manner that they may be analogous to that of influenza orthomyxoviruses (Bishop *et al.*, 1983; Eshita *et al*, 1985). The synthesis of bunyavirus mRNA does not occur in the nucleus of infected cells, unlike the transcription of influenza mRNA (Bishop, 1986).

The first published CCHF virus sequence data available were from the Chinese sheep isolate C68031, which has been passaged several times in cell culture. Figure 2 shows the nucleotide sequence of CCHF S segment (Marriott & Nuttall, 1992).

```
1     TCTCAAAGAA ACACGTGCCG CCTACGCCCA CAGTGTTCTC TTGAGTGCTA
      GCAAAATGGA GAATAAAATC GAGGTGAATA ACAAAGATGA AATGAACAAG 100
      TGGTTTGAAG AGTTCAAGAA AGGAAATGGA CTTGTGGATA CTTTCACAAA
      CCCCTACTCC TTTTGTGAGA GTGTTCCAAA TCTGGAAAGG TTTGTGTTTC 200
      AGATGGCCAG TGCCACCGAT GATGCACAAA AGGATTCCAT CTACGCATCA
      GCTCTGGTGG AAGCAACCAA ATTTTGTGCA CCCATATACG AGTGTGCCTG
300   GGTTAGCTCC ACTGGCATTG TGAAGAAGGG ACTGGAGTGG TTCGAAAAAA
      ATGCAGGAAC CATTAAATCT TGGGATGAAA GCTACATTGA GCTGAAAGTT 400
      GAGGTCCCTA AAATAGAACA GCTTGCCAAT TACCAACAGG CTGCTCTCAA
      GTGGAGGAAG GACATAGGTT TTCGTGTCAA TGCAAATACG GCAGCCTTAA 500
      GCCACAAGGT CCTTGCAGAG TACAAGGTCC CTGGCGAAAT TGTAATGTCC
      GTCAAAGAAA TGTTGTCAGA TATGATTAGA AGAAGGAACT TGATTCTCAA
600   CAGAGGTGGC GATGAAAATC CACGAGGCCC AGTGAGCCGT GAACATGTGG
      AGTGGTGCAG GGAATTTGTC AAAGGCAAGT ACATCATGGC TTTCAACCCG 700
      CCCTGGGGGG ACATCAACAA GTCAGGCCGA TCAGGAATAG CACTTGTTGC
      AACAGGCCTT GCCAAGCTCG CAGAGACTGA GGGGAAGGGA GTGTTTGATG 800
      AAGCCAAAAA GACTGTAGAG GCTCTCAACG GGTACCTTGA CAAACACAAG
      GACGAAGTTG ACAAAGCAAG TGCCGACAAC ATGATAACAA ACCTTCTCAA
900   ACACATTGCT AAGGCACAAG AGCTTTACAA AAACTCGTCT GCACTTCGTG
      CACAGGGTGC ACAGATTGAC ACTGCTTTCA GCTCATACTA CTGGCTCTAC 1000
      AAGGCCGGCG TGACTCCAGA AACCTTCCCG ACTGTCTCAC AGTTCCTTTT
      TGAGCTAGGG AAACAACCAA GGGGTACCAA GAAAATGAAG AAGGCACTCT 1100
      TGAGCACCCC AATGAAGTGG GGTAAGAAGC TTTATGAGCT CTTTGCTGAT
      GACTCATTCC AGCAAAACAG GATCTACATG CACCCTGCCG TGTTGACAGC
1200  TGGCAGAATC AGTGAGATGG GTGTCTGCTT TGGAACAATC CCTGTGGCCA
      ATCCCGATGA TGCTGCCCAG GGATCTGGAC ACACCAAGTC CATTCTTAAC 1300
      CTACGGACAA ACACCGAAAC CAACAATCCG TGTGCCAAGA CAATTGTCAA
      GTTGTTTGAA ATTCAAAAAA CAGGATTTAA TATACAAGAC ATGGACATTG 1400
      TAGCCTCTGA GCACCTGCTG CACCAATCCC TTGTCGGCAA GCAGTCTCCA
      TTCCAGAATG CCTACAACGT CAAGGGCAAT GCCACCAGTG CCAACATCAT
1500  CTGAAGCTCC AAATGCTTTG CATTCAGCTT TCCTCCCTTT TGCATTGCTA
      TCTATGATTG TAACCATCAA CAATGTTTAT TTTAACTGCT TATATAATCC 1600
      TGTTTTATTA ACTTCTTCTT GTTTCTTTCG TTTAAACACT TAAAGGGCTG
      GCGGCAACGA TATCTTTGAG A
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Figure 2: Nucleotide sequence of S RNA segment of CCHF C68031 virus (Marriott & Nuttall, 1992).

1.2.2 HOST RANGE

1.2.2.1 Vertebrates

CCHF virus has been isolated from small wild mammals, domestic vertebrates and humans (Shepherd *et al.*, 1985; Swanepoel *et al.*, 1987), cattle (Camicas *et al.*, 1990), goats (Causey *et al.*, 1970), sheep (Wilson *et al.*, 1991) and hares (*Lepus sp.*) (Shepherd *et al.*, 1987a). In the USSR, Bulgaria (Hoogstraal, 1979) and South Africa (Swanepoel *et al.*, 1983; Shepherd *et al.*, 1987a), the CCHF virus has also been isolated from hedgehogs (*Atelerix albiventris*) and from multimammate mice (*Mastomys spp.*) in the Central African Republic (Causey *et al.*, 1970). Serological evidence of CCHF viral infections has also been demonstrated in these and other species of wild vertebrates, humans and domestic animals (Watts *et al.*, 1988).

1.2.2.2 Invertebrates

CCHF virus has been demonstrated only in ticks. Attempts to infect *Aedes aegypti* mosquitoes experimentally were unsuccessful. More than 25 000 mosquitoes (6 species) from the Astrakhan CCHF focus were tested for viruses during 1967 and 1969. All results were negative and sentinel laboratory mammals on which mosquitoes fed in this focus showed no evidence of CCHF infection. There is no evidence to suggest that insects play a role in the natural history of CCHF virus (Hoogstraal, 1979).

1.2.3 VARIOUS DETECTION METHODS

In the past, CCHF virus has been propagated and titrated most commonly by intracerebral inoculation of suckling mice. The virus is non-pathogenic for other laboratory animals, including rabbits, guinea pigs and monkeys (Swanepoel, 1998a). Isolation of the CCHF virus by inoculation of infant mice is generally slow, with an

incubation period of 5 to 10 days (Hoogstraal, 1979; Swanepoel *et al.*, 1983; Shepherd *et al.*, 1985; Shepherd *et al.*, 1986). Diagnostic results can be achieved more rapidly (1 to 6 days) by cell culture, but cell cultures are less sensitive than cultures in mice. Attempts to isolate virus from clinical specimens are often unsuccessful (Shepherd *et al.*, 1986; Swanepoel *et al.*, 1987). The CCHF virus can be grown in a wide variety of primary and line cell cultures, including Vero, CER, BHK-21 and SW13 cells. The CCHF virus has a poor cytopathic effect and hence infectivity the virus is titrated by plaque production or demonstration of immunofluorescence in infected cells (Swanepoel, 1998a). It is important to note that most of the patients who succumb to infection fail to develop an antibody response (Swanepoel *et al.*, 1987). In a number of limited studies, diagnosis of CCHF infection has been achieved by detection of antigen in sera or organ homogenates by reversed (or indirect) passive haemagglutination (RPHA) (Klisenko *et al.,* 1984; Shepherd *et al.*, 1985). Enzyme-linked immunosorbent assays (ELISAs) have been described for the detection of CCHF virus antigen (Donets *et al.*, 1982). ELISAs have also been used for the detection of CCHF virus antigen in suspensions of naturally infected ticks (Shepherd *et al.*, 1988). A rapid reverse transcription-polymerase chain reaction (RT-PCR) method have been described as a method to detect CCHF in both human and tick samples. This methodology, followed by DNA sequencing and phylogenetic analysis of amplimeres, enabled the efficiently identification of infected ticks and humans, analyse the genetic characteristics of the CCHF viruses and determine the possible origin of these infections (Rodriguez *et al.*, 1997). CCHF virus is regarded as a class 4 agent and biosafety level four (BSL-4) containment facilities are required for isolation.

1.2.4 DIAGNOSTIC PROCEDURES

1.2.4.1 Clinical diagnosis

The incubation period of CCHF virus infection is generally short, ranging from 1 to 3 days (maximum 9 days) following infection by tick bite and is usually 5-6 days (maximum 13 days) in person exposed to infected blood or other tissues of livestock or human patients (Swanepoel, 1998a).

When presenting with an illness that might be CCHF, the patient must be immediately hospitalised in isolation for proper care and appropriate investigating (Altaf *et al.*, 1998). It is often difficult to make a diagnosis of CCHF virus infection during the pre-haemorrhagic period (1 to 7 days post infection) as well as in mild cases. Moderate and severe disease courses are often easily diagnosed, but only during the haemorrhagic period (Burt *et al.*, 1998).

CCHF virus infection must be distinguished from other viral haemorrhagic fevers which partially overlap in distribution with CCHF: Lassa fever, Marburg disease, Ebola fever, Omsk haemorrhagic fever, Kyasanur Forest disease and the haemorrhagic fevers with renal syndrome (HFRS) group of diseases associated with Hantavirus infections. Other febrile illnesses which can be acquired from contact with animal tissues within the same geographic range as CCHF include Rift Valley fever, Q-fever, brucellosis and systemic anthrax, while diseases which can be acquired from ticks include Q-fever and tick-borne typhus (*Rickettsia conorii* infections, commonly known at tick-bite fever). However, severe forms of many other common infections may resemble CCHF, including the various types of viral hepatitis, malaria and bacterial septicaemia's (Swanepoel, 1998a).

1.2.4.2 Serological and virological diagnosis

Specimens to be admitted for laboratory conformation of a diagnosis of CCHF include blood from live patients. In order to avoid performing full autopsies, heart blood and liver samples taken with a biopsy needle from deceased patients. On account of the propensity of the virus to cause laboratory infections, and the severity of human disease, investigations of CCHF is generally undertaken in maximum security laboratories (Swanepoel, 1998a).

According to Casals (1973a & 1973b), as cited in Hoogstraal (1967), the haemagglutination-inhibition (HI) test has not been generally applied for the detection of the CCHF virus. Casals (1973b) also confirmed that HI and neutralisation tests are considered vital for conducting antigenic relationship studies on arboviruses. The results of serodiagnosis and sero-epidemiological surveys are difficult to evaluate due to cross-reactions. Saidi *et al.* (1975) found a good relationship between the modified agar gel diffusion precipitation (AGDP), neutralisation and haemagglutination-inhibition (HI) tests in Iranian sheep sera. The complement-fixation (CF) test revealed only one third as many positives as the other tests. The results of Saidi *et al.* (1975) with human sera were inconclusive and differ from those with sheep sera. None of the human sera, positive in the modified AGDP test, were positive in the neutralisation and CF tests and only 5 out of 31 were positive at low titres in the HI test. In a CCHF survey done by Zarubinsky *et al.* (1975) as cited in Watts *et al.* (1988) the indirect haemagglutination-inhibition (IHI) test produced 7 times as many positives in cattle sera as the AGPD test. Sera from 4 persons that tested positive in the IHI test, tested negative in the AGPD test. With the aid of IHI tests, CCHF virus antibodies were detected in humans 6 to 9 years after infection.

In many sero-surveys, the AGDP and CF tests have been widely employed, but the results are not readily interpretable due to problems related to the sensitivity of these techniques and possibly the low and transient nature of antibodies produced by CCHF viral infections. Serological techniques such as the HI test, routinely employed for most arboviruses, have not been used extensively because not all strains yield agglutinating antigen (Hoogstraal, 1979). The CF test is useful for the diagnosis of current cases and possibly for surveys. The AGPD test should be applied mainly to determine strain differences of the virus. The HI test is mainly used for diagnosis and surveys and for studying related CCHF virus strains and other arboviruses. The IHI test is presumed to have the same applications as the HI test. The neutralisation test, whether in the form of mouse neutralisation, plaque reduction, or reduction of foci of infection, is important for all studies. Neutralisation tests have not been considered acceptable diagnostic methods, because of non-specific antiviral activity associated with serum of both human and lower vertebrates. These non-specific factors were eliminated by acetone-ether treatment of human sera (Watts *et al.*, 1988). The direct and indirect fluorescent antibody techniques (FAT) are mainly used to diagnose disease in humans, for surveys, and for detecting the virus in vectors (Hoogstraal, 1979). The addition of the IgM and antigen ELISA detection for CCHF has greatly enhanced the ability to identify acute cases. The antigen detection test has significantly enhanced the ability to identify the CCHF virus in vector tick species (Khan *et al.*, 1997).

1.2.5 ANTIGENIC RELATIONSHIPS

The CCHF virus is a member of the genus Nairovirus of the family Bunyaviridae, which at present contains 33 viruses arranged in seven serogroups on the basis of antigenic affinities (Mathews, 1982; Calisher, 1992). An antigenic relationship

among members of the genus has been demonstrated by cross-immune precipitation (Clerx *et al.*, 1981). Negatively stained electron microscopic preparations have revealed that the surface units of the CCHF virions are smaller than representative viruses of other genera of the family Bunyaviridae (Martin *et al.*, 1985). Nairoviruses are organised into related antigenic serogroups as have been shown in Table 1 (Calisher, 1992).

Table 1: Related antigenical serogroups of Nairoviruses

ANTIGENIC SEROGROUPS	VIRUS
CCHF virus	CCHF Hazara Khasan Abu Hammad Abu Mina Dera Ghazi Khan Kao Shuan Pathum Thani Pretoria
Huges virus group	Farallon Frazer Point Great Saltee Huges Puffin Island Punta Salinas Raza Sapphire II Soldado Zirqa
Nairobi Sheep disease virus group	Dugbe Nairobi sheep disease
Qalyub virus group	Bandia Omo Qalyub
Sakhalin virus group	Avalon Clo Mor Kachemak Bay Paramushir Sakhalin Taggert Tillamook
Thiafora virus group	Erve Thiafora

The antigenic relationship among these groups was demonstrated by complement-fixation (CF), haemagglutinationinhibition (HI), indirect fluorescent antibody (IFA) and neutralisation (N) tests (Casals, 1980).

1.2.6 STRAIN VARIATION AMONG CCHF VIRUSES

In spite of the wide geographic distribution of CCHF virus and the diversity of invertebrate and vertebrate hosts, kinetic neutralisation (N) tests failed to demonstrate significant differences among CCHF viral strains (Tignor *et al.,* 1980). Earlier studies employing modified agar gel diffusion precipitation (AGDP), mouse neutralisation, cell-culture interference and complement fixation (CF) tests demonstrated that there were no apparent antigenic differences among strains from several different geographic locations in Russia and Africa (Casals, 1969; Casals *et al.*, 1970). More detailed molecular comparisons among CCHF viral strains have been hindered by the need for BSL-4 containment facilities when working with the agent and difficulties in producing adequate concentrations of the virus (Watts *et al.*, 1988).

Different genotypes of CCHF virus were identified within the Bandia area in Senegal during an epizootic. Several genotypes of the CCHF virus are circulating simultaneously in this area. One genotype appears localised in this specific region and has endured for 20 years, whilst the cycle involves different tick species as vectors, with both rodents and ruminants as hosts of immature and mature stages. The cycles of the other two CCHF genotypes that are scattered widely in Senegal involve *Hyalomma sp.* ticks as vectors, with predominantly birds as hosts for immature ticks and cattle for adult ticks (Zeller *et al.*, 1997). With the use of a nested RT-PCR, sequence analysis of amplified cDNA products identified at least

three phylogenetically different CCHF virus variants causing an outbreak of CCHF virus infection in the United Arab Emirates (Schwartz *et al.*, 1996).

1.2.7 STABILITY

Little information about the stability of the CCHF virus is available. The infectivity of the CCHF virus is destroyed by low concentrations of formalin or β-prppriolactone. Being enveloped, the virus is sensitive to lipid solvents. The CCHF virus is labile in infected tissues after death, presumably due to a fall in pH. Infectivity of the CCHF virus is retained for a few days at ambient temperature in separated serums, for up to 3 weeks at 4°C. Infectivity is stable at temperatures below -60°C, but the CCHF virus is rapidly destroyed by boiling or autoclaving (Swanepoel, 1998a)

1.3 EPIDEMIOLOGY

1.3.1 GEOGRAPHICAL LOCATION

The CCHF virus is the most widespread among the tick-borne viruses associated with human disease and occurs in three major biotic zones of the world (Watts *et al.*, 1988). Sporadic distributed enzootic foci of CCHF virus infection have been described throughout southern Eurasia and have also been recognised in western China and other countries in southern Europe. A similar focal distribution pattern extends southward and spans a vast portion of the Middle East region, possibly including India and a large portion of Africa extending into the Southern Hemisphere. The evidence of CCHF virus enzootic foci for most countries is based on virus isolations from humans or ticks, and/or antibody detection in humans and domestic animals (Hoogstraal, 1979).

Historically, the recognition of CCHF virus enzootic foci has been characterised by an unpredictable and sudden occurrence of human cases in presumably non-

enzootic areas. While this phenomenon is not understood, evidence indicates that CCHF virus persists in silent cycles involving ticks and non-human vertebrate hosts. It is also possible that new enzootic foci may be established by infected ticks introduced by parasitized vertebrates, particularly birds and livestock that can disperse ticks within and outside of enzootic foci (Watts *et al.*, 1988). Adding to this confusing epidemiological picture are extensive movements of infected livestock (and ticks) between countries and continents and the potential role that migratory birds might play in the spread of the virus between distant geographic areas (Gonzalez-Scarano & Nathanson, 1996).

1.3.2 OCCURRENCE OF CCHF VIRUS INFECTION

The incidence of CCHF during epidemics in Eurasia was described as merely "sporadic" (Hoogstraal, 1979). As there was no systematic surveillance system, the estimated number of cases was based on different methods that varied from region to region nation-wide (Watts *et al.*, 1988). Goldfarb stated that since 1975, cases of CCHF virus infection have increased. Of all the reported CCHF virus cases, 12.5% were reported in the Rostov Oblast (Goldfarb *et al.*, 1980). The incidence of CCHF virus epidemics in other parts of the world has also been characterised by sporadic outbreaks and episodes, including cases acquired from tick bites and by contagion. Nosocomial cases occurred during the CCHF outbreak in Iraq. Apparently most cases were attributed to tick bite (Al Tikriti *et al.*, 1981).

The first recognised case of CCHF infection in South Africa occurred after a boy was bitten by a *Hyalomma sp.* tick in the Transvaal province during February 1981 (Gear *et al.*, 1982). Since then CCHF virus infection has become increasingly recognised as an important human disease in South Africa (Gear *et al.*, 1982; Shepherd *et al.*, 1987a). Recently, increased surveillance and greater awareness of the disease

have resulted in the diagnosis of an increasing number of human cases in several countries of the Middle East and Africa (Suleiman *et al.*, 1980; Swanepoel *et al.*, 1987).

In 1984 a nosocomial outbreak at Tygerberg hospital in the Western Cape Province, South Africa, resulted in seven cases and two deaths, including the index case (Van Eeden *et al.*, 1985). The increasing awareness of CCHF virus infection has led to the laboratory confirmation by the Special Pathogens Unit at the National Institute for Virology of 141 cases in southern Africa up to the end of 1998, with 28 deaths (Swanepoel *et al.*, 1998b). The fatality rate in South Africa is approximately 30% with deaths occurring between days 5 through 14 after onset of symptoms (Hoogstraal, 1979; Swanepoel, 1994, 1995). The most recent outbreak of CCHF virus occurred in November 1996 among workers at an ostrich abattoir in the Western Cape Province of South Africa, during which a total of 17 cases were confirmed (Burt *et al.*, 1997).

1.3.3 SEASONAL ACTIVITY AND DISTRIBUTION

Enzootic foci of CCHF virus occur in certain areas which are characterised by warm summers and relatively mild winters (Hoogstraal, 1979). These areas range from the arid desert and semi-deserts of Eurasia and North Africa to the wet Central African forests of Zaire, Uganda and the semi-arid high-altitude areas of eastern South Africa (Watts *et al.*, 1988).

