A COMBINED RAPID TEST FOR DETECTING ARBOVIRUS ANTIBODIES IN BLOOD SPECIMENS

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A COMBINED RAPID TEST FOR DETECTING ARBOVIRUS ANTIBODIES IN BLOOD SPECIMENS

by

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ABBREVIATIONS

Ae.	Aedes
An.	Anopheles
AP	Alkaline phosphatase
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate *p*-Toliudine salt
BSA	Bovine serum albumin
CCID	Cell culture infectious dose endpoint
CF	Complement fixation
CPE	Cytopathic effect
Cx.	Culex
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
HI	Haemagglutination inhibition
HRP	Horseradish peroxidase
i.c.	intracerebral
IF	Immunofluorescence
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
i.p.	intraperitoneal
JE	Japanese encephalitis
LPB	Lactose-phosphate buffer
MEM	Minimum essential medium
NBT	Nitro Blue Tetrazolium
NC	Nitrocellulose
NT	Neutralisation test
PBS	Phosphate buffered saline
PRNT	Plaque reduction neutralising test
PSN	Penicillin, Streptomycin, Neomycin
RF	Rheumatoid factors
RIA	Radio-immunoassay
RNA	Ribonucleic acid
RVF	Rift Valley Fever
SIN	Sindbis
SLE	St. Louis encephalitis
WN	West Nile
WSL	Wesselsbron

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CHAPTER 1

INTRODUCTION

Arboviruses (arthropod-borne viruses) are viruses that are maintained in nature through transmission between susceptible vertebrate hosts by haematophagous arthropods. These viruses multiply and produce viraemia in the vertebrates, replicate in the tissue of arthropods, and are then passed on to new vertebrates by the bites of these arthropods (Simpson, 1984).

There are 504 arboviruses registered in the International Catalogue of Arboviruses (Labuda, 1991) of which approximately 100 are capable of infecting man. The majority of arboviruses have been serologically classified in the families; *Togaviridae, Flaviviridae, Reoviridae, Bunyaviridae,* and *Rhabdoviridae.*

Mosquitoes are the most important arbovirus vectors, followed by ticks. Phlebotomines, *Culicoides* species and *Cimex* species are also involved in the transmission of some arboviruses (Simpson, 1984).

Studies have shown that Sindbis (SIN), West Nile (WN), Rift Valley fever (RVF) and Wesselsbron (WSL) viruses are the most prevalent arboviruses in the Orange Free State (McIntosh, 1980) and with generalised clinical symptoms such as fever, headache, general malaise, muscle or joint pains and a macular or maculopapular rash (Lennette & Schmidt, 1979) a conclusive diagnosis for arbovirus infections can only be made by the laboratory.

Standard tests for identifying viruses or detecting viral antibodies are often time consuming, expensive and necessitate sophistication. No commercial diagnostic kits are available for detecting antibodies to certain arboviruses, therefore, it was necessary to develop a simple, cost-effective test for screening and determining specific antibodies of the immunoglobulin G (IgG) and immunoglobulin M (IgM) class against RVF, SIN, WN and WSL viruses.

In the conventional enzyme-linked immunosorbent assays (ELISA) for detecting viral antigens and antibodies, polystyrene or other materials (which have a rather low binding capacity for proteins) are generally used as the solid phase. On the other hand, the superior binding capacity of nitrocellulose (NC) for the adsorption of proteins from a polyacrylamide gel following electrophoresis by the Western blot technique, has been demonstrated and implicated the advantageous use of NC instead of polystyrene as the solid phase (Towbin *et al.* 1979).

The aim of this study was to develop a dot-ELISA by using a NC membrane as solid phase on which to spot antigens for later visualisation of antigen-antibody complexes of RVF, SIN, WN and WSL with enzyme-conjugated anti-human immunoglobulins of the IgG, as well as the IgM class, using a substrate which gives an insoluble colour reaction.