In South Africa, a study has shown that adults of two of the *Hyalomma species*, *H. marginatum rufipes* and. *H. truncatum* are very common during summer, while the immature stages were active during winter and also demonstrated a second peak of activity in November. Larvae of *H. truncatum* and *H. marginatum rufipes* collected from vegetation demonstrated different patterns of activity. Larvae of the two

species showed two peaks in numbers, one peak in July and a second in November. The presence *H. marginatum rufipes* and *H. truncatum* during winter and summer indicate that the species go through two generations a year in the western Transvaal. Immature ticks were found on hares throughout the year, peaking in July and November. Larvae and nymphs of *H. truncatum* were more common on hares than immature stages of *H. marginatum* rufipes.

The results of adult ticks collected from the ground showed that *H. marginatum rufipes* males and females are found from September/October until February/March with a peak during December/January. Similar activity was present in adult *H. truncatum*. However, it appears that the adult activity of this species starts earlier in the season when compared with *H. marginatum rufipes* and lasts until March/April, with a peak in January/February. Adult ticks removed from game animals showed that *H. marginatum rufipes* was more active during December/January, while *H. truncatum* was more abundant during February/March (Rechav, 1986).

In South Africa, CCHF cases have occurred more commonly during the spring and summer seasons of the Southern Hemisphere, but cases have been reported for every month of the year except June. However, all the cases recognised during September were acquired by contact with a patient hospitalised during late August 1985 (Van Eeden *et al.*, 1985).

The pattern seen in South Africa therefore closely parallels the seasonal feeding activity period and the peak population density of the suspected CCHF virus tick vectors (Rechav, 1986). According to Fabiyi (1973), as cited in Watts *et al.* (1988) CCHF virus was isolated from wild, domestic animals and ticks throughout the year in Nigeria, but most isolates were obtained during October, November and December. Thus, the data clearly demonstrate the potential for the occurrence of

CCHF virus transmission to humans throughout the year in milder climatic regions where ticks may remain active.

1.3.4 RISK FACTORS

Shepherds, campers, agricultural workers, veterinarians, abattoir workers and other persons in close contact with live-stock and ticks are at risk for CCHF virus infection (Saluzzo *et al.*, 1984; Swanepoel *et al.*, 1983; Swanepoel *et al.*, 1985a; Chapman *et al.*, 1991). In addition too zoonotic transmission, CCHF virus can be spread from person to person and has caused many nosocomial outbreaks (Suleiman *et al.*, 1980; Van Eeden *et al.*, 1985; Fisher-Hoch *et al.*, 1995). Most of the CCHF virus infections have occurred among agricultural workers. Agricultural practices, particularly those allied with large domestic animals, are important risk factors for CCHF contracted from the bite of infected ticks. Exposure after crushing infected ticks and butchering infected animals has also been a frequent source of CCHF viral infection among these workers (Hoogstraal, 1979; Suleiman *et al.*, 1980; Swanepoel *et al.*, 1983; Swanepoel *et al.*, 1985a; Swanepoel *et al.*, 1985b; Van Eeden *et al.*, 1985). During many outbreaks a large proportion of the cases are among health care workers and the relatives of patients (Rodriguez *et al.*, 1997). Numerous contagion-acquired cases have been documented among medical workers and others who care for CCHF patients, as well as laboratory workers who handle material containing virus. All ages and both sexes appear equally susceptible to CCHF viral infection. An unequal distribution of cases among males and females is not uncommon. This phenomenon can be attributed to specific occupational activities that allow for differential exposure to the sources of CCHF viral infection (Hoogstraal, 1979; Suleiman *et al.*, 1980; Swanepoel *et al.*, 1983; Swanepoel *et al.*, 1985a; Van Eeden *et al.*, 1985). Infection occurs by contact with infected blood,

blood-containing vomit or respiratory secretions and possibly by aerosol from patients in advanced stages of the disease (Suleiman *et al.*, 1980). However, it is becoming increasingly evident that the greatest risk is in areas where adults of one or two species of ticks of the genus *Hyalomma* are predominant (Watts *et al.*, 1988). In endemic areas, sheep and cow antibodies appear to be one of the best indicators of risk to humans (Wilson *et al.*, 1990b; Gonzalez *et al.*, 1990).

1.4 TRANSMISSION CYCLES

1.4.1 VECTORS

During epidemics in Eurasia, ticks, mainly of the genus *Hyalomma*, had been circumstantially implicated as vectors for CCHF virus (Hoogstraal, 1979). A vector role was suspected on the basis of a temporal and spatial association between the seasonal distribution, population density and adult activity period of *Hyalomma* ticks and the occurrence of CCHF cases. Of greater significance was the observation that the patients revealed evidence of being bitten by *Hyalomma* or other tick species, or they had crushed ticks with their fingers. These observations supported a tick-borne route of transmission for CCHF virus, but it was not until the late 1960's that CCHF virus was isolated from adult *Hyalomma* ticks as well as from several other tick species (Hoogstraal, 1979). An exceptional biological feature of ticks is their potential to act as reservoirs of arboviruses and to transmit arboviruses transovarially (Burgdorfer & Varma, 1967). Immature and adult ticks can be infected with CCHF virus as a result of transstadial transmission and feeding on viraemic vertebrates (Logan *et al.*, 1989).

According to Chumakov (1969), as cited in Watts *et al.* (1988) evidence of transovarial and transstadial transmission of CCHF virus has been demonstrated after viral isolates were obtained from field-collected eggs and unfed immature

stages of *H. m. marginatum*. CCHF virus has also been isolated from field-collected, unfed *H. m. marginatum* nymphs and adults (Watts *et al.*, 1988). Haematophagous arthropods other than ticks have not been implicated as vectors of CCHF virus. A certain amount of progress has been made to postulate what the role of the tick species/subspecies, as vector of the CCHF virus will be. This can be attributed not only to the enormous number of suspected vectors, but to the extremely complex and diverse ecological and biological features of ticks. In addition, technological difficulties and the human health risk posed by working with CCHF virus have definitely hindered progress in understanding the relative importance of ticks as vectors of this virus. The extensive review referred earlier, provides an excellent overall coverage of the current understanding of the vector status of most species/subspecies of ticks (Hoogstraal, 1979). Despite much research documenting the widespread distribution of CCHF virus, its possible vectors and potential vertebrate reservoirs, the understanding of the transmission cycle(s) of the CCHF virus remains inadequate (Wilson *et al.*, 1991).

1.4.2 VERTEBRATE HOSTS

Vertebrates are fundamental as source of blood for the development and growth of ticks. Tick species associated with the CCHF virus affect a wide variety of vertebrates. However, the qualitative and quantitative roles, if any, of vertebrates in the maintenance and transmission cycle of the CCHF virus are poorly understood.

A variety of small animal species have been involved as hosts. The role, if any, of humans in the continuation of the natural cycle of CCHF virus is unknown. Whether CCHF viral infection of humans produces a sufficient viraemia to infect ticks has not been determined, but a human-to-human transmission cycle can be initiated by contact with blood or tissues of CCHF virus infected patients or domestic animals.

Viral isolation and serological evidence of infection have demonstrated evidence of CCHF viral infection among domestic animals, particularly livestock. Antibody prevalence among livestock has varied according to the time after infection. Despite the documented incidence of viraemia in livestock, CCHF virus transmission to ticks and the ability of various ticks to allow replication and transmit the virus in nature, still remain unclear (Watts *et al.*, 1988).

Among the vertebrate species known to be susceptible to CCHF virus, small mammals appear to have the greatest potential for a contribution to the maintenance and transmission cycles of the virus. Evidence of CCHF viral infection has been demonstrated by isolation of the virus from hares in the USSR, hedgehogs in Nigeria and a multimammate mouse in the Central African Republic (Causey *et al.*, 1970; Kemp *et al.*, 1974). Antibody responses were only detected in South African hedgehogs, highveld gerbils, Namaqua gerbils, 2 species of multimammate mouse (*Mastomys natalensis* and *M. coucha*) and Syrian hamsters (Shepherd *et al.*, 1989a). Hares are the most important mammalian host for the immature stages of all 3 *Hyalomma spp.*, which occurred, in southern Africa (Shepherd *et al.*, 1987a; Rechav *et al.*, 1987). Hares were also involved as important hosts of the CCHF virus during outbreaks in Eurasia (Hoogstraal, 1979) and more recently they were considered important hosts in South Africa (Swanepoel *et al.*, 1983). Serological evidence of CCHF virus infection has been demonstrated in these, as well as several other vertebrates. The large wild vertebrates are utilised as a source of blood supply for adult ticks. Sero-epidemiological surveys indicate different percentages of positive reactions for CCHF antibodies in sera of domestic cattle, horses, donkeys, sheep, goats and pigs in Eurasia and Africa (Hoogstraal, 1979).

1.4.3 EXPERIMENTAL INFECTION

Although CCHF virus has an extensive geographical distribution and the disease has serious consequences, there are comparatively little experimental data on the possible role of vertebrates as virus-amplifying hosts. Early Soviet studies found many infected nymph ticks on rooks (Hoogstraal, 1979). After experimental inoculation of chickens and doves with CCHF virus, the birds remained healthy and evidence of a viraemia or an immune response was not demonstrable. CCHF virus was unable to replicate in chickens, as shown by the absence of viraemia and antibody response and the failure of these birds to transmit the virus to immature *H. marginatum rufipes* ticks. However, a significant antibody response was obtained in red-beaked hornbills and glossy starlings. The antibody response indicated some viral replication that would permit the infection of ticks. Four months later antibodies were still detectable in these birds. Transmission of CCHF virus to larvae/nymphs was obtained with these birds even though the birds had an undetectable viraemia. The virus was subsequently transmitted transstadially to nymphs, adult ticks and infected rabbits that were used as experimental hosts of the adult stages. The CCHF virus was recovered from the offspring of these ticks. Transovarial transmission of CCHF virus was successful and larvae were able to infect other birds (Zeller *et al.*, 1994a).

In the past, birds were not thought to be important reservoirs of the CCHF virus, because they did not develop a significant level of viraemia. The Russian investigators were unable to re-isolate the CCHF virus and did not obtain serological evidence of infection in rooks and rock doves (Hoogstraal, 1979). In guinea fowls, viraemia of low intensity was demonstrated, followed by a transient antibody response. A case of CCHF virus infection in a worker who was infected while slaughtering ostriches on a farm in South Africa has been reported. Antibodies to

CCHF virus were detected in 23.9% of the ostriches tested (Shepherd *et al.*, 1987b). Calves were infected with a CCHF virus strain from a Nigerian goat and showed demonstrable viraemias (Causey *et al.*, 1970). Cattle, sheep and goats have been suspected to be a source of virus during epidemic manifestations (Watts *et al.*, 1988). In a study done by Gonzalez sheep were infected with the CCHF virus, either by intraperitoneal inoculation or by the bite of experimental infected ticks (Gonzalez *et al.*, 1989, 1992). A fever has been found as a clinical symptom associated with a period of onset in adult CCHF virus-infected sheep. The persistence of fever reflects the effectiveness of virus infection and replication (Gonzalez *et al.*, 1998). Following an outbreak of CCHF among workers at an ostrich abattoir in South Africa in 1996, 9 susceptible young ostriches were infected subcutaneously with the virus in order to study the nature of the infection. The ostriches developed viraemia, which was demonstrable on days 1 to 4 following infection. The CCHF virus was detectable in visceral organs such as spleen, liver and kidney up to day 5 post-inoculation. No infective virus was detected in samples of muscle, but viral nucleic acid was detected by RT-PCR in muscle from a bird sacrificed on day 3 following infection (Swanepoel *et al.*, 1998b).

1.4.4 HIBERNATION

Epidemiologically, the long survival of arboviruses in ticks is an important factor. This is especially important where populations of short-lived, small-sized bird or mammal hosts of long-lived ticks have a rapid turnover in the ecosystem. These hosts rapidly develop antibodies to CCHF virus infection acquired in the nest during their first few days of life (Hoogstraal, 1973a). In general, data suggest that in nature CCHF virus often survives throughout the life of the tick and may be transovarially (or vertically) transmitted from one tick generation to the next.

Chumakov has reported that ticks served as hibernation hosts of the CCHF virus in unfed nymph and female *Hyalomma m. marginatum* which were collected in the field during spring in the Crimean, Rostov and Astrahan Oblast (Watts *et al.*, 1988).

1.4.5 TRANSSTADIAL SURVIVAL AND TRANSOVARIAL TRANSMISSION

In argasid and ixodid ticks the transstadial survival of the CCHF virus (from larva to nymph to adult) is an important epidemiological factor. This phenomenon is rare in haematophagous insects. The reason for this biological difference lies in the relatively insignificant structural changes of the tick during moulting, when ectodermal derivates and certain muscle groups are practically the only structures to undergo histolysis. Only the tick salivary gland alveoli are completely replaced while moulting. Throughout the entire life cycle of the tick vector, the midgut, malpighian tubules and other organs that are intensely invaded by micro-organisms, are gradually replaced. The phenomenon of transovarial transmission of pathogens is more common in ticks than insects and requires more precise investigation (Hoogstraal, 1979).

Large wild and domestic mammals cannot be excluded, but limited experimental data suggest that ticks are not readily infected by feeding on large domestic mammals during the viraemic phase of CCHF viral infections (Watts *et al.*, 1988). The persistence of the CCHF virus during hostile climatic conditions in subtropical and tropical regions is likely to rely on similar mechanisms of infection of the vector tick species, but the climatic conditions may be permissive for a continuous transmission cycle involving ticks and vertebrates (Rechav, 1986).

If applicable to CCHF virus, it is likely that small mammals, e.g. hares, serve as principal virus-amplifying hosts in the proposed maintenance cycle involving *Hyalomma* ticks as vector/reservoirs (Figure 3).

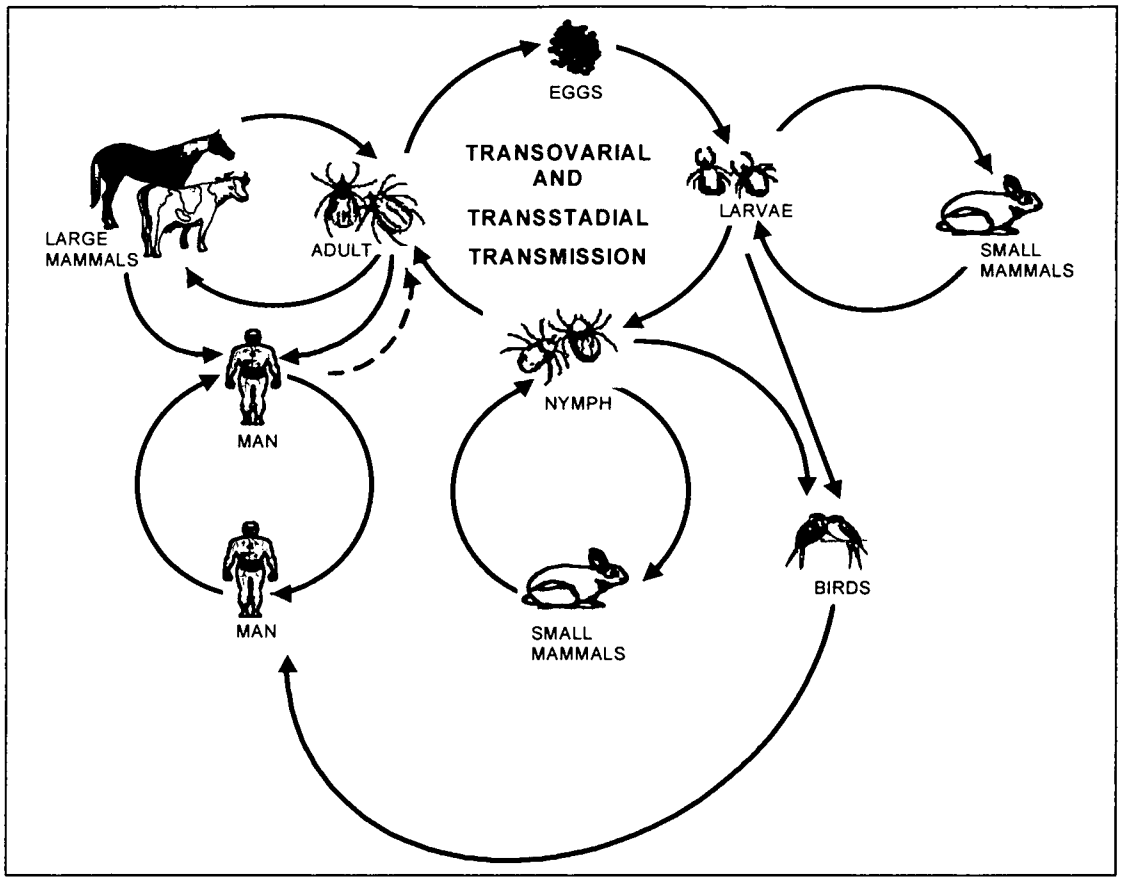

Figure 3. CCHF virus maintenance and transmission cycles involving *Hyalomma marginatum marginatum* and associated vertebrate hosts (Gear *et al.*, 1982; Watts *et al.*, 1988)

1.5 TICK ECOLOGICAL DYNAMICS

The CCHF virus has been isolated from tick species associated with CCHF virus infection in Eurasia (Hoogstraal, 1979). Despite the documented incidence of CCHF viraemia in livestock, virus transmission to ticks and the capability of various ticks to replicate and transmit the virus in nature, remain uncertain (Watts *et al.*, 1988). Much more needs to be learned regarding interaction among a variety of vectors, hosts and even virus strains (Gonzalez *et al.*, 1991). The tick species associated with the CCHF virus in Eurasia are listed below (Hoogstraal 1979; Camicas *et al.*, 1997; Zeller *et al.*, 1997).

Family Argasidae

Argas (Persicargas) persicus (Oken)

Family Ixodidae

Ixodes (Ixodes) ricinus (Linnaeus)

Haemaphysalis (Aboimisalis) punctata Canesrini and Fanzago

Hyalomma (Hyalomma) anatoliticum anatoliticum Koch

Hyalomma (Hyalomma) asiaticum asiaticum Schulze

Hyalomma (Hyalomma) detritum Schulze

Hyalomma (Hyalomma) marginatum marginatum Koch [= *H. P. plumbeum* (Panzer)]

Hyalomma (Hyalomma) marginatum turanicum Pomerantsev

Hyalomma dromedaii Koch

Dermacentor (Dermacentor) daghestanicus Olenev

Dermacentor (Dermacentor) marginatus (Sulzer)

Rhipicephalus (Digineus) bursa Canestrini and Fanzago

Rhipicephalus (Rhipicephalus) pumilio Schulze

Rhipicephalus (Rhipicephalus) rossicus Yakimov and Kohl-Yakimova

Rhipicephalus (Rhipicephalus) sanguineus Latreille

Rhipicephalus (Rhipicephalus) turanicus Pomerantsev and Matikashvili

Rhipicephalus evertsi evertsi Neumann

Rhipicephalus guilhoni Morel & Vassiliades

Boophilus annulatus (Say) [= B. calcaratus Birula]

Boophilus microplus (Canestrini)

Boophilus decolaratus Koch

Boophilus geigy Aesclimann and Morel

The CCHF virus has been isolated from the following 9 ixodid species in Africa (Hoogstraal, 1979).

Hyalomma (Hyalomma) anatoliticum anatoliticum Koch

Hyalomma (Hyalomma) impeltatum Schultz and Schlottke

Hyalomma (Hyalomma) impressum Kock

Hyalomma (Hyalomma) marginatum rufipes Koch

Hyalomma (Hyalomma) nitidum Schulze

Hyalomma (Hyalomma) truncatum Koch

Amblyomma (Theileriella) variegatum (Fabricius)

Rhipicephalus (Lamellicauda) pulchellus Gerstäcker

Boophilus decoloratus (Koch)

1.5.1 BITING ACTIVITY AND HOST PREFERENCE OF VECTOR TICKS

Cattle represent the most sensitive indicator of a low level of CCHF virus circulation, because they can be infested 10 times more heavily than small ruminants by *Hyalomma* ticks (Camicas *et al.*, 1990). In view of the high prevalence and the wide distribution of antibodies in cattle, it is pertinent to ask why the disease has not assumed greater medical significance. To some extent, the answer may lie in failure to recognise the disease in the past. On the other hand, results from antibody tests on farm residents and veterinary personnel suggest that the infection is rare even in population groups with occupational exposure to the virus. The host preferences of tick vectors of the virus must obviously play an important role in determining which vertebrates become infected (Swanepoel *et al.*, 1983). Local species of the genus *Hyalomma* are considered to be the main vectors of the CCHF virus (Clarke & Casals, 1985). As immature ticks, this species feeds exclusively on small mammals and ground-feeding birds. As adult ticks they feed on large mammals such as cattle,