CHAPTER 2

LITERATURE REVIEW

2.1. RIFT VALLEY FEVER VIRUS

2.1.1. CLASSIFICATION

RVF virus is a member of the genus *Phlebovirus* of the family *Bunyaviridae.* For many years after its isolation, RVF virus was an unclassified arbovirus. It was demonstrated by Murphy *et al.* (1973) that RVF virus was morphologically similar to the bunyaviruses and its classification, as a member of the family *Bunyaviridae*, was confirmed by basic molecular studies (Rice *et al.*, 1980). Serological tests indicated that RVF virus was antigenically related to the phlebotomus fever viruses and therefore belonged to the *Phlebovirus* genus (Shope *et al.*, 1980).

2.1.2. EPIDEMIOLOGY

2.1.2.1. GEOGRAPHICAL DISTRIBUTION

According to Daubney *et al.* (1931) RVF was first recognised in the Rift Valley of Kenya in 1930 where it primarily affected sheep, causing an increased abortion rate in pregnant ewes and mortality among lambs. Fatal disease occurred predominantly in young sheep, cattle and goats, manifesting with focal liver necrosis. Human infection resulted in a mild febrile illness. The illness was thought to be probably caused by an arthropod-borne virus. It appeared, retrospectively, that the disease had been present for about 20 years in the area of the Great Rift Valley in Kenya.

Major epizootics of RVF occurred in Kenya in 1931 and 1978, South Africa in 1951 and 1975, Namibia in 1955 and 1974, Zimbabwe in 1957, 1969 and 1978, Sudan in 1973, Zambia in 1973 and 1978, and Egypt in 1977. Limited outbreaks were also reported from Kenya, Mozambique, South Africa, Sudan, Tanzania and Uganda. Serological evidence confirmed that the virus was present in numerous sub-Saharan African countries (Meegan & Bailey, 1989). The first confirmed outbreak in West Africa occurred in Mauritania in 1987 (Ksiazek *et al.*, 1989), and the first fatal case of RVF in Madagascar was reported by Morvan *et al.* (1992).

During the summer of 1950 to 1951 the first known epizootic of RVF occurred in South Africa (Alexander & Dickson, 1951; Mundel & Gear, 1951). This epizootic mainly involved areas of the western and south-western Orange Free State and the north-western Cape Province (Kimberley area), with isolated outbreaks occurring in the western and southern areas of the Transvaal (Gear *et al.*, 1951).

According to Weiss (1957), several laboratory workers contracted the disease, 20 000 humans were infected and over 100 000 sheep and cattle died during this outbreak. Serological evidence of RVF virus infection was also found in a number of herds of cattle in the Knysna district of the southern Cape Province.

RVF re-appeared in South Africa in the late autumn of 1953 in epizootic form in an area near Luckhoff in the Orange Free State (Van der Linde, 1953).

During April, 1955, an outbreak of RVF occurred on farms in the Marienthal district, Namibia, and in the late summer of 1956 an epizootic of RVF was reported from farms in the Kroonstad district in the Orange Free State (Weiss, 1957).

After an apparent absence of 13 years, RVF re-appeared in the summer of 1968 and 1969 in a widespread epizootic in Zimbabwe with smaller outbreaks in the coastal lowlands of Natal, South Africa and Mozambique, involving only cattle. Further outbreaks occurred during 1970 in several districts of the Orange Free State and south-eastern Transvaal (McIntosh, 1972).

After exceptionally heavy rains over the Karroo and the western region of the Cape Province in 1974 and 1975, an epizootic of RVF occurred in the sheep and cattle-farming districts, causing the loss of many thousands of livestock. Associated with this epizootic many cases of human infections were reported, and for the first time ever, human deaths due to haemorrhagic complications of RVF were recognised (Van Velden *et al.*, 1977). Later in the 1977 to 1978 Egyptian epidemic, extensive human involvement, with fatality estimates of up to 600 persons dying of haemorrhagic fever associated with acute hepatitis was reported (Shope *et al.*, 1982).

Epidemiological studies in South Africa revealed an extensive epizootic area for RVF. It includes the Karroo, the Cape Province north of the Orange River, the southern half of Namibia, the Orange Free State Province, and the southern half of the Transvaal Province, excluding the eastern Lowveld region of that province. In the coastal lowlands of Natal, cattle are enzootically infected with RVF virus, with a minimal infection rate in humans (McIntosh & Jupp, 1981).