in preference to man or smaller mammals. *Amblyomma variegatum* might intensely parasitize a wide variety of vertebrate hosts. Occasionally ground-dwelling birds were found with intense infestations of larvae *H. marginatum rufipes, H. impeltatum, H. truncatum* and *Amblyomma variegatum* ticks (Camicas *et al.*, 1990).

Studies on the biting activity of larval, nymph and adult ticks usually examine the seasonal incidence of the ticks on vegetation or ticks attached to their hosts. Data concerning host preference is of crucial epidemiological importance in understanding CCHF viral maintenance cycles. It is also important to understand the activity of the ticks and the number of hosts that have been parasitized by an individual tick during its lifetime. The potential variety of hosts and the degree of host specificity are biological parameters that must be understood (Watts *et al.*, 1988).

Hoogstraal (1979) has reviewed the general features of the one-, two-, three-, and multi-host types of ixodid life cycle.

1.5.1.1 One-host ticks

All the *Boophilus* species are one-host ticks and the developmental stages are completed on the same vertebrate host. The feeding period of the *Boophilus* population extends over weeks. Their role in the ecology and epidemiology of the CCHF virus is not very clear. The *Boophilus* tick species is biologically adapted to wandering hoofed mammals and seldom attacks man (Hoogstraal, 1973b).

1.5.1.2 Two-host ticks

Two-host ticks can be divided into two subgroups based on their feeding habits. The first group includes those that utilise similar vertebrate host species for the development of immature and adult stages. Unlike one-host ticks, the immature

stages complete their development on a host and detach as blood-engorged nymphs. The nymphs moult and reattach as adults on a similar vertebrate host species. This pattern is characteristic of *R. d. bursa, H. detrium, H. a. anatoliticum* and possibly *H. truncatum*. The second group includes those species that depend on two dissimilar host species for their development i.e.; the vertebrate host species utilised by immature stages differs from that of the adult stage. Several of the *H. marginatum* complex species exhibit this feeding pattern. Of these two subgroups, all the *Hyalomma* ticks are considered to be especially important as epidemic and enzootic CCHF virus vectors and possible reservoirs for the CCHF virus (Hoogstraal, 1979).

1.5.1.3 Three-host ticks

Three different vertebrates serve as hosts for the 3 developmental stages of these ticks. The three-host system exposes the tick to three possible environmental extremes. If more ixodids had been able to convert to a two- or one-host pattern, tick populations might be more ubiquitous than they are in the modern world. CCHF virus has been isolated from six of three-host ticks in Africa, including *H. impeltatum, .H. nitidum, H. truncatum, A. variegatum, R. pulchellus* and *R. appendiculatus.* Existing data suggest that three-host ticks are primarily involved as enzootic vectors of the CCHF virus (Hoogstraal, 1979).

1.5.1.4 Multi-host ticks

Argasid ticks typically feed once as larvae, 2 to 4 times as nymphs, and several times as adults. From 5 to 20 hosts may be utilised as a source of blood during the life cycle of these ticks (Hoogstraal, 1973c).

1.5.2 MACRO- AND MICRO-ENVIRONMENT

Hyalomma ticks seem to be essential for the enzootic distribution of CCHF virus in the Palearctic, Oriental and Ethiopian regions (Hoogstraal, 1979). Within enzootic foci, *Hyalomma* ticks appear to be restricted to areas with long dry seasons. The most intense viral activity is in restricted areas where the variety and population density of the ticks and their natural hosts are the greatest. Factors influencing transmission of CCHF virus include the density of competent vectors and the relative abundance of vertebrates that serve as both hosts of these ticks and possible reservoirs (Watts *et al.*, 1988).

A study done by Swanepoel (1985a) showed that antibodies to CCHF virus were widely distributed in cattle sera in South Africa. The only area that appears to have a consistently low prevalence of antibodies is along the southern coast of South Africa. This could indicate that there is either a low level of virus transmission in this area or that natural transmission does not occur or that the few cattle with antibodies originate in the interior (Swanepoel *et al.*, 1985a). Reasons for this low level of CCHF virus activity could also include the absence of one of the suspected tick vectors, *H. m. rufipes*, from the area (Howell *et al.*, 1978) or lack of one or more of the small mammals suspected of being a reservoir host of the CCHF virus (Swanepoel *et al.*, 1983).

1.5.3 VECTOR OVIPOSITION

Ticks of the family Ixodidae oviposit only once during their lifetime. Eggs are laid without regard to location after detaching from the host. Usually numerous eggs are laid at a time, sometimes more than 10 000 eggs (Nuttall, 1915). A single *H. m. marginatum* female can lay from 4300 to 15000 eggs six days after detaching from the host and *H. m. rufipes* laid an average of 6867 eggs (Knight *et al.*, 1987).

Oviposition starts in summer 3 to 10 days after feeding, but is delayed for weeks or months during winter (Watts *et al.*, 1988).

1.5.4 DENSITY, FERTILITY AND LONGEVITY OF VECTOR TICKS

Epidemic transmission of CCHF virus coincides with an increased population density and feeding of the vector tick species (Hoogstraal, 1979). *H. m. rufipes and H. truncatum* have been implicated as vectors of CCHF virus in South Africa where they exhibit peak population densities during summer. While seasonal periodicity in density is characteristic of tick population, fertility and activity can be affected by temperature, humidity, predators, fire, flooding, and host availability (Watts *et al.*, 1988).

The fertility rate of a tick population is the product of the total number of eggs oviposited and the number of generations completed during that particular year. The number of eggs produced by individual females of different species varies extremely. Four to five months are required for a tick generation to reach adulthood. The climatic conditions and host availability may drastically increase generation time and decrease the overall fertility of a tick population.

In South Africa, in the western Transvaal, both *H. m. rufipes* and *H. truncatum* probably complete two generations per year. In the south eastern parts of Zimbabwe, *H. m. rufipes* completes only one generation per year (Rechav, 1986).

It is well known that ticks survive for months or years without a blooodmeal in the absence of a host and that they may hibernate. The time required for the completion of the life cycle may therefore vary considerably and be much prolonged under unfavourable conditions (Nuttall, 1915). The longevity of a tick, due to its extended development cycle, may also reduce its chances of survival. On the other hand

longevity can maximise blood feeding that is necessary for viral transmission (Watts *et al.*, 1988).

1.5.5 VERTEBRATE HOSTS AND SEROLOGICAL BACKGROUND OF CCHF VIRUS

1.5.5.1 Horizontal transmission

The horizontal transmission of CCHF virus in ticks may occur by various means. Classically it occurs when vectors feed, during the time vertebrate hosts are viraemic. However, other mechanisms are suspected, as host viraemia is often undetectable. CCHF virus transmission has been observed when non-infected ticks co-feed with infected ones (Logan *et al.,* 1989). The effective horizontal transmission of CCHF virus was demonstrated in *H. truncatum* ticks feeding on a non-viraemic, pre-immunised and infected sheep (Wilson *et al.,* 1991).

A serological survey in southern Africa has shown that CCHF virus antibodies were widely distributed among wild mammals, which reflected the feeding preferences of immature and adult *Hyalomma* ticks (Shepherd *et al.,* 1987a). The need for a sufficient number of vertebrate hosts to support the population of vector tick species, is a fundamental requirement for the maintenance of the CCHF virus. The biological or ecological factors which determine the apparently close relationship between the CCHF virus and ticks of the genus *Hyalomma* are still unknown, nor is it known to what extent ticks participate in the natural circulation cycle of the virus. The maintenance cycle of CCHF virus is still not fully understood, particularly in southern and western Africa (Watts *et al.*, 1988). Antibodies to CCHF virus infection were found in numerous species of vertebrates. Few of these vertebrate species are thought to develop CCHF viraemias high enough to be involved in the transmission of the CCHF virus (Causey *et al.*, 1970; Saidi *et al.*, 1975; Shepherd *et al.*, 1987a;

Shepherd *et al.*, 1989a; Gonzalez *et al.*, 1992). Most of the vertebrates infected with the CCHF virus are thought not to be important reservoirs because few developed high viraemias (Gordon *et al.*, 1993). The virus is present in the blood for up to seven days. A few days later antibodies to the CCHF virus appear. It appears that adult ticks do not acquire the virus from their hosts. The larvae probably contract the virus from hares on which they feed (Rechav, 1986).

A novel mode of arbovirus transmission of Thogoto virus to ticks while co-feeding (4 to 6 days) with infected ticks on a guinea pig that did not have a detectable viraemia was described by (Jones *et al.*, 1987). Classically, CCHF virus is acquired by an arthropod during a bloodmeal from viraemic vertebrate hosts. However, many infected hosts apparently exhibit a brief, sometimes undetectable, viraemia (Gonzalez *et al.*, 1989). It is more likely that the CCHF virus, in the absence of tick vectors, is not horizontally transmitted within a sheep herd (Camicas *et al.*, 1990) and horizontal transmission from sheep to sheep appears nearly impossible (Gonzalez *et al.*, 1998).

1.5.5.2 Vertical transmission

Vertical transmission of the CCHF virus has been demonstrated by transovarial transmission (Lee & Kemp, 1970). CCHF virus appeared via transovarial transmission in a few larvae, but not in nymphs. The variable rates of transovarial transmission depend on numerous factors related to the biological characteristics of the virus as well as vector physiology (Gonzalez *et al.*, 1992).

According to Shepherd, transstadial and transovarial transmission of the virus was reported in *H. m. marginatum* ticks in the USSR and Bulgaria (Shepherd *et al.*, 1989b). In Nigeria *H. marginatum rufipes* was intracoelomically inoculated with CCHF virus and the ticks demonstrated both transstadial and transovarial

transmission of the CCHF virus (Lee & Kemp, 1970). The CCHF virus replicated rapidly when ticks were infected, reaching a plateau a few days post inoculation. These ticks remained infected for several months, though virus replication varied among individuals (Gonzalez *et al.*, 1991).

Transovarial transmission of the CCHF virus in the principal *Hyalomma sp.* tick vectors could serve as a maintenance mechanism. However, transmission rates reported in the literature may be too low to have an impact on virus maintenance (Hoogstraal, 1979). CCHF virus was detected in nymphs and adults arising from ticks exposed to the CCHF virus as either larvae or nymphs and this phenomenon demonstrates that the ticks became infected and that the virus was transmitted transstadially (Gordon *et al.,* 1993). This type of transmission may be an important factor in the ecology of the CCHF virus because the distribution and population densities of *Hyalomma sp.* are closely associated with the occurrence of CCHF virus, particularly in Africa (Hoogstraal, 1979). According to Gonzalez (1992), uninfected female *H. truncatum* became infected while co-feeding with infected males on rabbits. Two examples of mechanisms that might play a role in increasing the rate of CCHF virus infection of ticks in nature, are transovarial transmission to the next generation and direct transmission from male to female during mating. Pre-infected female ticks, however, did not sexually transmit the CCHF virus to males (Gonzalez *et al.*, 1992). The abundance of infected ticks suggests some sexual or co-feeding transmission, or both, of the virus between ticks as well as transmission directly from animal to ticks (Jones *et al.*, 1987). The relative role of vertical (transovarial) and horizontal transmission of the CCHF virus in the tick population has not been adequately studied. If horizontal transmission of the virus between vertebrates and the various stages of the tick is essential for maintenance of the virus, the density of susceptible vertebrate species is an important factor. It

determines the prevalence of ticks capable of transmitting CCHF virus to susceptible hosts. If vertical transmission of this virus occurs with the same efficiency as demonstrated for experimentally infected familial "lines" of mosquitoes (Tesh, 1980), the role of vertebrates may be secondary in regard to viral amplification, but essential as a source of blood for aiding the vector population. The nature of tick-borne seasonal epidemiology differs because ticks are relatively immobile and enjoy an extended life cycle and longevity of each stage. The virus might survive in areas for fairly extensive periods of time by relying on vertical transmission among ticks and survival of already infected ticks (Watts *et al.*, 1988).

With few exceptions, the CCHF disease in humans seems to occur at irregular intervals and in low numbers. Environmental and climatic conditions may sporadically favour increased densities of vertebrates and their associated tick species. If humans are unfortunate enough to be undertaking major projects in these areas, larger numbers of cases may occur, as illustrated by the epidemic in the Crimea during 1944 (Hoogstraal 1979).

The immune status of the vertebrate hosts may play a twofold role in the maintenance and transmission of the CCHF virus. Firstly, if horizontal transmission of the virus is a critical factor, flourishing populations of young and susceptible animal hosts may amplify the prevalence of the virus among the susceptible tick population. Secondly, immune mechanisms of vertebrates have been shown to reduce the feeding success of attaching ticks. This phenomenon may hinder the ability of the tick population to expand and to effectively serve as a vector and/or reservoir for the CCHF virus. However, the dynamics of these tick populations remain unclear. Sustained transmission is found only where *Hyalomma sp.* ticks are present and epizootic transmission occurred during periods of increased abundance of these ticks (Watts *et al.*, 1988). Vertical transmission in ticks appears more likely

to reduce the rate of infection during the successive stages of the ticks (Watts *et al.*, 1988; Logan *et al.*, 1989; Wilson *et al.*, 1991; Gonzalez *et al.*, 1992). The maintenance of the virus will depend mainly on the efficiency of the vertebrate host in multiplying the virus and in becoming highly infested by potential vectors (Gonzalez *et al.*, 1998).

1.5.6 VECTOR CAPABILITY

No quantitative data have been reported on the vector capability of ticks for the CCHF virus. The biological features of ticks in general are particularly suitable and unique among haematophagous arthropods regarding their potential competence to serve as effective vectors and reservoirs for the CCHF virus (Hoogstraal, 1979). It is not surprising that the ability of a virus to replicate depends on the vector. Two major factors could influence intrinsic virus replication in ticks: firstly the gut barrier limiting passage of the virus out of the gut and secondly the ability of tick haemocytes, haemolymph or target organs to favour virus replication. Tick species vary in the composition of the haemolymph (Neitz *et al.*, 1978) and only a few of the more than 30 species that have been found to be naturally infected by CCHF virus, favour active virus replication (Hoogstraal, 1979; Camicas *et al.*, 1990). Under favourable ecological and climatic conditions, ticks are long-lived, extremely productive, absorb large quantities of blood from a wide variety of animal species and can adapt to many different ecological environments. All stages of both male and female ticks are parasitic. Ixodid ticks attach to their hosts for extended periods of time. This provides a mechanism for the distribution of ticks over long distances and allows the colonisation of new areas. More importantly, as potential vectors, the extended period of attachment to a host is likely to increase the probability of ingesting an infective bloodmeal or viral transmission to the host. Arboviral

infections persist throughout the lives of ticks and vector potential and efficiency are likely to be enhanced by different transmission routes, including the possibility of transmission from male to female ticks during copulation (Watts *et al.*, 1988).

1.5.7 MOVEMENT AND MIGRATION OF VECTORS AND HOSTS

Different avian species are responsible for both intra- and intercontinental dissemination of ticks associated with the CCHF virus. Distribution of ticks may be restricted to short distances during local post-breeding flights or long distances during migration flights of the migrating birds. Studies done on tick parasites found on birds migrating through Egypt between 1955 and 1973, provided important information on the intercontinental distribution of ticks. More than 90% of immature ticks found on birds migrating south between 1959 and 1981 were species which had been associated with the CCHF virus. On birds migrating north from sub-Sahara and Africa to Eurasia, the parasite *H. m. rufipes* was exclusively found. The movement of domestic animals to new pasture lands, markets, abattoirs, as well as the migration of wild mammals, may also contribute to the dissemination of CCHF virus from enzootic foci. Both the infected animals and their ticks may serve as a source of CCHF virus in new areas (Hoogstraal, 1979).

1.5.8 HUMANS IN DISEASE ECOLOGY

The virus survives the moulting process to persist in adult ticks, thus humans can be infected if bitten by an adult tick (Rechav, 1986). During enzootic foci humans become infected sporadically with CCHF virus when they inadvertently interrupt ongoing viral circulation between the tick vector and the natural host. Unusual human activities can alter the natural vector-host cycle and create unnatural conditions predisposing to an outbreak of CCHF virus infection (Hoogstraal, 1979).

Ticks transmit most human CCHF virus infections. The CCHF virus can also be spread by exposure to infected tissues. These agents therefore put medical, nursing and veterinary staff at great risk. It has been shown that with CCHF virus, the case fatality rate is higher following nosocomial infection than natural infection (Shope, 1985).

1.5.9 ASSOCIATION OF CCHF VIRUS WITH CERTAIN TICK SPECIES

CCHF virus has been isolated from at least 16 of the 17 tick species associated with the disease in Eurasia. Some of the *Hyalomma sp.* ticks are efficient laboratory vectors (Hoogstraal, 1979; Watts *et al.,* 1988; Logan *et al.*, 1989).

Currently there is insufficient knowledge concerning the potential for soft ticks to transmit CCHF virus. Results of laboratory transmission tests indicate that the soft tick *Ornithodoros sonrai* failed to transmit CCHF virus infection to naïve suckling mice. The reason for the failure to infect *O. sonrai* with CCHF virus indicates that this tick might possess a midgut virus barrier or that a midgut CCHF virus receptor is absent. However mechanical transmission of the CCHF virus may occur because of the relatively high viral titres that were detected in ticks immediately after they had engorged on viraemic suckling mice and because of the tendency of soft ticks to re-feed frequently (Durden *et al.,* 1993). The CCHF virus also failed to replicate in 3 Argasid tick species, *A. walkerae*, A. *p. percicus*, and A. *savignyi,* after intracoelomic inoculation (Shepherd *et al.,* 1989b).

Ticks of the species *Hyalomma truncatum* and *Amblyomma variegatum* were infected with the CCHF virus by intra-anal inoculation. Gradually the amount of total viral antigen detected by antigen capture (AC) and the percentage of infected cells, increased. The CCHF virus replicated when ticks were infected, reaching a plateau a few days post-inoculation. The stability of the CCHF virus titre approximately 15

days after intrinsic inoculation suggests that a lifelong stable persistence of the virus may occur in certain susceptible ticks (Gonzalez *et al.*, 1991). Kondratenko reported that the CCHF virus was isolated up to 335 days following infection of *H. m. marginatum* ticks (Shepherd *et al.*, 1989b). An increase in the CCHF viral titres for experimentally infected nymph and adult *H. m. rufipes* were reported after bloodmeals (Okorie & Fabiyi, 1980). If ticks infected with CCHF virus were not allowed to feed constantly on hosts, their viral titre decreased with time. CCHF virus positive ticks did not always transmit the CCHF virus to the host during feeding (Logan *et al.*, 1989).

1.6 CCHF VIRUS DISEASE ASSOCIATIONS

1.6.1 HUMANS

Clinical signs of CCHF virus infection have only been confirmed in humans (Swanepoel *et al.*, 1989). Ticks of the genus *Hyalomma* are the main vectors of the virus (Hoogstraal, 1979). Humans usually acquire the disease through tick bite or contact with infected blood or other tissues from patients or livestock. Nosocomial infections arise from contact with blood or vomit of infected patients. Contact with blood appears to be an important factor during contraction of the CCHF virus infection, particularly where broken skin or pricked fingers are exposed to infectious material (Shepherd *et al.*, 1985). Human risk for disease in rural areas showed a direct relationship with contact with ruminants (Chapman *et al.*, 1991).

Symptoms of CCHF infection become evident after an incubation period of 2 to 9 days (Swanepoel *et al.*, 1989). The clinical course of CCHF virus infection can vary from mild to severe and is characterised by sudden onset of headache, fever, nausea, dizziness, photophobia and stiffness (Hoogstraal, 1979; Swanepoel *et al.*, 1983 & Swanepoel *et al.*, 1989). Leg and back pains as well as general myalgia are

severe. The illness may progress after 3 to 6 days to a haemorrhagic state. Haemorrhagic manifestations include petechial rash in mild cases, or ecchymosis and massive haemorrhage in severe cases. Bleeding in the form of melaena, haematemesis and epistaxis is common and occurs 4 to 5 days after the onset of symptoms. The majority of patients who develop severe illness are reported to develop hepatorenal failure approximately 5 days post-onset and become progressively drowsy, stuporous, and eventually comatose. Most of the CCHF patients who have died in South Africa developed multiple organ failure including cerebral and kidney failure with cardiac and pulmonary insufficiency (Watts *et al.*, 1988). The case fatality rate in South Africa is 22%, with deaths occurring on days 5 through 14 after onset of illness (Burt *et al.*, 1997).

Recovery from CCHF virus infection starts around day 9 to 10 with abatement of the rash and a general improvement of the patient. Recovery may be extended and may be accompanied by weakness, confusion and asthenia. Hair loss and local neuralgia have been observed in some patients (Watts *et al.*, 1988).

There are no pathognomonic lesions for CCHF; lesions in fatal cases seem to be primarily of vascular origin (Baskerville *et al.*, 1981). Liver samples from those who died in the recent South African outbreaks have showed varying degrees of necrosis of hepatocytes. Other organs, including the central nervous system and the kidneys, showed congestion, focal haemorrhage and necrosis (Watts *et al.,* 1988). At present are no data available on the cellular targets and distribution of CCHF virus in human tissues and little is known about the pathogenesis of the disease (Burt *et al.*, 1997).

1.6.2 DOMESTIC ANIMALS

There is a possibility that domestic animals may acquire the CCHF virus infection either on their home range, at collecting depots, or on trips through diverse

ecological zones. In such movements, the mingling of imported and resident animals may provide a means for virus dissemination through interchange of parasites and infections of new vectors (Causey *et al.*, 1970). Domestic ungulates are the principal host of most adult *Hyalomma* ticks and the prevalence of antibodies against CCHF virus in these animals indicates that they are frequently infected (Watts *et al.*, 1988; Gonzalez *et al.*, 1990; Wilson *et al.*, 1990a, 1990b). Sheep play a role in the horizontal transmission of the CCHF virus as they are among the most abundant tick-infested animals in many regions where CCHF virus circulates (Wilson *et al.*, 1991). In spite of a high turnover of local sheep herds (median age 3 years) and long-term CCHF antibody persistence (>3 years), sheep can be infected and efficiently transmit the virus at least once in a lifetime (Gonzalez *et al.*, 1998).

1.6.3 LABORATORY ANIMALS

The presence of CCHF virus in the urine of infected laboratory mice is an observation of possible importance in the epidemiology of animal infections and may constitute a risk for laboratory personnel (Causey *et al.*, 1970). A viraemia was observed in one out of 17 infected rabbits inoculated by various routes, but results of other studies indicate that the response of the laboratory rabbits to the CCHF virus may be heterogeneous (Levi & Vasilenko, 1972). Several reports suggest that small laboratory animals are susceptible to CCHF virus infection, but precise information on the nature of the viraemic response is lacking (Shepherd *et al.*, 1987b).

1.6.4 SMALL AND MEDIUM-SIZED WILD MAMMALS

CCHF virus antibodies have been found in hares *(Lepus saxatilis)* in the USSR, Bulgaria (Hoogstraal, 1979) and South Africa (Swanepoel *et al.*, 1983; Shepherd *et al.*, 1987a), as well as in long-eared hedgehogs (*Hemiechinus auritus*) and several

species of rodents in the USSR (Hoogstraal, 1979). Hedgehogs act as important hosts during the immature stages of many of the tick species from which the virus has been isolated. However, the response of individual hedgehog species to the CCHF virus appears to vary considerably. Blagoveshchenskaya and Zgurskaya reported that long eared hedgehogs *(H. auritus)* develop a viraemia which is of sufficient intensity to infect feeding ticks, while European hedgehogs are clearly refractory to infection (Shepherd *et al.*, 1989a). The west African hedgehog *(A. albiventris)* appears to be susceptible to CCHF virus infection, as the virus has been isolated from individuals trapped in the wild (Causey *et al.*, 1970).

A study done in South Africa indicates that the South African hedgehog (*Atelerix frontalis*) is not an amplifying host of the CCHF virus (Shepherd *et al.*, 1989a). Hares are one of the most important mammalian hosts during the immature stages of all 3 *Hyalomma spp.* that occur in southern Africa (Shepherd *et al.*, 1987a; Rechav *et al.*, 1987).

Table 2: Summary of viraemia and antibody responses of small wild mammals to CCHF virus infection

SPECIES	NO. BLED/DAY	VIRAEMIA		ANTIBODY	
		DURATION (DAYS)	MAXIMUM INTENSITY*	DAY OF APPEARANCE	MAXIMUM TITRE
Lepus saxatilis	4	7 (1-7)	4.2	6	512
Xerus inauris	1-2	7 (2-8)	3.2	8	512#
Mystomys albicaudatus	3-4	6 (1-6)	3.7	7	512
Aethomys chrysophilus	2-3	3 (1-3)	4.0	8	128
Tatera leucogaster	3-4	7 (1-7)	2.7	9	256
Rhabdomys pumilio	1-2	1 (5)	1.5	-⊗	-
Tatera brantsii	2-3	--	-	14	128
Mastomys coucha	4	--	-	7	256
Mastomys natalensis	4	--	-	5	1024
Desmodillus auricularis	1-2	--	-	7	256
Atelerix frontalis	2-5	--	-	8	256

*** Log $_{10}$ LD_{50}/ml**

⊗ Antibody was not detected in animals killed up to day 8

 # Tested by RPHI: all other species were tested by IF (Shepherd et al., 1989a)

This study indicates that a proportion of scrub hares *(Lepus saxatalis)* develop a CCHF viraemia which adequately infects feeding ticks. Hares of the genus *Lepus* appear to be the only vertebrates in southern Africa that meet the accepted criteria for definition as amplifying hosts of the CCHF virus (Shepherd *et al.*, 1987a).

1.6.5 BIRDS

The role of birds as a potential host reservoir has been considered, but their ability to transmit the virus was not clear. The immature stages of the *Hyalomma spp.* are known to be parasites of migrating birds (Hoogstraal, 1979).

In guinea fowls a viraemia of low intensity was demonstrated, followed by a transient antibody response (Shepherd *et al.*, 1987b). The most recent outbreak of CCHF virus occurred in November 1996 among workers at an ostrich abattoir in the Oudtshoorn district, South Africa. During this outbreak a total of 17 cases was confirmed (Burt *et al.*, 1997). A much higher prevalence of antibody to CCHF virus was detected in ostriches than in wild guinea fowl, the implication being that ostriches may undergo a more intense infection than do other birds, with a stronger and more durable antibody response (Shepherd *et al.*, 1987b; Burt *et al.*, 1993; Swanepoel, 1994). Ostriches developed a viraemia which was demonstrable on days 1 to 4 following subcutaneous infection with CCHF virus. Furthermore, virus was detectable in visceral organs such as spleen, liver and kidneys up to day 5 (Swanepoel *et al.*, 1998b).

It has also been postulated that non-viraemic transmission is mediated by factors secreted in the saliva of feeding ticks (saliva-activated transmission) (Jones *et al.*, 1989). Other modes of transmission, such as co-feeding and sexual transmission have also been described (Gonzalez *et al.*, 1992; Labuda *et al.*, 1993; Gordon *et al.*, 1993). These observations indicate that vertebrates that do not develop any

detectable viraemia can serve as important maintenance and amplifying hosts of the CCHF virus (Zeller *et al*, 1994a). Subsequently it was established that a few species of wild birds tested in West Africa fail to develop demonstrable viraemia following experimental infection (Zeller *et al.*, 1994b).

CHAPTER 2

MATERIALS AND METHODS

2.1 SOURCE OF BIOLOGICAL MATERIALS

2.1.1 CCHF VIRUS

Positive control CCHF viral RNA was prepared from Vero 76 cells infected with CCHF virus SPU 497/89 which was isolated at the National Institute for Virology (NIV), Sandringham, South Africa in 1989 from a human patient and used throughout the study.

2.1.2 TICKS

From December 1999 to January 2000, 35 *Hyalomma marginatum rufipes* and *15 Hyalomma truncatum* ticks were collected in the Boshof and Petrusburg districts, Free State Province, South Africa. The ticks were collected from cattle, sheep and goats. Each tick was individually placed in an Eppendorf tube. Two different methods were used for the extraction of the tick RNA, as described in section *2.2.1.2.1* and *2.2.1.2.2.*

2.1.3 ESCHERICHIA COLI TRANSFORMATION

Competent cells were prepared using calcium chloride and transformed as described by Sambrook *et al.,* (1989). The pGEMEX-I Vector with the cloned CCHF virus cDNA fragment (Promega Corporation) was transformed into competent *Escherichia coli* cells and incubated overnight at 37°C on SOB-agar plates containing ampicillin.

2.1.4 REAGENTS

All the reagents were of analytical grade and their supplier's name, city, country and catalogue number are shown in appendix A. A list of buffers and reagents are shown in appendix B.

2.2 EXTRACTION OF NUCLEIC ACIDS

2.2.1 RIBONUCLEIC ACID (RNA) EXTRACTION

To obtain a good preparation of eukaryotic mRNA, it is necessary to minimise the activity of RNases liberated during the lysis by using inhibitors of RNases or by using methods that disrupt cells and inactivate RNases simultaneously. Consequently, it is also important to avoid the accidental introduction of RNase from other sources in the laboratory. The single–step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform (AGPC) extraction was used for the isolation of RNA. This method of extraction provides high yield and the extracted RNA is both pure and undegraded. Due to the simplicity and the elimination of ultracentrifigation, the AGPC method allows simultaneous processing a large number of samples. The method proved to be particularly useful for RNA isolation from a few as 10^6 cells or 3 mg of tissue. The degradation and loss of RNA is minimised by the limited handling involved in this technique (Chomczynski & Sacchi, 1987).

2.2.1.1 Preparation of CCHF viral nucleic acid

Total CCHF viral RNA was extracted from the infected Vero 76 cell monolayers using the AGPC method (Chomczynski and Sacchi, 1986). All work with potential infectious materials was performed in a biosafety level four (BSL-4) containment facility at the NIV. The CCHF virus RNA was from strain SPU 497/89. The RNA was stored in 2.5 M ammonium acetate and ethanol at -20°C until used. It was recovered

by centrifuging at 8 000 x g for 20 minutes and washed with 70% ethanol, dried in a vacuum centrifuge (Speedvac Sc 110, Savant) and resuspended in 20 µl nuclease-free water. The total RNA concentration was determined spectrophotometrically and quality of the isolated RNA was tested by agarose electrophoresis. Five hundred ng of the purified RNA was used for first strand cDNA synthesis.

2.2.1.2 Total RNA isolation from ticks

Two different approaches were followed for the isolation of the total RNA of the *Hyalomma* ticks.

2.2.1.2.1 RNeasy™ Total RNA Kit

The RNeasy™ Total RNA Kit (Qiagen) was used for the isolation of total RNA from the *15 Hyalomma truncatum* ticks. Each tick was individually homogenised in a Tenbroeck grinder. The homogenisation in lysis buffer RLT instantaneously lyses cells, disrupts tissue, rapidly creates a complete denaturing environment and shears high molecular weight genomic DNA. Complete homogenisation is very important to obtain high yields of RNA and to avoid clogging of the RNeasy spun column. The buffer, the homogenate was centrifuged at 13 000 x g for 3 minutes. The supernatant was withdrawn and placed in a clean Eppendorf tube. To purify the RNA, 350 µl of a 70% ethanol solution was added to the lysate and centrifuged at 8 000 x g for 60 seconds. Wash buffer, RW I (700 µl), was subsequently pipetted into the spun column and centrifuged, when after the spun column was placed in a clean 1.5 ml collection tube. Wash buffer, RPE (500 µl), was pipetted into the spun column and centrifuged at (13 000 x g) for 2 minutes. The supernatant was discarded and the spin column was transferred to a new 1.5 ml collection tube. RNA was eluted with 10 µl diethyl pyrocarbonate-treated water (DEPC) and centrifuged at 8 000 x g

for 60 seconds. The RNA product was run on a 2% agarose gel to demonstrate the presence of RNA. The purified RNA was frozen in liquid nitrogen and kept at -70°C until tested.

2.2.1.2.2 TRIZOL®

The 35 *Hyalomma marginatum rufipes* ticks that have been collected were placed into 500 µl TRIZOL (Life Technologies) in a 2 ml Eppendorf tube. Each tick was homogenised using a power homogeniser (Kinematica AG, Lasec). The homogenised sample was incubated for 5 minutes at room temperature after which 100 µl chloroform was added. The tube was vigorously shaken for 15 seconds, incubated at room temperature for 3 minutes and centrifuged at 13 000 x g for 15 minutes at 4°C. The reaction mixture separated into a lower red phenol-chloroform phase, an interphase and an upper, colourless, aqueous phase. The aqueous phase was transferred to a fresh tube. RNA was precipitated by adding 0.5 volume isopropanol, incubation at room temperature for 10 minutes and centrifugation at 13 000 x g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with one volume of 70% ethanol. The sample was mixed by vortexing and centrifuged at 7 500 x g for 5 minutes at 4°C. The supernatant was aspirated, the RNA briefly dried in a vacuum centifuge and resuspended in 10 µl DEPC-treated water. The RNA extracts were kept at -70°C until tested.

2.2.2 DEOXYRIBONUCLEIC ACID (DNA)

2.2.2.1 The pGEMEX-I plasmid vector

During this study the pGEM Express Systems (pGEMEX-I Vector) kit was used for cloning of the RT-PCR cDNA CCHF fragment.

2.3 CONSTRUCTION OF THE POSITIVE INTERNAL CONTROL

2.3.1 *GENERAL RECOMBINANT DNA METHODS*

Methods that are not described in detail were performed as described by Sambrook *et al.* (1989) and Ausubel *et al.* (1990).

2.3.2 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

2.3.2.1 cDNA synthesis from the CCHF virus

For first–strand cDNA synthesis, 1 picomol (pmol) F3 and F2 primer were added to 0.5 µl purified total cellular CCHF virus RNA, in a total volume of 10 µl, in two separate tubes. The tubes were heated for 3 minutes at 95°C and then cooled for 3 minutes at 42°C. The reaction mixture was briefly centrifuged and returned to 42°C. A 10 µl volume of stock solution containing 25 U (0.6 µl) RNase-Inhibitor (Boehringer Mannheim), 4 µl 10 x reaction buffer, 2 µl dNTP mix, (10 mM each), 2 µl DTT (0.1 M stock) and 20 U (1 µl) M-MuLV Reverse Transcriptase was added to each reaction tube. The tubes were incubated for 30 minutes at 42°C, heated for 3 minutes at 95°C, transferred to ice for 3 minutes, briefly spun in a microcentrifuge and returned to ice.

2.3.2.2 PCR amplification

For DNA amplification, the following reaction mixtures were set up in four different Eppendorf tubes on ice.

Tube 1 = F2 & R2
Tube 2 = F2 & R3
Tube 3 = F3 & R2
Tube 4 = F3 & R3

The sequences of the four primers used for the PCR were as follows:

Table 3: Oligonucleotide primer sequence used for the first round reverse transcription-polymerase chain reactions

PRIMER	NUCLEOTIDE SEQUENCE	MAP POSITION
F2*	5' >TGG ACA CCT TCA CAA ACT C< 3'	135-153
F3*	5' >GAA TGT GCA TGG GTT AGC TC< 3'	290-309
R2#	5' >GAC ATC ACA ATT TCA CCA GG< 3'	549-530
R3#	5' >GAC AAA TTC CCT GCA CCA< 3'	670-653

*** Primers F2 and F3 are in the sense orientation**

 # Primers R2 and R3 are in the antisense orientation

Primers manufactured by Biochemistry department University of Cape Town, South Africa

REACTANT	VOLUME
Primers (100 pmol)	0.8 µl each
10 x reaction buffer	10 µl
dNTP	4 µl
dH_2O	81 µl
cDNA	3 µl
Taq DNA polymerase	0.4 µl
Total reaction volume	***100 µl***

The DNA was amplified in a Gene Amp PCR System 2400 thermocycler (Perkin-Elmer Corporation) for 30 cycles. Each cycle consisted of 30 seconds at 95°C, 30 seconds at 48°C, 30 seconds at 72°C followed by a 5 minute incubation at 72°C.

2.3.2.3 Analysis of PCR-amplification products on agarose gel

Twenty µl aliquots of the PCR products were electrophoresed on a 2% agarose D1/LE gel (Whitehead Scientific) in 1 x TBE buffer containing 0.5 µg/ml ethidium bromide. The DNA bands were visualised in a 350 nm UV-transilluminator and photographed on Polaroid film (Fuji instant black and white film FP-3000B).

2.3.2.4 *Purification of PCR products*