2.1.2.2. VECTORS AND HOSTS

Numerous species of haematophagous arthropods are incriminated as possible vectors of RVF virus. These incriminations are based on the isolation of virus from these species during or following peak periods of transmission. Different species of mosquitoes were suggested to be involved as vectors or reservoirs in the various geographic areas of Africa where the disease is

endemic (Table 1) (Meegan & Bailey, 1989). Meegan *et al.* (1980) also implicated a *Rhipicephalus* tick as a vector or host for RVF virus.

TABLE 1. **ARTHROPODS FOUND NATURALLY INFECTED WITH RVF VIRUS**

Species	Locality
Aedes	
aedimorphus	
cumminsii	Burkina Faso
dalzieli	Kenya, Senegal
dentatus	Zimbabwe
tarsalis	Uganda
durbanensis	Kenya
Neomelaniconion	
circumluteolus	Uganda, South Africa
lineatopennis	Zimbabwe, South Africa, Kenya
palpalis	Central African Republic
Ochlerotatus	
caballus	South Africa
juppi	South Africa
Stegomyia	
africanus	Uganda
dendrophilus	Uganda
furcifer	Burkina Faso
Anopheles	
Anopheles	
coustani	Zimbabwe
coustani and fusicolor	Madagascar
Cellia	
pauliani and squamosus	Madagascar
christyi	Kenya
pharoensis	Kenya
Culex	
antennatus	Kenya,Nigeria
antennatus and annulioris gp.	Madagascar
simpsoni and vansomereni	Madagascar
antennatus, simpsoni and vansomereni	Kenya, Madagascar,
theileri	Kenya, South Africa, Zimbabwe
zombaensis	Kenya
Eumelanomyia	
rubinotus	Kenya
Eretmapodites	
quinquevittatus spp.	South Africa, Uganda
Coquillettidia	
fuscopennata	Uganda
grandidieri	Madagascar
Mansonia	
uniformis	Madagascar
Mansonioides	
africana	Uganda, Central African Republic
uniformis	Uganda
Other Diptera	
Culicoides spp.	Nigeria
Simulium	South Africa

(Meegan & Bailey, 1989)

RVF epizootics occurred following unusually heavy rainfall and the presence of large numbers of mosquitoes, giving merit to the hypothesis that the virus may be transmitted and maintained transovarially in floodwater mosquitoes. Linthicum *et al.* (1985) recovered RVF virus from both male and female *Aedes lineatopennis* reared in the laboratory, supporting the hypothesis that

transovarial transmission is the most likely mechanism for maintaining the virus during environmental conditions that are unsuitable for active transmission between vector and susceptible vertebrate hosts.

In South Africa Gear *et al.* (1955) isolated RVF virus from 6 pools of *Ae. caballus* and 3 pools of *Culex theileri* during the 1953 outbreak. It was also shown that RVF could be transmitted by naturally infected *Ae. caballus*.

In 1956, RVF virus was again isolated from *Cx. theileri* during an outbreak of disease among sheep in the Wesselsbron district, Orange Free State, according to unpublished data of the Arbovirus Research Unit (McIntosh, 1972).

The isolation of RVF virus during field investigations implicated *Cx. theileri* as the main vector in Zimbabwe, as well as in the Kroonstad and Johannesburg districts of South Africa during the 1969 epizootic. *Ae. lineatopennis* was also suggested to play a role as vector in Zimbabwe, while *Ae. circumluteolus* was mentioned as a possible vector in the northern parts of Natal (McIntosh, 1972).

During an outbreak of RVF in the coastal region of Natal in 1981, RVF virus was isolated from both *Cx. zombaensis* and *Cx. neavei*. Laboratory tests revealed that *Cx. zombaensis* was the main vector during this outbreak and that *Ae. circumluteolus* was possibly a vector during the earlier stages (McIntosh *et al.*, 1983).