Fifty µl of each cDNA PCR product was run on a 2% agarose gel. The bands were cut out of the agarose gel and equilibrated for 30 minutes in 10 ml of 300 mM sodium acetate, 1 mM EDTA (pH 7.0) in the dark. The cDNA products were centrifuged at (13 000 x g) for 5 minutes through the glass wool to purify them and stored at -70°C for 30 minutes. The products were then centrifuged at 13 000 x g for 30 minutes. The final volume of cDNA was precipitated with 1/100 volume 1 M magnesium chloride, 10% acetic acid (0.85 µl) and 2.5 volume 100% ethanol (212 µl). The precipitate was stored at -70°C for 15 minutes and then centrifuged at 12 000 x g for 15 minutes. The ethanol solution was aspirated and the cDNA pellet briefly rinsed in 250 ml 70% ethanol solution. The ethanol was aspirated, the cDNA pellet dried in a vacuum centrifuge, after which it was eluted in 20 µl TE

2.3.2.5 *Labelling of cDNA with [γ-^{32}P]ATP*

REACTANT	VOLUME
cDNA	6 µl
[γ-^{32}P]ATP	3 µl
PNK 10 x phosphorylation buffer	2 µl
T4 polynucleotide kinase	2 µl
dH_2O	7 µl
Total reaction volume	***20 µl***

The reaction mixture was incubated for 30 minutes at 37°C. Five µl loading buffer were added to the reaction mixture. The blunt-end cDNA, labelled with [γ-^{32}P]ATP, was run through a column with Sephadex G-50. Every 5 drops (approximately 100 µl) were captured in a 1.5 ml tube. Radioactivity in each tube was determined. Pooled aliquots with high radioactivity was precipitated with 50 µl ammonium acetate and 200 µl ethanol. This reaction mixture was stored at -20°C for 120 minutes and

then centrifuged in a microcentrifuge at 13 000 x g for 20 minutes. The ethanol solution was carefully aspirated with a micropipette and the labelled cDNA rinsed with 250 µl 70% ethanol. The ethanol was aspirated and the pellet dried in a vacuum centrifuge. The labelled cDNA pellet was resuspended in 10 µl TE.

2.3.2.5 Ligation

The ligation of *Eco*R I linkers to cDNA labelled with [γ-^{32}P]ATP.

REACTANT	VOLUME
Blunt-ended cDNA	9 µl
L/K buffer	2 µl
Enzyme enhancer	5 µl
*Eco*R I linkers (100pmoles/µl)	2.5 µl
dH_2O	1.5 µl
Total reaction volume	***20 µl***

The reaction mixture was gently mixed for a few seconds, spun in a microcentrifuge for a few seconds and 2 µl T4 DNA ligase added. The reaction mixture was gently mixed and incubated at 16°C for 30 minutes. Two µl of 0.25 M EDTA were added to stop the reaction. Seventy-six µl STE was added to the reaction mixture prior to removal of free linkers. The reaction mixture was passed through a spin column, with a gel matrix, to remove all remaining *Eco*R I linkers. Two ml STE buffer were added to the column. The column was inverted several times to resuspend the gel and then allowed to drain. This was repeated three times. The meniscus just reached the top of the gel bed and the column was placed in a 15 ml conical centrifuge tube (Elkay tube). The column was centrifuged for 2 minutes at approximately 400 x g in a swing-out bucket rotor. The column was removed and placed upright in a rack. Hundred µl of the ligated sample was slowly applied to the centre of the flat surface, on top of the compacted bed of the column. The column was placed in a clean 15 ml

conical centrifuge tube and centrifuged at 400 x g for 2 minutes. This step was repeated eight times, each time with 100 µl STE and in a different conical centrifuge tube. The eluate was collected and contained labelled cDNA. The radioactive count rate of [γ-^{32}P]ATP in the aliquot was measured by scintillation counting and the total amount of cDNA calculated. The cDNA was precipitated as described using in section *2.3.2.5* and resuspended in 90 µl TE.

2.3.2.7 Kinase treatment of labelled cDNA

The ends of the labelled cDNA are unphosphorylated and were treated with kinase prior to ligation to allow covalent insertion. This was done because the pGEMEX-I has dephosphorylated vector arms.

REACTANT	VOLUME
Labelled cDNA	90 µl
L/K buffer	20 µl
T4 polynucleotide kinase	4 µl
dH_2O	96 µl
Total reaction volume	***200 µl***

The reaction mixture was incubated for 30 minutes at 37°C. The cDNA pellet was extracted twice with an equal volume of phenol/chloroform, followed by two chloroform/isoamyl alcohol extractions. For each extraction, the contents of the tube was briefly vortexed, centrifuged in a microcentrifuge for 1 minute at 13 000 x g and transferred the aqueous phase to a clean tube. The extracted cDNA was precipitated with ethanol. The reaction mixture was stored at -70°C for 30 minutes, centrifuged at 13 000 x g for 15 minutes and the ethanol solution carefully aspirated. The cDNA pellet was briefly rinsed in 70% ethanol solution and the ethanol solution

aspirated. The cDNA pellet was then briefly dried in a vacuum centrifuge and resuspended in 20 µl TE.

2.3.3 PREPARATION OF THE pGEMEX-I PLASMID VECTOR

The pGEMEX-I vector was digested with *Eco*R I restriction enzyme.

REACTANT	VOLUME
dH_2O	38 µl
Buffer H	5 µl
pGEMEX-I vector	5 µl
*Eco*R I enzyme	2 µl
Total reaction volume	***50 µl***

The reaction mixture was mixed in a sterile 1.5 ml tube, briefly centrifuged in a microcentrifuge and incubated for 3 hours at 37°C. Five µl (0.3 µg) of the pGEMEX-I DNA was mixed with 8 µl bromophenol blue loading buffer and analysed as described in section *2.3.2.3.*

2.3.3.1 Dephosphorylation reaction of pGEMEX-I

REACTANT	VOLUME
Digested pGEMEX-I DNA	45 µl
CIAP 10 x buffer	10 µl
CIAP (0.1 U)	1 µl
dH_2O	43 µl
Total reaction volume	***100 µl***

The reaction mixture was incubated for 30 minutes at 37°C to dephosphorylate the 5'-termini. Another 1µl CIAP was added to the reaction mixture. The reaction

mixture was incubated for another 30 minutes at 37°C. The reaction was stopped with the addition of 2 µl of 0.5 M EDTA, pH 8.0 and heated at 65°C for 20 minutes.

2.3.4 CLONING OF PCR PRODUCT INTO THE pGEMEX-I PLASMID

2.3.4.1 Ligation of pGEMEX-I vector and Insert cDNA

The insert cDNA was ligated into the *Eco*R I cloning site of the pGEMEX-I plasmid vector.

REACTANT	VOLUME TUBE 1	VOLUME TUBE 2
	Ligation of cDNA	Self-ligation
Cdna fragment	20 µl	-
PgemEX-I plasmid DNA	1 µl	1 µl
T4 DNA ligase	1 µl	1 µl
10 x ligase buffer	3 µl	3 µl
dH2O	5 µl	25 µl
Total reaction volume	***30 µl***	***30 µl***

The reaction mixtures were incubated at 10°C for 19 hours.

2.3.5 E. COLI TRANSFORMATION AND COLONY HYBRIDISATION

Competent cells were prepared using calcium chloride as described by Sambrook *et al.* (1989). Ten µl of the ligated cDNA fragment was mixed with 200 µl competent cells.

REACTANT	VOLUME TUBE 1	VOLUME TUBE 2	VOLUME TUBE 3
	Ligated DNA	Pos. Control	Neg. Conrol
Competent cells	200 µl	200 µl	200 µl
pUC 18	-	1 µl	-
Ligated DNA	10 µl	-	-
Total volume	***210 µl***	***201 µl***	***200 µl***

The reaction mixtures were gently mixed, incubated on ice for 30 minutes, then briefly incubated for 5 minutes at 37°C, after which 800 µl SOC-medium were added and the mixtures incubated for 30 minutes at 37°C. The transformation mixtures were streaked out on SOB agar plates containing 250 µl ampicillin and the agar plates were incubated overnight at 37°C. Since the pGEMEX-I plasmid does not have an insert selection system, colony hybridisation had to be performed to select positive colonies. The colonies were transferred to Hybond-N^+ nylon filters and incubated at 37°C. They were then relysed for 15 minutes with 0.5 M sodium hydroxide and 1.5 M sodium chloride. Renaturation was done for 5 minutes with 0.5 M Tris-HCl (pH 7.4), followed by 1 M sodium chloride for 5 minutes. The DNA was fixed to the membrane by exposing the Hybond-N^+ nylon filters to an UV-transilluminator for 3 minutes.

2.3.5.1 Labelling of the probe with [α-^{32}P]dATP

The Megaprime™ labelling kit was used to label the CCHF specific DNA probe with [α-^{32}P]dATP. The reaction mixture was boiled for 3 minutes and placed on ice.

REACTANT	VOLUME	PROCEDURE	PROCEDURE
DNA	10 µl	Reaction mixture boiled for 3 minutes and placed on ice	Total reaction mixture incubated at 37°C for 10 minutes
F3 primer	5 µl		
H_2O	11 µl		
Subtotal reaction mixture	***26 µl***		
Buffer	5 µl		
(dCTP, dGTP and dTTP) mix	12 µl		
[α-^{32}P]dATP	5 µl		
Enzyme	2 µl		
Total reaction volume	***50 µl***		

The hybridisations were performed as described by Maniatis *et al.*, (1984). Visualisation of the bound probe was accomplished by exposing the Hybond-N^+ nylon filters to MP-Hyperfilm overnight. After exposure, the film was developed and the positive hybridisation signals were marked on the plates, each positive colony identified and streaked out. Single colonies were picked with a toothpick and placed on an agar plate. A second hybridisation was done to identify positive colonies. These colonies were picked and cultured in 10 ml Terrific Broth (TB) medium and 10 µl ampicillin was added to the TB medium. The reaction was incubated overnight in an incubator at 37°C.

2.3.5.2 Plasmid extraction and purification

A high pure plasmid isolation kit (Boehringer Mannheim) was used for to isolate and purify the plasmid. Two ml of the overnight reaction culture was centifuged at 13 000 x g for 2 minutes. The pellet was resuspended in 250 µl suspension buffer and mixed well. Lysis buffer (200 µl) was added, reaction mixture gently mixed and incubated for 5 minutes at room temperature, during which the solution became viscous and clear. Chilled binding buffer (350 µl) was added and the reaction mixture was gently mixed by inverting the tube 6 times, incubated on ice for 5 minutes and centrifuged at 13 000 x g for 10 minutes. A compact white pellet formed. A high pure filter tube was placed in a collection tube, the supernatant was pipetted into the upper reservoir and centrifuged at 13 000 x g for 60 seconds. The flowthrough was discarded. Wash buffer I (350 µl) was added and centrifuged for 60 seconds at maximum speed. The flowthrough was discarded again, 700 µl wash buffer II were added and centrifuged for 60 seconds at maximum speed. The flowthrough was discarded and centrifuged for an additional at 13 000 x g for 60 seconds. The centrifuge step was repeated to remove residual wash buffer. The collection tube

was discarded and the filter was inserted in a clean 1.5 µl tube. Elution buffer (100 µl) was added and centrifuged at 13 000 x g for 30 seconds. Five µl of the purified plasmid with cloned CCHF fragment (pHEN I) were mixed with 8 µl bromophenol blue loading buffer and analysed as described in section *2.3.2.3.*

2.3.5.3 Determination of the optical density of pHEN I DNA

Two and a half µl of purified pHEN I DNA was mixed with 97.5 µl distilled water. The optical density of the pHEN I plasmid was determined at 260 nm in an UV1201 (Shumatzu) spectrophotometer.

2.3.5.4 Ten-fold dilution series of pHEN I DNA

A ten-fold dilution series was made of the purified pHEN I plasmid DNA. One µl plasmid DNA was mixed with 9µl distilled water. One µl of this dilution was transferred to the next tube mixed with 9 µl distilled water. A new pipette tip was used for each dilution. This step was repeated 9 times. PCR was performed as described in section *2.3.2.2* with each dilution. Each PCR reaction contained the following:

REACTANT	VOLUME
Primer F3	0.8 µl
Primer R3	0.8 µl
10 x reaction buffer	10 µl
dNTP	4 µl
dH_2O	81 µl
cDNA	3 µl
Taq DNA polymerase	0.4 µl
Total reaction volume	***100 µl***

Fifteen µl of the 100 µl PCR amplification product fom each of the dilution series was mixed with 8 µl bromophenol blue loading buffer and analysed as described in section *2.3.2.3.*

2.3.6 *SEQUENCING OF THE CLONED CCHF VIRUS RT-PCR PRODUCT*

Amplified PCR product cloned into pGEMEX-I was analysed with the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli*Taq*® DNA Polymerase Fs (Perkin-Elmar Corporation).

REACTANT	VOLUME TUBE 1	VOLUME TUBE 2	VOLUME TUBE 3	VOLUME TUBE 4
Terminator ready mix	4 µl	4 µl	4 µl	4 µl
PCR product	5 µl	5 µl	5 µl	5 µl
Primer	1 µl (T3)	1 µl (Sp6)	1 µl (F3)	1 µl (R3)
Total reaction volume	***10 µl***	***10 µl***	***10 µl***	***10 µl***

The reaction mixtures were overlaid with 40 µl light mineral oil and amplified on the same thermocycler as mentioned before; 25 cycles of 10 seconds each at 96°C, 5 seconds at 50°C, 4 minutes at 60°C and a rapid thermal ramp to 4°C. The PCR extension products were purified with an ethanol precipitation. For each reaction, a 1.5 ml tube was prepared and the following reagents were added.

REACTANT	VOLUME
Nucleotide sequence mixture	20 µl
3 M Sodium acetate, pH 4.6	2 µl
95% Ethanol	50 µl
Total reaction volume	***72 µl***

The reaction mixture was vortexed and placed on ice for 10 minutes, centrifuged at maximum speed for 20 minutes and the ethanol aspirated. The pellet was rinsed with 70% ethanol and the ethanol aspirated. The pellet was dried in a vacuum

centrifuge. Sequences were analysed using the PC Gene program package (Intelligenetics).

2.3.7 CONSTRUCTION OF THE DELETION VARIANT

2.3.7.1 Plasmid isolation

The colonies were individually selected and the plasmids isolated using standard miniprep procedures. This technique was used to isolate small amounts of recombinant DNA for evaluation. The insert cDNA was digested with *Eco*R I. The quality of the product was tested by agarose gel electrophoresis to demonstrate the PCR insert. The pHEN I vector was digested with *Bln* I restriction enzyme, which digested the cloned DNA fragment at given restriction area.

REACTANT	VOLUME
DNA	40 µl
Buffer H	5 µl
Bln I enzyme	5 µl
Total reaction volume	50 µl

The reaction mixture was vortexed and incubated overnight in a 37°C water bath. The cDNA pellet was extracted with an equal volume of phenol/chloroform 1:1, 50 µl (twice), followed by chloroform/isoamyl alcohol 24:1, 50 µl (twice). Before each extraction the content of the tube was briefly vortexed for 1 minute, centrifuged at 13 000 x g for 5 minutes and the aqueous phase separated into a clean tube. The extracted cDNA was precipitated with 12 µl 7.5 M ammonium acetate and 100 µl ethanol. The reaction mixture was stored at -70°C for 30 minutes and centrifuged at 13 000 x g for 15 minutes. The ethanol was aspirated and the cDNA pellet briefly rinsed with 70% ethanol solution. The ethanol was then aspirated and the cDNA pellet briefly dried in a vacuum centifuge and resuspended in 40 µl dH_2O. The buffer

H was removed from the DNA by a phenol-chloroform extraction. *Bst*E II restriction enzyme was used to digest the cloned DNA fragment at a second restriction area.

REACTANT	VOLUME
DNA	40 µl
*Bst*E II enzyme	5 µl
Buffer D	5 µl
Total reaction volume	***50 µl***

The reaction mixture was vortexed and incubated at 60°C for 60 minutes. A phenol-chloroform extraction was done to clean the DNA from buffer D. Agarose gel electrophoresis was performed as previously described for DNA products. After the DNA was digested with *Bln* I and *Bst*E II, the overhanging ends of the cloned DNA fragment were filled with dNTP's by using *Taq* DNA polymerase.

REACTANT	VOLUME
DNA	48 µl
5 x dNTP mix	1 µl
Taq DNA polymerase	1 µl
Total reaction volume	***50 µl***

The reaction mixture was vortexed and then incubated at 72°C for 2 minutes. The PCR product, which was cloned into the pGEMEX-I vector, was digested with *Bst*E II and *Bln* I.

REACTANT	VOLUME TUBE 1	VOLUME TUBE 2	VOLUME TUBE 3
	DNA insert	Positive control	Negative control
pGEMEX-I plasmid DNA	20 µl	-	-
pUC 18 DNA	-	1 µl	-
T4 DNA ligase	1 µl	1 µl	1 µl
10 x ligase buffer	3 µl	3 µl	3 µl
dH_2O	6 µl	25 µl	26 µl
Total reaction volume	***30 µl***	***30 µl***	***30 µl***

After ligation at 10°C for 19 hours the DNA insert was transformed into *E. coli* as described in section *2.3.5.*

2.3.7.2 Transcription of the DNA insert and pGEMEX-I positive control

The pHEN II plasmid was digested with *Hind* III, which provided a linear DNA template used for the transcription of the DNA insert. The linear DNA template was analysed on an agarose gel as described in section *2.3.2.3* to ensure that all the plasmids were linear. The following reagents were added at room temperature and incubated for 60 minutes at 37°C.

REACTANT	VOLUME TUBE 1	VOLUME TUBE 2
	Plasmid	+ Control
Transcription optimising 5 x buffer	40 µl	4 µl
100mM DTT	20 µl	2 µl
Rnasin ribonuclease inhibitor	5 µl	0.5 µl
ATP, GTP, UTP and CTP (2.5 mM of each)	40 µl	4 µl
pGEM Express positive control template	10 µl	1 µl
Linearised template DNA	50 µl	-
T3 RNA polymerase	10 µl	-
SP6 RNA polymerase	-	1 µl
Nuclease-free H_2O	75 µl	8 µl
Total reaction volume	***200 µl***	***20 µl***

2.3.7.3 Removal of DNA template following transcription

RNase-Free DNase was used to digest the DNA.

REACTANT	VOLUME
Transcripted RNA and DNA template	200 µl
RQI buffer	30 µl
RQI RNase-Free Dnase	10 µl
Nuclease-free dH_2O	70 µl
Total volume	***310 µl***

The reaction mixture was incubated for 30 minutes at 37°C. The activity of the DNase was stopped with 10 µl RQI stop solution and the mixture incubated for 10 minutes at 68°C. Agarose gel electrophoresis was performed (see section *2.3.2.3*) to make sure that the DNA was digested.

2.3.7.4 Isopropyl alcohol precipitation of the transcribed RNA internal control

Isopropyl alcohol (186 µl) and sodium acetate (31 µl) were added to the reaction mixture. The mixture was incubated for 3 hours at -20°C and centrifuged at 7 500 x g for 15 minutes at 4°C. The supernatant was removed and the RNA pellet briefly dried in a vacuum centrifuge and resuspended in 50 µl nuclease-free water. Aliquots of 10 µl of the resuspended internal control was made and kept at -70°C.

2.3.7.5 Determination of the optical density of internal control RNA

Ten µl RNA internal control was mixed with 90 µl distilled water. An UV1201 (Shumatzu) spectrophotometer was used to determined the optical density of the internal control RNA.

2.3.7.6 Ten-Fold dilution series of internal control RNA

A ten-fold dilution series were made as described in section *2.3.5.4.* RT-PCR was done with each of this series. The first step of the RT-PCR reactions was prepared as follows and the second step as described in section 2.4. Twenty µl of the cDNA product dilution series were mixed with 8 µl bromophenol blue loading buffer and analysed as described in section *2.3.2.3.*

REACTANT	VOLUME
Internal control RNA	5 µl
F3 Primer (1 ρmole)	0.8 µl
Nuclease-free H_20	4.2 µl
Total volume	***10 µl***

2.3.7.7 Determination of the optical density of tick RNA

Initially, the RT-PCR did not work when 5 µl tick RNA was used. Different volumes of tick RNA i.e. 4 µl, 3 µl, 2µl and 1µl were added to the RT-PCR reactions. One µl tick RNA showed the best results. One µl tick RNA negative for CCHF virus was mixed with 99 µl distilled water and an UV1201 (Shumatzu) spectrophotometer was used to determined the optical density of the RNA. The concentration of tick RNA was calculated as 1.08 µg/µl from its optical density. A 0.1 µg/µl dilution, the same as the internal control RNA, was made of the tick RNA.

2.4 INTERNAL CONTROL USED TO SPIKE THE RT-PCR

An access RT-PCR system kit (Promega Corporation) was used for the RT-PCR reactions that were done on the isolated tick RNA samples. The RT-PCR was done in two steps (single tube) in a final volume of 20 µl. The first step was the mixture of the tick RNA, internal control RNA, F3 primer and water with a final volume of 10 µl in each reaction tube.

REACTANT	VOLUME TUBE 1	VOLUME TUBE 2
	0 control	Sample
Tick RNA	-	1 µl
Internal control RNA	-	1 µl
F3 Primer (1 ρmole)	0.8 µl	0.8 µl
Nuclease-free H_20	9.2 µl	7.2 µl
Total volume	***10 µl***	***10 µl***