In experimental studies McIntosh *et al.* (1980(a)) demonstrated that *Cx. theileri, Cx. univittatus, Ae. juppi* and *Ae. lineatopennis* (plateau fauna), *Ae. aegypti, Eretmapodites quinquevittatus, Cx. zombaensis and Cx. neavei* (Natal coast) and *Cx. quinquefaciatus* (both regions), can transmit RVF virus, although some of these species are not efficient vectors. It was evident that *Cx. theileri* was very capable of transmitting virus to sheep and cattle, therefore acting as the main vector on the inland plateau.

Numerous studies have been conducted to determine the role of vertebrate hosts in the natural cycle of RVF virus (Smithburn *et al.*,1948; Davies & Onyango, 1978; Davies & Addy, 1979; Hoogstraal *et al.*, 1979; Davies & Linthicum, 1986). Although the possible role of wild rodents has been investigated, results have shown that they are probably not involved as amplification hosts of the virus (Weinbren & Mason, 1957; Davies, 1975; Swanepoel *et al.*, 1978). However, Weinbren & Mason (1957) suggested that the field rat *Arvicanthis abyssinicus nubilans* can fulfil all the requirements necessary to act as a natural host. Swanepoel *et al.* (1978) have shown that four species of murids (Rodentia: Muridae) were capable of circulating amounts of RVF virus likely to

be infective for mosquitoes. Pretorius (1992) reported on viraemia studies performed on *Aethomys namaquensis* (Rodentia: Muridae) and concluded that this species can act as asymptomatic carriers of RVF virus in nature.

2.1.3. DISEASE ASSOCIATIONS

2.1.3.1. ANIMALS

Disease in Africa appears to be limited to domestic animals and outbreaks are most obvious in sheep and cattle, and are characterised by widespread abortion in pregnant animals and a high fatality rate in neonates (Daubney *et al.*, 1931; Alexander & Dickson, 1951).

The severity and progression of the disease is inversely proportional to age, and lambs, calves, and kids are most susceptible to RVF (Meegan & Bailey, 1989).

According to Dickson (Alexander & Dickson, 1951) the clinical symptoms of RVF can be classified in 4 stages:

The peracute form

This form is common in lambs. The incubation period is about 12 h and death follows within 36 h as a result of liver necrosis. The mortality rate varies between 95 and 100 %.

The acute form

This form can occur in lambs and to a lesser extent in adult sheep. The symptoms appear suddenly with a rapid rise in temperature, vomiturition, a mucopurulent discharge from the nose, rapid pulse, unsteady gait, and abortion in ewes. Death usually takes place 24 to 48 h after the onset of symptoms. The mortality rate can vary from 20 to 30 %.

The sub-acute form

This form is common in adult sheep and cattle. A temperature rise, lasting from 24 to 96 h, can be detected. Inappetence and general weakness are present. In pregnant animals abortion may be the only symptom. Milk production decreases rapidly. The mortality rate is low and in cattle it can be less than 10 %.

The mild or inapparent form

This form usually occurs in adult sheep and cattle. There is only a slight febrile reaction and the disease can only be diagnosed serologically.

Swine, horses, domestic buffaloes and camels do not become ill, although abortions due to RVF have been reported in camels and buffaloes. Inoculation studies in wild rodents revealed a brief, low-level viraemia, with development of protective antibodies. Both wild and domestic fowls appeared refractory to infection (Meegan & Bailey, 1989).

In susceptible animals, the liver is the primary site of viral replication and on post-mortem it often appears a light brown-yellowish colour. Small white spots of necrotic tissue, surrounded by small haemorrhages under the capsule, can be seen. The liver becomes congested, haemorrhagic and soft. The kidneys can display congestion of the cortical and medullary blood vessels. The digestive tract can be affected and in acute cases a severe haemorrhagic gastro-enteritis can be present (Alexander & Dickson, 1951).

2.1.3.2. HUMANS

During an outbreak, disease usually appears first in livestock, while humans are secondarily infected (Meegan, 1979; McIntosh *et al.*, 1980(a)). RVF infection occurs mainly in farmers and others who come in close contact with animals infected with the disease (Daubney *et al.*, 1931; Mundel & Gear, 1951; van Velden *et al.*, 1976).