The tubes were heated for 3 minutes at 95°C and then cooled for 3 minutes at 42°C, briefly centrifuged and kept on ice. Step two was the addition of the following reagents, a master mix was made of the following reagents and 10 µl was added to every reaction tube.

REACTANT	VOLUME
Nuclease-free H_2O	2.8 µl
AMV/Tfl 5 x reaction buffer	4 µl
dNTP	0.8 µl
R3 Primer (100 ρmole)	0.4 µl
F3 Primer (100 ρmole)	0.4 µl
$MgSO_4$	0.8 µl
Tfl *Taq* DNA polymerase	0.4 µl
AMV reverse transcriptase	0.4 µl
Total reaction volume	***10 µl***

The DNA was incubated in a thermocycler for 30 minutes at 42°C, for 5 minutes at 95°C and amplified for 30 cycles of 30 seconds each at 95°C, 30 seconds at 48°C, 30 seconds at 72°C for each cycle and followed by a 5 minute incubation period at 72°C.

2.5 SUMMARY OF THE DEVELOPMENT OF THE METHOD FOR THE DETECTION AND ESTIMATION OF CCHF VIRUS RNA IN TICK SPECIES

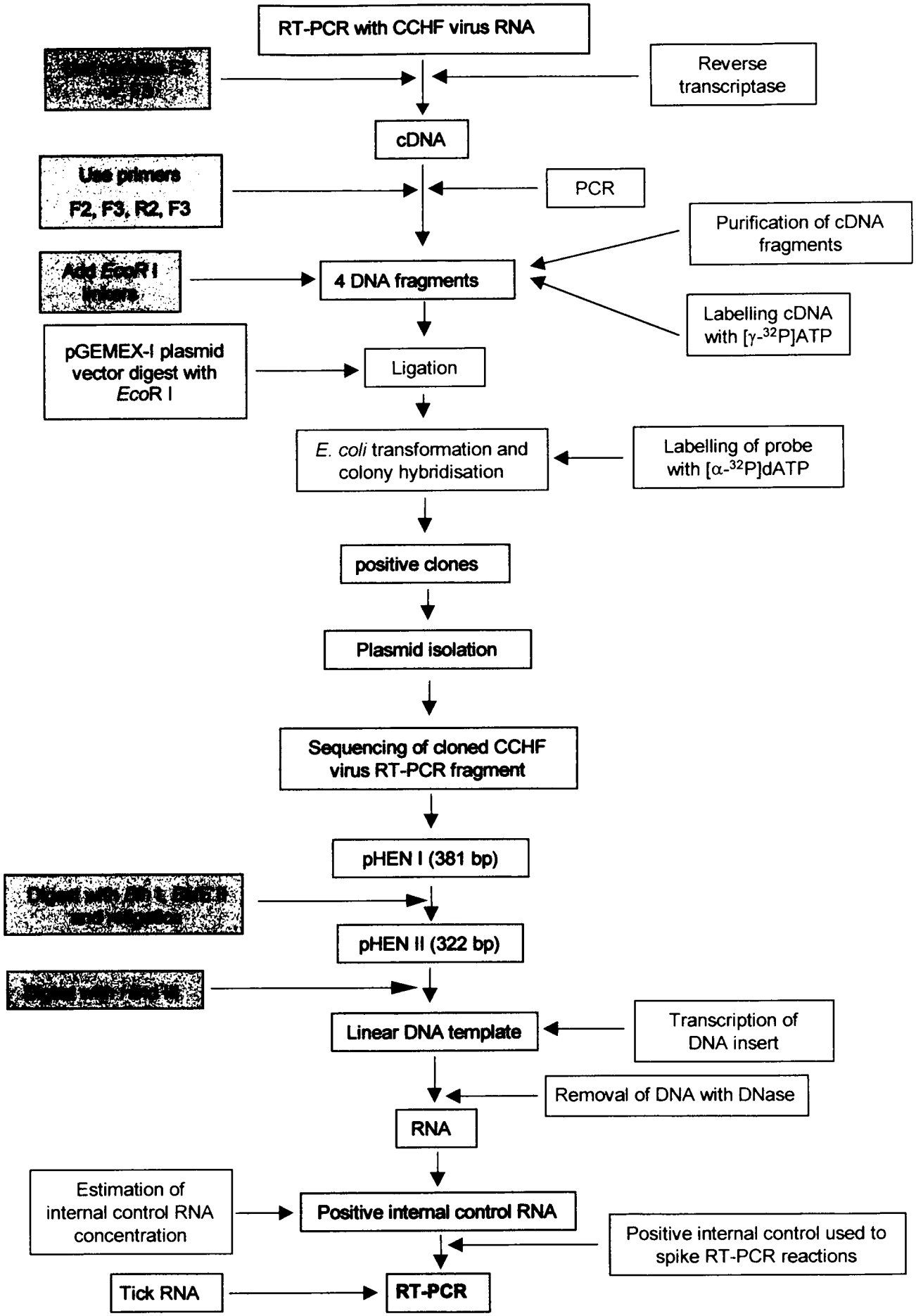

Figure 4: Summary of the development of the method for the detection and the estimation of CCHF virus RNA in tick species

CHAPTER 3

RESULTS AND DISCUSSION

3.1 INTRODUCTION

There is a definite need to improve the serological diagnosis of CCHF. The clinical diagnosis is currently confirmed in the laboratory by virus isolation from cell cultures, which is less sensitive than mouse brain inoculation (Swanepoel *et al.*, 1998b). These methods are time consuming and not possible in remote areas where infections are endemic. In general the Ag-ELISA performs well and remains the method of choice for screening large numbers of suspected acute-phase samples (Logan *et al.*, 1993). These techniques also require a BSL-4 containment laboratory, are time consuming and expensive.

In addition, timely diagnosis of CCHF infected patients is important, since it is seldom recognised or suspected before 3-5 days after the onset of the illness. Rapid diagnosis will also protect medical staff and perhaps reduce cost of treatment and management of patients. To achieve these goals, RT-PCR has been developed to detect genomic CCHF virus RNA (Burt *et al.*, 1997). Results are available within less than 5 hours after receiving a specimen (Rodriguez *et al.*, 1997).

The purpose of this study was to establish a RT-PCR method that allows rapid detection and estimation of the concentration of genomic CCHF virus RNA in humans and in *Hyalomma* ticks. The method entails a reverse transcriptase reaction using a specific downstream primer, followed by PCR with the addition of the upstream primer. As internal control for the reaction and as aid for quantification, a

variant CCHF RNA produced *in vitro* is used. This variant CCHF RNA must differ in size from the natural viral RNA, but still be able to be amplified by the same primers. This was achieved by the use of two unique restriction sites, which allowed a small fragment to be dropped out of the original cloned CCHF RNA fragment.

An important aim of this variant is to aid in quantification of natural CCHF virus RNA found in ticks. This internal control RNA was added to samples in a dilution series to determine the point of equal intensity with a positive sample. As the internal control has been made by *in vitro* transcription, its concentration is known and the amount of viral RNA in the sample can be determined.

3.2 RT-PCR

The DNA used in this study was generated from PCR products amplified from the S segment of the CCHF virus genome using primers with CCHF virus-specific sequences. The sequences of the oligonucleotide primers, shown in Table 3 were selected from a region of the nucleocapsid protein gene in the S RNA segment of the CCHF virus. The selection was made on the basis of maximal consensus sequence determined for seven CCHF virus isolates from diverse geographic areas, including Nigeria, Pakistan, China, Russia, Senegal, South Africa and Uganda (Lofts *et al.*, 1991).

The primer sequences were selected so that no more than two mismatches occurred for any primer/template combination according to the seven S segment sequences available at the time. A polygenetic investigation was performed on virus derived from infected cell culture fluids and showed that the primers used could be applied successfully to RT-PCR of 57 CCHF virus isolates from southern Africa (Burt *et al.*, 1998).

Two forward (F2/F3) and two reverse (R2/R3) primers shown in Table 4, targeting the S genome segment of the CCHF virus were used. The two forward primers are complementary to the viral RNA, i.e. the negative sense strand, while the two reverse primers are complementary to the message sense RNA, i.e. the positive sense strand.

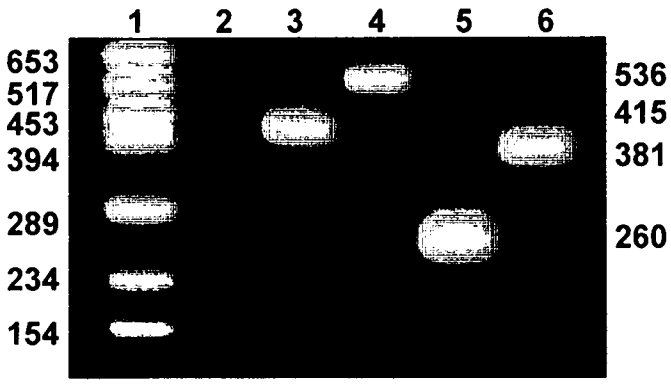

Figure 5: Agarose gel electrophoresis of the RT-PCR products of the four different primer pair combinations. Lane 1, molecular weight marker VI (Boehringer Mannheim); Lane 2, negative control; Lane 3, primer pair F2/R2 (415 bp); Lane 4, F2/R3 (536 bp); Lane 5, F3/R2 (260 bp) and Lane 6, F3/R3 (381 bp). Values in base pairs

For this study, all the necessary steps to avoid contamination during the RT-PCR were undertaken i.e. separation of pre- and post-PCR work areas and limited use of pipettes and other equipment. The CCHF virus isolate SPU 497/89 received from the NIV was used throughout the study. Reverse transcription was performed on the CCHF virus isolate first using the two downstream primers F2 and F3 in separate reactions. This was followed by PCR with the four different primer combinations F2/R3, F2/R2, F3/R3 and F3/R2 to synthesise a large quantity of cDNA.

The products obtained from the four different primer pair combinations were run on a 2% agarose gel (Figure 5) and the expected lengths of the four PCR products are shown in Table 4.

Table 4: PCR product lengths of the four different primers

FORWARD PRIMER	REVERSE PRIMER	PCR PRODUCT LENGHT (bp)
F_2	R_3	536
F_2	R_2	415
F_3	R_3	381
F_3	R_2	260

cDNA was synthesised on large scale because a large amount of cDNA was lost during the purification process. After purification of the blunt-ended cDNA products they were radioactively labelled with [γ-^{32}P]ATP, which allowed the cDNA to be followed through the various processing steps.

3.3 CLONING AND CHARACTERIZATION OF PCR PRODUCTS

The blunt ends of the cDNA were converted to *Eco*R I cohesive termini following a rapid single step ligation with *Eco*R I linkers (Sartoris *et al.*, 1987 & Stover *et al.*, 1987). The pGEMEX-I expression vector, based on the T7 expression system (Studier & Moffat, 1986), was used for cloning of the CCHF virus fragment. The vector contains dual opposed SP6 and T3 RNA polymerase promoters (shown in Figure 6) which flank the multiple cloning region and allows the production of *in vitro* single-stranded RNA from any strand of the cloned insert. This vector was digested with *Eco*R I and subsequently used to clone 4 different CCHF virus S RNA PCR fragments. Three out of the 4 PCR fragments were successfully cloned, but despite several attempts, the largest fragment was never present as an insert in the vector. The inability to clone this large fragment could be due to the production of a lethal protein product formed by a low level of *in vivo* transcription from one of the flanking promoters. Failure to clone the large fragment meant that it was not possible to develop a nested PCR, which would have greatly enhanced the sensitivity of

detection of viral RNA. Thus a single-round RT-PCR method using an internal control appeared to be the best option.

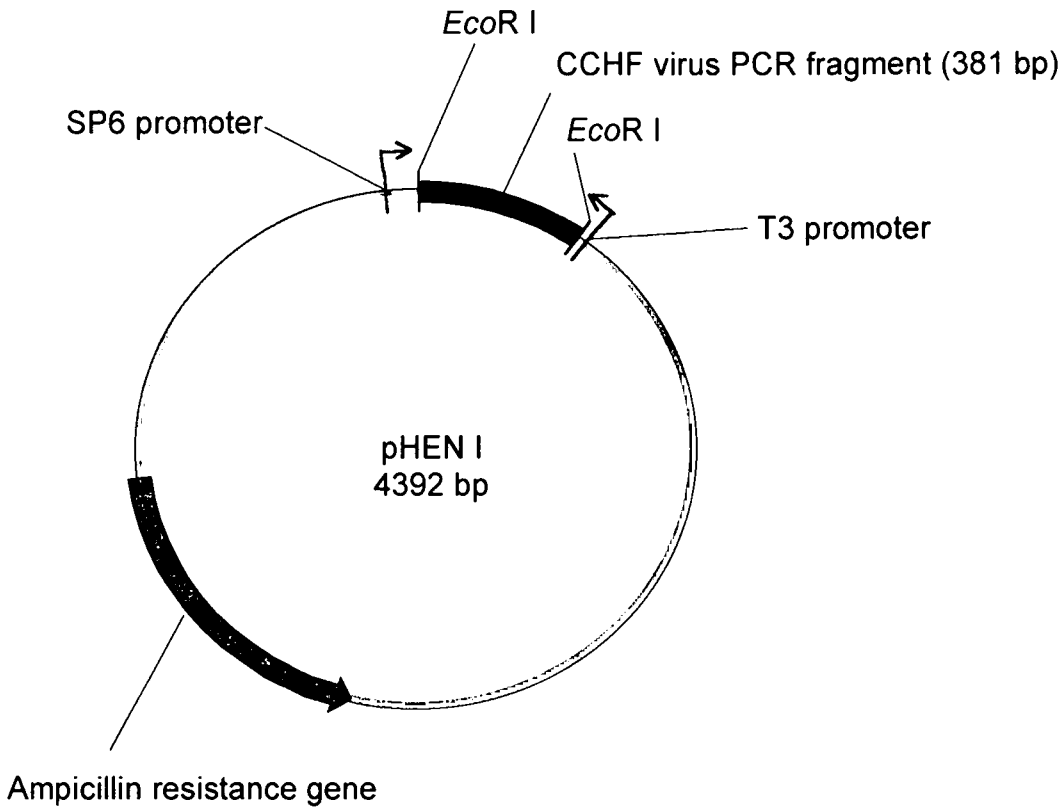

Figure 6: CCHF virus PCR product (381 bp) cloned into the pGEMEX-I plasmid vector at the *Eco*R I cloning site to create pHEN I

The pGEMEX-I plasmid does not have an insert selection option and colony hybridisation had to be performed with an [α-^{32}P]dATP labelled probe, which revealed 20 positive clones. Plasmid extraction of 8 randomly chosen positive clones followed by *Eco*R I digestion, revealed only 2 true positive clones. The reason for the high percentage of false-positive signals found after colony-hybridization is not clear, but since two insert-containing plasmids were found, this was not investigated further. The inserts of these 2 clones were subsequently sequenced from both sides to determine the orientation and fidelity of the vector-insert junction. These fragments were both 381 bp long and they gave identical sequences. One fragment was chosen at random for further work. The nucleotide sequence of the cloned

CCHF virus fragment with the sequences of the *Eco*R I linkers that were added to the blunt-ended DNA fragment are shown in Figure 7. Three primers, i.e. the upstream, forward primer (F3) and the two downstream, reverse primers, R2 and R3, and two unique restriction sites used for construction of the deletion variant are also shown in Figure 7 and Table 5.

Table 5: Oligonucleotide primer sequence and their positions in CCHF virus genome

PRIMER DESIGNATION	NUCLEOTIDE POSITIONS #		PRIMER SEQUENCE *
	5'	3'	
F3	290	309	5'***GAATGTGCATGGGTAGTCTC***CA CTGGCATTGTAAAAAAGGGA3'
R2	549	530	5'***CCTCGCAGAATACAAAGTCC***CT GGTGAGATTGTGATGTCT3'
R3	670	653	5'***TGGTGCAGGGAATTTGTC***CCATG AGGACCCGGATCCTCGAATTGG*3'*

 # Nucleotide positions relative to the positive-sense strand of CCHF virus strain SPU 497/89.

*** The underlined sequences contain the F3 forward primer (negative sense strand) and R2 and R3 reverse primers (positive sense strand).**

AATTCGAGGATCCGGGTACCATGG***GAATGTGCATGGGTAGTCTC***CACTGGCAT
TGTAAAAAAGGGACTTGAATGGTTCGAGAAAAATGCAGGAACCATTAAGT**C↓CT**
GGGATGAAAGTTATACTGAGCTAAAGGTCGACGTCCCGAAAATAGAGCAGCTTA
CC**G↓GTTACC**AACAAGCTGCCTTGAAGTGGAGAAAAGACATAGGTTTCCGTGTC
AATGCCAACACAGCAGCTCTGAGCAACAAAGT***CCTCGCAGAATACAAAGTCC***C
TGGTGAGATTGTGATGTCTGTCAAAGAGATGCTGTCAGACATGATTAGGAGAAG
GAACCTGATTCTAAACAGGGGTGGTGATGAGAACCCACGTGGCCCAATGAGCC
ATGAGCATGTAGAC***TGGTGCAGGGAATTTGTCC*****CATGGTACCCGGATCCTCGA**
ATT

AATTCGAGGATCCGGGTACCATGG: ***Eco*R I linker**
CCATGGTACCCGGATCCTCGAATT: ***Eco*R I linker**
GAATGTGCATGGGTAGTCTC: **Forward primer F3**
CCTCGCAGAATACAAAGTCC: **Reverse primer R2**
TGGTGCAGGGAATTTGTC: **Reverse primer R3**
C↓CTGGG: ***Bln* I restriction site (80 bp)**
G↓GTTACC: ***Bst*E II restriction site (139 bp)**

Figure 7: Nucleotide sequence of S RNA segment of CCHF virus (strain SPU 497/89). The PCR product was cloned into pGEMEX-I vector at the *Eco*R I cloning site. Restriction sites of *Bln* I (80) and *Bst*E II (139) are shown.

The sequence of the cloned 381 bp fragment was compared to CCHF virus sequences stored in the Genbank® database and the results are shown in Figure 9. The sequences start right after the F3 forward and end right before the R3 reverse

primer. Several single nucleotide differences between the 15 variants and the isolate SPU 497/89 can be seen. On the basis of all 16 CCHF virus variants that were compared, a consensus nucleotide sequence was determined which is also shown in Figure 9. The consensus sequence of the 16 CCHF virus variants showed a divergence of 33.3% when compared with the reference strain SPU 497/89. The divergence in amino acid sequence between SPU 497/89 and strain CCHFSRNA was 7.4% (Figure 10). Although sizeable divergence was found at the nucleic acid level, it did not influence the amino acid sequence of the viral gene very much. Since the structure/function relationship of this protein is not known at this stage, it is impossible to predict functional differences between the different virus strains based on the differing primary structure.

3.4 ESTIMATION OF THE SENSITIVITY OF THE PCR STEP

The concentration of the plasmid DNA was calculated as 0.48 µg/µL from its optical density. A ten-fold dilution series was made of the stock DNA and used for PCR. An aliquot of each PCR product was electrophoresed on a 2% agarose gel and PCR bands of the expected size, 381 bp, were obtained (Figure 11).

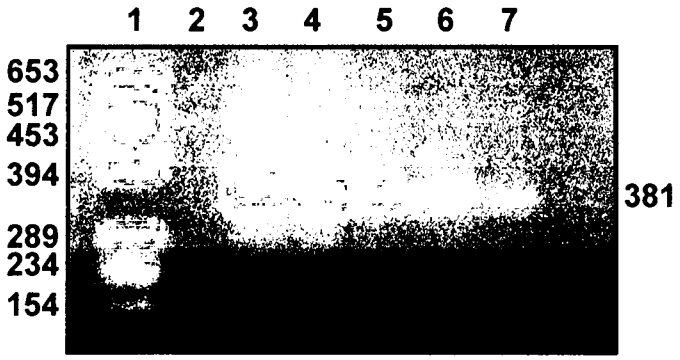

Figure 8: Agarose gel electrophoresis of the PCR products of the dilution series. Lane: 1, molecular weight marker VI (Boehringer Mannheim); Lane 2, negative control; Lane 3, 4.8 x 10^{-2} µg; Lane 4, 4.8 x 10^{-3}µg; Lane 5, 4.8 x 10^{-4} µg; Lane 6, 4.8 x 10^{-5} µg and lane 7, 4.8 x 10^{-6} µg.

The last visible band in the dilution series was seen in lane 7, corresponding to a concentration of 4.8×10^{-6} µg. The minimum concentration of target DNA detectable by this PCR was estimated to be 3×10^{-5} pmol plasmid DNA, translating into about 10^6 molecules. The water control (lane 2, Figure 8) did not give false positive results. The stringency of the PCR assay can possibly be increased by prolonging the annealing time to approximately 1 minute in the first five cycles, followed by a short annealing time (not longer than 30 seconds), at the highest possible temperature. By increasing the amount of *Taq* polymerase to 4 - 6 U/100 µl in the reaction mixture could also improve the sensitivity (Rolfs *et al.*, 1992). The PCR assay may require the addition of a cosolvent for optimal efficiency. The addition of a denaturant such as dimethyl sulfoxide (DMSO) at final concentrations of 10% has proved to be advantageous in certain systems requiring high sensitivity (Kogan, 1987). The primers used were each 20 bp in length, which could also influence the sensitivity of the PCR. Increasing the length of each primer to between 22 and 30 bp can increase the sensitivity of the PCR assay (Rolfs *et al.*, 1992). The sensitivity of the PCR assay can decrease when the amount of the starting material is increased (Carman *et al.*, 1989). This may result from inhibition of the reverse transcriptase reaction, or interference with the hybridisation step by excess cellular nucleic acid (Carman & Kidd, 1989).


1
50
100
130
SPU 497/89 CACTGGCATT GTAAAAAAGG GACTTGAATG GTTCGAGAAA AATGCAGGAA CCATTAAGTC CTGGGATGAA AGTTATACTG AGCTAAAGGT CGACGTCCCG AAAATAGAGC AGCTTACCGG TTACCAACAA
CCFSRNA CACTGGCATT GTGAAGAAGG GACTGGAGTG GTTCGAAAAA AATGCAGGAA CCATTAAATC TTGGGATGAA AGCTACATTG AGCTGAAAGT TGAGGTCCCT AAAATAGAAC AGCTTGCCAA TTACCAACAG
CCU04958 TACAGGCATT GTAAAGAAAG GTTTGGAGTG GTTTGAAAAG AACACGAGCA CTATCAAATC CTGGGACGAG AGCTACACTG AGCTGAAGGT TGACATTCCC AAAATAGAGC AGCTCTCTAG TTATCAGCAG
CHU15010 CACAGGTATA GTGAAGAGAG GCCTTGAGTG GTTTGAAAAA AACACAGGAA CCATTAAGTC TTGGGATGAG AGCTACACTG AGCTGAAAGT GGATGTGCCC AAAATTGAAC AGCTTGCCAA CTATCAGCAG
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Identity :: :: : :: :: : : : :: :: ::: :: :: :: : : : : :: :: :: : ::: :: : :: : : :: :: :: :: :: :: : :: ::::: :: : : :: : :: :: ::
Consensus yACAGGCATT GTGAAGAAGG GGCTTGAGTG GTTCGAAAAG AATACAGGAA CCATTAAGTC CTGGGATGAG AGCTACACTG AGCTGAAAGT GGATGTGCCC AAAATTGAAC AGCTTGCCAA TTACCAACAG
131
260
SPU 497/89 GGTGCGTTGA AGTGGAGAAA AGACATAGGT TTCCGTGTCA ATGCCAACAC AGCAGCTCTG AGCAACAAAG TCCTCGCAGA ATACAAAGTC CCTGGTGAGA TTGTGATGTC TGTCAAAGAG ATGCTGTCAG
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CHU15113 GCCGCTCTCA AGTGGAGGAA GGACATAGGC TTCCGCGTTA ATGCAAACAC AGCGGCCCTA AGCAACAAAG TCCTCACTGA GTACGAGGTT CCTGGTGAGA TTGTGATGTC TGTCAAAGAG ATGCTTTCAG
CHU15114 GCTGCTCTAA AGTGGAGGAA AGAAGTAGGC TTCCGTGTCA ATGCAAACAC GGCAGCTCTG AGTAACAAAG TCCTTGCAGA ATACAAAGTT CCTGGCGAAA TTATAATGTC TGTCAAAGAG ATGTTGTCAG
CHU15099 GCTGCCTTGA AATGGAGAAA GGACATAGGT TTCCGTGTCA ATGCAAACAC AGCGGCTCTG AGTAACAAAG TCCTCGCAGA GTACAAAGTT CCTGGCGAGA TTGTAATGTC TGTCAAAGAG ATGCTGTCAG
CHU15090 GCTGCCTTGA AATGGAGAAA GGACATAGGT TTCCGTGTCA ATGCAAATAC AGCGGCTCTG AGCAACAAAG TCCTCGCAGA GTACAAAGTT CCTGGCGAGA TTGTAATGTC TGTCAAAGAG ATGCTGTCAG
CHU15091 GCTGCCTTGA AATGGAGAAA GGACATAGGT TTCCGTGTCA ATGCAAATAC AGCGGCTCTG AGCAACAAAG TCCTCGCAGA GTACAAAGTT CCTGGCGAGA TTGTAATGTC TGTCAAAGAG ATGCTGTCAG
CHU15092 GCCGCCTTAA AGTGGAGAAA AGACATAGGT TTCCGTGTCA ATGCCAACAC AGCAGCTCTG AGCAACAAAG TCCTCGCAGA ATACAAAGTC CCTGGTGAGA TTGTAATGTC TGTCAAAGAG ATGCTGTCAG
CHU15093 GCTGCCTTGA AGTGGAGAAA AGACATAGGT TTCCGTGTCA ATGCCAACAC AGCAGCTCTG AGCAACAAAG TCCTCGCAGA ATACAAAGTC CCTGGTGAGA TTGTGATGTC TGTCAAAGAG ATGCTGTCAG
CHU88410 GCTGCCTTGA AGTGGAGAAA AGACATAGGT TTCCGTGTCA ATGCCAACAC AGCAGCTCTG AGCAACAAAG TCCTCGCAGA ATACAAAGTC CCTGGTGAGA TTGTGATGTC TGTCAAAGAG ATGCTGTCAG

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CHU88411  GCCGCTCTCA AGTGGAGGAA GGACATAGGC TTCCGCGTTA ATGCAAACAC AGCGGCCCTA AGCAACAAAG TCCTCTCTGA GTACAAGGTT CCTGGTGAGA TTGTGATGTC TGTCAAAGAG ATGCTTTCAG
CHU88412  GCTGCTCTCA AATGGAGGAA GGACATAGGT TTCCGTGTCA ATGCAAACAC GGCAGCCTTA AGCAACAAGG TCCTTGCAGA ATATAAAGTC CCTGGCGAAA TTGTGATGTC TGTTAAAGAA ATGCTGTCAG
Identity  :: ::  : : : ::::: ::  ::  ::::  :: ::  : : :::: :: ::   : ::  :  ::  :::: : : ::  : ::  ::  : ::  :: :: :: : :  :  :: : :: :::::  ::: : ::::
Consensus GchGCyyTvA ArTGGAGrAA rGAmrTAGGy TtyCGyrTyA ATGChAAyAC rrCrGCbyTr AGymACAArG TsCTydCwGA rTAyrArGTy CcwGGhGArA TydTrrTGyC yGTyAAAGAr ATGyThTCAG

          261                                   300                                   343
SPU 497/89  ACATGATTAG GAGAAGGAAC CTGATTCTAA ACAGGGGTGG TGATGAGAAC CCACGTGGCC CAATGAGCCA TGAGCATGTA GAC
  CCFSRNA   ATATGATTAG AAGAAGGAAC TTGATTCTCA ACAGAGGTGG CGATGAAAAT CCACGAGGCC CAGTGAGCCG TGAACATGTG GAG
  DQ004959  ACATGATAAG GAGAAGGAAC ATTATCCTTA ACAGAGGCAG CGATGAGAAT CCACGAGGCC CAGTAAGTCA TGAGCACATT GAG
  CHU15020  ACATGATTAG AAGGAGGAAC CTGATCCTTA ACAGAGGAGG TGATGAGAAC CCAAGAGGCC CAGTAAGCAA GGAGCACATA GAA
  CHU15021  ACATGATTAG AAGGAGGAAC CTGATCCTTA ACAGAGGAGG TGATGAGAAC CCAAGAGGCC CAGTAAGCAA GGAGCACATA GAA
  CHU15022  ACATGATTAG AAGGAGGAAC CTGATCCTTA ACAGAGGAGG TGATGAGAAC CCAAGAGGCC CAGTAAGCAA GGAGCACATA GAA
  CHU15023  ACATGATTAG AAGGAGGAAC CTGATCCTTA ACAGAGGAGG TGATGAGAAC CCAAGAGGCC CAGTAAGCAA GGAGCACATA GAA
  CHU15024  ATATGATCAG AAGAAGGAAC CTAATTCTCA ACAGAGGTGG TGACGAAAAC CCACGTGGCC CAGTTAGCCG TGAACATGTG GAG
  CHU15 89  ACATGATTCG GAGAAGAAAC CAAATTCTAA ACAGGGGTGG TGATGAGAAT CCACGTGGCC CTGTGAGCCG TGAGCATGTG GAC
  CHU15090  ACATGATTCG GAGAAGAAAC CAAATTCTAA AGAGGGGCGG TGATGAGAAT CCACGTGGCC CTGTGAGCCG TGAGCATGTG GAC
  CHU15091  ACATGATTCG GAGAAGAAAC CAAATTCTAA ACAGGGGCGG TGATGAGAAT CCACGTGGCC CTGTGAGCCG TGAGCATGTG GAC
  CHU15092  ACATGATTAG GAGAAGGAAC CTGATTCTAA ACAGGGGTGG TGATGAGAAC CCACGTGGCC CAGTGAGCCG TGAGCATGTA GAC
  CHU15 93  ACATGATTAG GAGAAGGAAC CTGATTCTAA ACAGGGGTGG TGATGAGAAC CCGCGTGGCC CAGTGAGCCG TGAGCATGTA GAC
  CHU88410  ACATGATTAG GAGAAGGAAC CTGATTCTAA ACAGGGGTGG TGATGAGAAC CCACGTGGCC CAGTGAGCCA TGAGCATGTA GAC
  CHU88411  ACATGATTAG AAGGAGGAAC CTGATCCTTA ACAGAGGAGG TGATGAGAAC CCAAGAGGCC CAGTAAGCAA GGAGCACATA GAA
  CHU88412  ACATGATTAG AAGGAGGAAT CTAATTCTCA ACAGGGGCGG TGATGAAAAT CCGCGTGGCC CAGTGAGCCG TGAACATGTG GAG
  Identity: ::::: :  :: :: ::     :: :: : : :: ::  :  :: :: ::  ::  : :::: :  : ::     :: ::  :  ::
  Consensus AyATGAThmG rAGrAGrAAy hwdATyCThA AsAGrGGhrG yGAyGArAAy CCrmGnGGCC CwrTdAGymr kGArCAyrTd GAv
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Figure 9: Nucleotide sequence of S RNA segment of CCHF virus (strain SPU 497/89) compared with 15 different CCHF variants from Genbank®. semicolons (:) indicate sequence identity.

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           1                   20                  40                  60          70
SPU497/89  ECAWVVSTGI VKKGLEWFEK NAGTIKSWDE SYTELKVDVP KIEQLTGYQQ AALKWRKDIG FRVNANTAAL
CCFSRNA    ECAWVSSTGI VKKGLEWFEK NAGTIKSWDE SYIELKVEVP KIEQLANYQQ AALKWRKDIG FRVNANTAAL
Consensus  ECAWVxSTGI VKKGLEWFEK NAGTIKSWDE SYxELKVxVP KIEQLxxYQQ AALKWRKDIG FRVNANTAAL

           71       80                  100                 121
SPU497/89  SNKVLAEYKV PGEIVMSVKE MLSDMIRRRN LILNRGGDEN PRGPMSHEHV D
CCFSRNA    SHKVLAEYKV PGEIVMSVKE MLSDMIRRRN LILNRGGDEN PRGPVSREHV E
Consensus  SxKVLAEYKV PGEIVMSVKE MLSDMIRRRN LILNRGGDEN PRGPxSxEHV x
```

Figure 10: Amino acid sequence of the S RNA segment of CCHF virus strain SPU 497/89 compared with strain CCFSRNA

A or a	A
C or c	C
G or g	G
T or t	T
U or u	U
R or r	either A or G
Y or y	either C or T or U
M or m	either C or A

K or k	either T or U or G
W or w	either T or U or A
S or s	either C or G
B or b	C ,T ,u, or G (not A)
D or d	A, T, U or G (not C)
H or h	A, T, U, or C (not G)
V or v	A, C, or G (not T or U)
N or n	any base (A, C, G, T, or U

IUPAC Codes for nucleic acids

For nucleic acid sequences, OMIGA uses the following characters:

3.5 CONSTRUCTION OF THE DELETION VARIANT

The construction of the deletion variant started with the search for two unique restriction sites that would allow a small fragment of the cloned CCHF virus DNA to be dropped from the pHEN I plasmid. Two such sites were identified, i.e. those for *Bln* I and *Bst*E II, which were used separately to digest the cloned CCHF virus PCR fragment. This resulted in the release of a 59 bp fragment. The overhanging ends of the plasmid were filled in with dNTPs using *Taq* DNA polymerase, religated to form pHEN II and transformed into *E. coli*. The result was a variant insert that differed in size from the natural virus RNA, but that could still be amplified by the same primers. The size difference of 59 bp is enough to distinguish between the full-length and the deletion variant inserts when visualised on an ethidium bromide stained gel after RT-PCR, as shown in Figure 11 lanes 3, 4 and 5. The RT-PCR assay was repeated with the CCHF virus RNA received from the NIV. The expected band of 381 bp was present together with the 322 bp internal control (Figure 9).