The virus can be transmitted by contact with tissue or blood of infected animals during care, autopsy, slaughter or disposal of infected carcases (Chambers & Swanepoel, 1980). Infection is postulated to result from transcutaneous or aerosol exposure (Hoogstraal *et al.*, 1979). During an epizootic, when there are many infected and dying animals, contact transmission may be a more important source of human infection than mosquito transmission (Joubert *et al.*, 1951). Laboratory-acquired infections, probably due to aerosols, have long been recognised and extreme precautions should be taken by those working with the virus (Sabin & Blumberg, 1947).

2.1.4. DIAGNOSIS

2.1.4.1. CLINICAL

RVF in man was considered to be an influenza-like disease from knowledge obtained from laboratory infections (Daubney *et al.*, 1931). Recovery from infection was usually complete. Schrire

(1951) described the loss of central vision in a few patients thought to be caused by RVF during an epizootic in South Africa in 1950. The first fatalities due to RVF were described by van Velden *et al.* (1977) during the 1975 epizootic in South Africa. Several patients were diagnosed with encephalitis. Five patients developed a haemorrhagic state with profuse bleeding and died soon after admission to hospital. A further 2 patients died from encephalitis.

It was only during the 1977 outbreak of RVF in Egypt that the full potential of RVF virus as a severe human pathogen was recognised. Four different forms of disease were recognised (Meegan *et al.*, 1981).

Uncomplicated RVF

Patients experienced the sudden onset of malaise and fever with an initial rigor, severe headache, 'backbreaking' myalgia and anorexia. Some experienced nausea and vomiting. On examination they were acutely ill and toxic, mostly with temperatures above 39 °C, with flushed faces and suffused conjunctivae. The febrile period lasted 4 to 7 days and a second temperature rise was observed during this period in a minority of cases. Patients improved symptomatically over the next 4 to 7 days and recovered completely within 2 weeks (Abdel-Wahab *et al.*, 1978).

RVF with ocular complications

Patients experienced the sudden onset of an acute febrile illness followed by a decrease in visual acuity within 5 to 15 days. The most common clinical findings were macular, paramacular or extramacular retinal lesions which frequently occurred bilaterally. Oedema, haemorrhage and vasculitis were observed. Patients with more severe macular lesions had permanent loss of central vision (Siam *et al.*, 1980).

RVF with meningoencephalitis

Patients described the acute onset of a febrile illness followed in 5 to 15 days by neurological symptoms which included disorientation, hallucination and vertigo. All exhibited meningismus. Hemiparesis and decerebrate posturing was noted in some cases. The cerebrospinal fluid showed cell counts of 20 to 600 cells/mm^3, mainly lymphocytes, but glucose and protein concentrations were normal. Recovery was slow and hemiparesis continued to manifest in some patients for up to 3 months (Laughlin *et al.*, 1979).

RVF with haemorrhage and jaundice

Patients experienced the sudden onset of a febrile illness, but 2 to 4 days later developed jaundice and haemorrhagic manifestations. Haematemesis, melaena, gingival bleeding and petechial and purpuric skin lesions were common. In fatal cases, death occurred within a week after the onset of jaundice. Shock and hepatic insufficiency were suspected as the cause of death. Virus was isolated from sera during both the febrile phase and the afebrile period which accompanied the onset of haemorrhagic signs. Patients with haemorrhages either died 3 to 6 days later or slowly convalesced and recovered completely (Laughlin *et al.*, 1979; Meegan, 1979).

RVF virus is highly abortive in many animals, and perhaps in humans. However, during the Egyptian epidemic, this possibility could not definitively be resolved (Abdel-Aziz *et al.*, 1980).