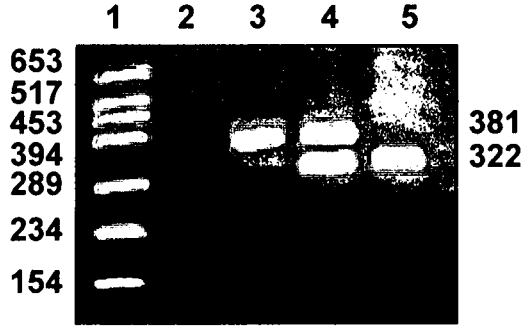

Figure 11: Agarose gel electrophoresis of the RT-PCR products. Lane 1, molecular weight marker VI (Boehringer Mannheim); Lane 2, negative control; Lane 3, CCHF virus RNA (381 bp); Lane 4, CCHF virus RNA (381 bp) with internal control (322 bp) and Lane 5, internal control (322 bp).

3.6 IN VITRO TRANSCRIPTION OF INTERNAL CONTROL RNA

With the use of T3 RNA polymerase, CCHF internal control RNA was synthesised on a large scale. The excess DNA from the pGEMEX-I plasmid vector and the cloned

PCR product were digested with DNase I. The *in vitro* synthesised control RNA was used as a positive control instead of Dugbe virus, a nairovirus antigenically related to CCHF virus (Burt *et al*, 1998).

3.7 ESTIMATION OF THE CONCENTRATION OF INTERNAL CONTROL RNA REQUIRED FOR RT-PCR

The concentration of the stock RNA solution was estimated as 0.1 µg/µL from the optical density. A ten-fold dilution series was made from the stock RNA solution and used for RT-PCR. The amplified cDNA products were separated on a 2% agarose gel and bands of the expected size, 322 bp, were observed (Figure 12). The last visible RT-PCR band was seen in lane 7, corresponding to a concentration of 10^{-6} µg RNA. The minimum concentration of target RNA the RT-PCR can detect was estimated to be 4 x 10^{-5} ρmol RNA, giving more or less the same sensitivity as the PCR alone. No false-positive results were obtained with the water control (Figure 12, lane 2).

Figure 12: Agarose gel electrophoresis of RT-PCR products of the internal control RNA dilution series. Lane 1, molecular weight marker VI (Boehringer Mannheim); Lane 2, negative control; Lane 3, 10^{-2} µg; Lane 4, 10^{-3} µg; Lane 5, 10^{-4} µg; Lane 6, 10^{-5} µg and lane 7, 10^{-6} µg.

The results of the RT-PCR of the dilution series in this study correlate well with published data of which the sensitivity of detection was 10^{-5} U, that is on the basis of the molecular weight equivalent to 3.4 x 10^{6} molecules of RT (Sears *et al*, 1999).

Since it is reported that 80 molecules of RT are present per HIV-I particle (Layne *et al.*, 1992), the sensitivity of detection of the method in this study was calculated to be 10^6 molecules.

Although RT-PCR assays generally detect about 10^5 to 10^6 virus particles and are not as sensitive as DNA-PCR assays, they are broadly reactive and have been used for the detection and isolation of different types of novel retroviruses (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984). Chang *et al.* (1997) showed that the sensitivity of a RT-PCR assay could be improved by lowering the pH of the RT-PCR reaction to pH 5.5, by reducing the reaction incubation time to 1 hour and by the addition of protease inhibitor to the reaction components. After these modifications RT-PCR assays were up to 10^6-fold more sensitive than conventional methods at detecting retroviruses (Chang *et al.*, 1997).

3.8 IMPLEMENTATION OF THE INTERNAL CONTROL WITH ISOLATED TICK RNA

The internal control with a concentration of 0.1 µg/µl was used to spike RT-PCR reactions performed with RNA isolated from *Hyalomma* ticks. The presence, after PCR amplification, of the internal control on an agarose gel would indicate that the RT-PCR had worked. Initially, no product was seen on the gels, even after many attempts. This problem was overcome by decreasing the volume of tick RNA solution in the reaction.

Since such a small amount of RNA is usually obtained from a single tick, it is impossible to accurately determine the concentration. The fifty ticks subsequently tested were all negative, an example of the results is given in Figure 13. Either the CCHF virus was not present in the tick RNA or was at levels below the detection cut-off of 4×10^{-5} pmol.

The detection of small amounts of retrovirus by RT-PCR assay may be made possible by either virus amplification in a susceptible cell line or by increasing the virus concentration in a sample by centrifugation (Sears *et al.*, 1999). The sensitivity of RT-PCR can be increased by using ^{32}P or ^{125}I radiolabelled nucleotide substrates (Gronowitz *et al.*, 1991; Willey *et al.*, 1988). Recently RT-PCR sensitivity has been increased using non-isotopically labelled nucleotides(Cook *et al.*, 1991; Eberle & Seibl, 1992; Suzuki *et al.*, 1995).

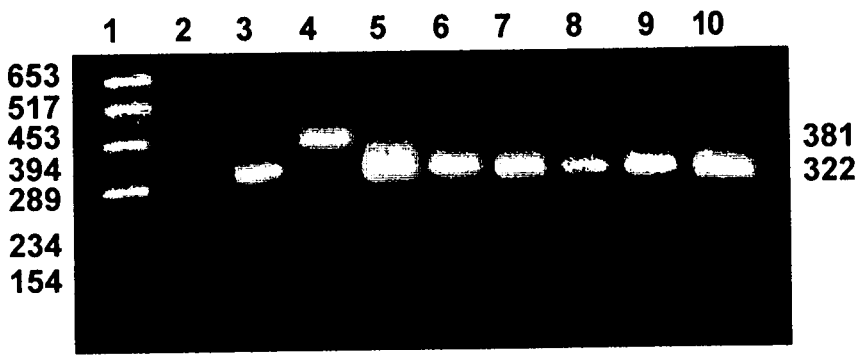

Figure 13: Agarose gel electrophoresis of the RT-PCR products. Lane 1, molecular weight marker VI (Boehringer Mannheim); Lane 2, negative control; Lane 3, internal control RNA (322 bp); Lane 4, CCHF virus RNA (381 bp); Lane 5 to Lane 11, tick RNA negative for CCHF virus spiked with internal control RNA (322 bp).

According to Ward *et al.* (1990b), the level of sensitivity of virus detection in blood samples, with RT-PCR was found to be 10 PFU of Dugbe virus, which is equivalent to the level of detection by plaque assay, but was 200-fold less sensitive than inoculation of suckling mice. Dugbe virus in the haemolymph of ticks is mostly associated with haemocytes, rather than acellular haemolymph (Booth *et al.*, 1990), which may account for the apparent low level of virus in ticks. For virus diagnosis in ticks, the level of sensitivity appeared to be comparable to that of biological assays. The combination of PCR and dot-blot analysis gave a weak positive signal with haemolymph from infected ticks (Ward *et al.*, 1990b).

3.9 CONCLUSION AND FUTURE APPLICATIONS

This study was successful as an exercise in the development and initial evaluation of a method, which includes an internal control for the rapid detection, and determination of the concentration of CCHF viral nucleic acid in *Hyalomma* ticks by means of a RT-PCR assay.

The major advantage of RT-PCR compared with infectivity assays is the time taken to obtain a result. The RT-PCR procedure described here can be carried out in less than 5 hours, compared to a week or more for biological amplification methods. Furthermore, if the sample contains different viruses, RT-PCR provides a means of identifying a particular virus. In contrast, biological systems will amplify any virus present in the sample and must rely on further serological characterisation of the amplified virus(es) before a positive diagnosis can be made. An additional advantage of the RT-PCR is that it is not affected by the presence of antibody in a sample, which may render the virus non-infectious in biological assays. Animals are used less for routine scientific studies because of pressure from animal rights activists (Webb, 1989) as well as the cost of animals and the need for facilities to handle infectious animals. RT-PCR provides an alternative to the use of animals and has the advantage of rendering a virus non-infectious at an early stage of the detection procedure. The ease of preparing samples and the convenience of a wide range of retrovirus RT-PCRs give the RT-PCR considerable potential for use as a screening procedure.

A possible explanation for the low detection rate of CCHF virus RNA in *Hyalomma* ticks in this study may be that the frequency of CCHF virus infested *Hyalomma* ticks can be as low as one in a thousand (Swanepoel, 2000). This is corroborated by the fact that CCHF is a scarce disease in South Africa, with only sporadic outbreaks. Using inoculated or naturally infected ticks can solve this problem, but this would

require BSL 4 facility to get the tick specimens and manipulation of the infectious material. This would allow a semi quantitative RT-PCR detection compared to detection in suckling mice or cell culture. The internal control allowed validation of only some steps of the RT-PCR technique using the internal control RNA as described in this thesis. It is impossible to distinguish between a negative sample and hydrolysed RNA. The internal control RNA can be placed in the same tube as the tick before it is homogenised. The absence of the internal control after RT-PCR would then be an indication of the presence of RNase and that the detection of CCHF virus in ticks may not be easy due to the presence of large amounts of RNase. Future research should involve the further optimisation of the RT-PCR assay because 10^6 molecules are far too high for clinical use and should aim to improve the sensitivity at least 100-fold, among others by including the use of radiolabelled primers and the design of a new set of primers. PCR detection could be enhanced using various techniques as nested PCR, northern blots for direct RNA and southern blots for cDNA detection.

CHAPTER 4

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APPENDIX A

LIST OF REAGENTS, THEIR SUPPLIER'S NAME, CITY AND COUNTRY

[γ-^{32}P]ATP (Amersham, Amersham, UK) Cat. No. PB 10474[(a)].

[α-^{32}P]dATP Megaprime ^{TM} labeling kit (Amersham, Amersham, UK) Cat. No. RPN 1606.

ABI Prism^{TM} Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer corporation Cat. No. P/N 402078).

Access RT-PCR system (Promega corporation, Madison, WI, USA)Cat. No. A1250.

Agarose MS-8 (Whitehead Scientific, Brackenfell, Cape Town) Cat. No. HO20600).

***Bln* I** (Boehringer Mannheim, Mannheim, Germany) Cat. No. 1556161.

***Bst*E II** Promega, Madison, WI, USA) Cat. No. R 6641.

dATP Li-salt (Boehringer Mannheim, Mannheim, Germany) Cat. No.1051440.

dCTP Li-salt (Boehringer Mannheim, Mannheim, Germany) Cat. No. 1051458.

dGTP Li-salt (Boehringer Mannheim, Mannheim, Germany) Cat. No. 1051466.

dTTP Li-salt (Boehringer Mannheim, Mannheim, Germany) Cat. No. 1051482.

***Eco*R 1** (Amersham, Amersham, UK) Cat. No E1040Y

***EcoR* 1 linkers** (Amersham, Amersham, UK) Cat. No. RPN 1712.

High Pure Plasmid Isolation Kit (Boehringer Mannheim, Mannheim, Germany) Cat. No. 1754785.

***Hind* III** (Amersham, Amersham, UK) Cat. No. E1060Y

Hybond-N$^+$ nylon filters (Amersham, Amersham, UK) Cat. No. RPN 203N.

Molecular marker VI (Boehringer Mannheim, Mannheim, Germany) pBR 328 DNA Bgl I + pBR 328 DNA Hinf I Cat. No. 1062590.

pGEM Express Systems kit (Promega, Madison, WI, USA) Cat. No. P2211.

Reverse transcriptase, M-MuLV (Boehringer Mannheim, Mannheim, Germany) Cat. No. 1062 603.

RNase-Inhibitor (Boehringer Mannheim, Mannheim, Germany) Cat. No. 799 017.

RQ1 RNase-Free DNase (Promega, Madison, WI, USA) Cat. No. M6101.

RNeasy^{TM} Total RNA Kit (Qiagen, Germany) Cat. No. 74106).

Sephadex G50 (Sigma, St Louis, USA) Cat.No. G/50/150.

TRIZOL® Reagent (Gibco BRL, Gaithersburg, USA) Cat. No. 15596-018.

Wizard ® Plus Maxipeps System (Promega, Madison, WI, USA) Cat. No. A7510.

APPENDIX B

LIST OF BUFFERS AND REAGENTS

2.5 M Ammonium acetate
192.7 g ammonium acetate in 200 ml distilled water
Adjust volume to 1000 ml with distilled water

Bromophenol blue loading buffer (Sucrose based buffer) 6x recipe
20 mg bromophenol blue
3 ml 0.5 M EDTA
10 ml glycerol
Adjust to volume of 20 ml with distilled water
Storage temperature 4°C

100 mM Calcium chloride ($CaCL_2$)
1.1 g calcium chloride
Adjust volume to 100 ml with distilled water

Chloroform/isoamyl alcohol (24:1)
10 ml isoamyl alcohol
240 ml chloroform
Mix
Add 10 ml TE

1 mM EDTA, pH 7.0
0.372 g $Na_2EDTA \bullet 2H_20$ (MW=372.24)
Bring volume to 800 ml with distilled water
Mix
Adjust to pH 7.0 with sodium hydroxide pellets
Adjust volume to 1 litre with distilled water

0.25 M EDTA, pH 8.0
93.05 g $Na_2EDTA \bullet 2H_20$ (MW=372.24)
Bring volume to 800 ml with distilled water
Mix
Adjust to pH 8.0 with sodium hydroxide pellets (approximately 20 g)
Adjust volume to 1 litre with distilled water

0.5 EDTA, pH 8.0
186.1 g $Na_2EDTA \bullet 2H_20$ (MW=372.24)
Bring volume to 800 ml with distilled water
Mix and adjust to pH 8.0 with sodium hydroxide pellets (approximately 20 g)
Adjust volume to 1 litre with distilled water

Ethidium bromide stock solution
1 g ethidium bromide
100 ml distilled water
Stir for several hours to ensure dye is dissolved
Store protected from light at 4°C

LB medium
10 g tTiptone
5 g yeast extract
10 g sodium chloride
Add 900 ml distilled water
Mix
Adjust pH 7.0 with 200 µl 5 N sodium hyroxide
Adjust volume to 1 litre with distilled water

1 M Magnesium chloride ($MgCl_2$)
20.3 g magnesium chloride
Add 50 ml distilled water
Mix
Add 10 ml acetic acid (CH_3COOH)
Adjust volume to 100ml with distilled water

1 M Sodium chloride (NaCl)
58.4 g sodium chloride in 800 ml distilled water
Adjust volume to 1 litre water with distilled water

3 M Sodium acetate
408.3 sodium acetate ($3H_2O$) in 800 ml distilled water
Adjust to pH 4.8 with 3 M acetic acid
Adjust volume to 1 litre with distilled water

0.5 M Sodium Hydroxide (NaOH)
20 g sodium hydroxide in 800 ml distilled water
Adjust volume to 1 litre distilled water

7.5 M Ammonium acetate (NH_4AC)
578.1 g ammonium acetate in 800ml distilled water
Adjust volume to 1 litre with distilled water

Phenol/chloroform (1:1)
5oml phenol
50 ml chloroform
Mix
Add 5 ml 1xTE

SOB-agar
Mix the following in 200 ml distilled water:
5 g triptone
1.25 g yeast extract
0.025 g sodium chloride
2.5 ml 250 mM potassium chloride
Adjust the volume to 250 ml with distilled water
3.75 g agar
Boil in microwave until the agar dissolved
Cool down and add 250 µl ampicillin
Pour 25 ml per petri-dishes and stall

SOC-medium
Mix the following in 80 ml distilled water:
2g triptone
0.5 g yeast extract
0.05 g sodium chloride
1 ml 250 mM potassium chloride
Adjust volume to 100 ml with distilled water
Divided into 10 ml aliquots in test tubes

3M Sodium acetate pH 4.6
408.3 g sodium acetate (3H20) in 800ml distilled water
Adjust to pH 4.6 with 3 M acetic acid
Adjust volume to 1 litre with distilled water

STE
5 ml 1 M tris
10 ml 5 M sodium chloride
1 ml 0.5 M EDTA
Adjust volume to 500 ml with distilled water.

Terrific Broth (TB) medium
2.4 g bacto-triptone
4.8 g yeast extract
1.6 ml glycerol
Adjust volume to 180 ml with distilled water.
Mix.
Put 8 ml aliquots in 25 ml test tubes.
Autoclave.
Cool down.
Add 1 ml each of sterile KH_2PO_4 and K_2HPO_4

TE, pH 7.4
10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0
10 ml M Tris-HCl
2 ml 0.5 M EDTA, ph 8.0
Adjust to pH 8.0 with HCl
Adjust volume to 1 litre with distilled water

Tris-borate (TBE) buffer (stock)
(1x =89 mM Tris base, 89 mM boric acid, 2 mM EDTA)

54.0 g tris base
27.5 g boric acid
3.27 g $Na_2EDTA \bullet 2H20$
Adjust to 1 litre with distilled water

0.5 M Tris-HCL, pH 7.4
60.55 g tris to 800 ml of distilled water
Adjust to pH 7.4 with concentrated HCl
Adjust volume to 1 litre with distilled water