2.1.4.2. LABORATORY

The virus circulates to a high titer in sheep and cattle and virus isolation is diagnostic of RVF virus infection. Whole blood or serum collected at the peak of pyrexia, specimens of liver tissue, placenta and fetus transported on wet ice, have been the most common samples used for virus isolation (Van Velden *et al.,* 1977; McIntosh *et al.,* 1980(b); Meegan & Bailey, 1989). RVF virus was also isolated from faecal specimens and throat washings from patients during the Egyptian epizootic (Imam *et al.*, 1979).

The virus can be isolated in a number of cell culture systems, including Vero (African green monkey kidney cell line), BHK-21 (baby hamster kidney cell line), and many primary cell cultures (Weiss, 1957). In cell cultures, the virus has grown to titers of 1 to 5 x 10^7 pfu/ml and produced complete cytopathic effect (CPE) within 3 to 5 days. The virus has grown to similar titers in mosquito cell cultures, but produced no CPE (Peters & Anderson, 1981; Oelofsen *et al.*, 1990).

Susceptible laboratory animals for virus isolation are suckling mice, adult mice, hamsters and 1 to 3 day old lambs. Isolation attempts should always include adult white mice and hamsters because RVF virus is one of the few viruses that will kill adult mice and hamsters within 1 to 4 days after inoculation by the intraperitoneal (i.p.) route. Histopathological examinations will reveal characteristic liver necrosis in all susceptible animals (Daubney *et al.*, 1931; Sabin & Blumberg, 1947; Smithburn *et al.*, 1948; Gear *et al.*, 1951; Alexander & Dickson, 1951).

Viral isolates can be identified as RVF by the plaque reduction neutralising test (PRNT) using reference anti-RVF virus polyclonal or monoclonal antiserum (Shope *et al.*, 1982).

Rapid diagnostic methods to demonstrate viral antigen in specimens have been used. Gel diffusion has demonstrated antigen in specimens, but the test is not sensitive. Immune electron microscopy, immunofluorescence (IF) and radio-immunoassay (RIA) tests utilising reference antibodies have proved to be successful for detecting antigen in specimens (Meegan & Bailey, 1989).

Niklasson *et al.* (1983) developed a double-antibody ELISA to detect RVF virus antigen in specimens. This test is suitable for field evaluations since it proved to give a rapid, specific and sensitive result in diagnostically relevant concentrations. Furthermore, this test can be prepared, standardised and performed with inactivated virus, increasing its utility in surveillance in non-endemic areas.

The following serological tests have been commonly used to demonstrate RVF virus antibody in domestic animals and humans: complement fixation (CF), agar gel diffusion, haemagglutination-inhibition (HI), mouse neutralisation, PRNT, IF and ELISA. In some of these tests, various low-titer, nonspecific cross-reactions do occur between RVF antigen and antibodies developed in response to infection with other viruses in the phlebotomus fever serogroup (Tesh *et al.*, 1982).

A seroconversion, or a high convalescent anti-RVF virus antibody titer obtained by serological tests, provides strong evidence of RVF infection. The detection of IgM antibodies in human sera is important for the early diagnosis of RVF infection, and the development of an ELISA IgM test could become an important tool in the future (Niklasson *et al.,* 1984; Soliman *et al.*, 1988).

2.1.5. PREVENTION AND CONTROL

Immunisation of susceptible animals is the most effective means of control of RVF. Two types of vaccine are currently used to immunise sheep and cattle (Shope *et al.*, 1982).

The attenuated live virus vaccine (Smithburn strain) is highly effective, but causes a small percentage of pregnant sheep to abort after immunisation. It is relatively inexpensive and has been extensively used in endemic areas. Since there is a theoretical potential for reversion of the vaccine to virulence, it is not recommended for non-endemic areas (Assaad *et al.*, 1983). Recommendations and requirements for preparing live attenuated RVF vaccine for veterinary use were formulated to ensure a degree of safety and potency of the vaccine, at least equal to that recommended by the national control authorities (WHO, 1984).

Formalin-inactivated vaccines have been used extensively in southern Africa, Egypt and Israel. These vaccines are effective and safe, but are more expensive and at least 2 doses must be given.

This type of vaccine is recommended for non-endemic areas or for animals being exported from endemic areas (Assaad *et al.*, 1983).

Ongoing research on RVF vaccines includes experiments on reassortant vaccines, gene cloning to produce recombinant DNA products and synthetic polypeptide vaccines (Meegan & Bailey, 1989).

A safe, effective formalin-inactivated cell culture-propagated vaccine for RVF is also available for humans. It should be used to immunise laboratory and field researchers and to protect persons at risk (Eddy *et al.*, 1981).

Since enforcement of quarantine of animals is difficult in Africa, quarantine is not generally an effective control measure. However, movement of animals from epizootic areas should be restricted to prevent the further spread of RVF (Shope *et al.*, 1982).

2.2. SINDBIS VIRUS

2.2.1. CLASSIFICATION

SIN virus is a member of the family *Togaviridae* and belongs to the genus *Alphavirus* ('group A' arboviruses) (Fraenkel-Conrat, 1985).

SIN virus is the prototype alphavirus which has served as a model virus in studies on viral replication, biochemistry, membrane biogenesis and murine viral encephalitis (Taylor *et al.*, 1955; Karabatsos, 1975; Rentier-Delrue & Young, 1980; Olson & Trent, 1985).

2.2.2. EPIDEMIOLOGY

2.2.2.1. GEOGRAPHICAL DISTRIBUTION

SIN virus was first isolated in Egypt in 1952 from *Culex* mosquitoes collected in the village of Sindbis, 30 km north of Cairo (Taylor *et al.*, 1955).

According to Niklasson (1989) the virus was isolated from humans for the first time in 1961 in Uganda. Since then SIN virus has been isolated in several countries in Europe, Africa, Asia and Australia. SIN virus is recognised as the most widely distributed of all known arboviruses.

Human infection by SIN virus is relatively common in the Nile valley of Egypt, due to the close proximity of humans, birds and *Cx. univittatus* and *Cx. pipiens* mosquitoes. An infection rate of 27% was demonstrated by Taylor *et al.* (1955) during a survey conducted in Egypt in 1952 to 1954. However, prevalence studies of antibodies to SIN virus conducted in 1969, showed an apparent decrease in infection rate to 6.3 % (Darwish & Ibrahim, 1975).

In other areas, such as India, Indonesia, New Guinea and Australia, the virus was readily isolated from mosquitoes and birds, but the antibody prevalence in humans was low (Niklasson, 1989).

Significant antibody presence in humans has been found in Kuwait, Botswana, Kenya, Uganda, Guinea Bissau, Nigeria and Senegal (Peters & Dalrymple, 1990).

In Europe, epidemics were reported from Finland, Sweden and the U.S.S.R. from 1981 to 1984. It was noted that human infections with SIN virus usually occurred during the summer months, with a peak incidence rate in mid-August in the region between the 60° and 65° north latitudes extending

from Sweden (Ockelbo disease), through Finland (Pogosta disease) to the Karelian, U.S.S.R. (Karelian fever) (Espmark & Niklasson, 1984; Peters & Dalrymple, 1990; Lvov *et al.*, 1984).

Complete ecological studies on SIN virus have been conducted in South Africa since the virus was first recognised in 1954. At that time three isolations of SIN virus were made from *Culex* mosquitoes collected on the Transvaal Highveld (Weinbren *et al.*, 1956).

A few years later SIN virus was isolated from *Cx. neavei* and *Mansonia uniformis* at Ndumu, Natal, but it was only in 1963 that SIN virus was proved to cause an illness in man in South Africa. This illness was somewhat similar to that caused by WN virus (Malherbe *et al.*, 1963; McIntosh *et al.*, 1964).

Epidemics of SIN virus were reported in 1963, 1967, 1974 and 1984 in South Africa after exceptionally high seasonal summer rainfall. Field and laboratory investigations during these epidemics implicated simultaneous infections with WN virus (McIntosh *et al.*, 1964; Findlay & Whiting, 1968; McIntosh *et al.*, 1976; Jupp *et al.*, 1986).

2.2.2.2. VECTORS AND HOSTS

During the initial studies on SIN virus in Egypt the virus was isolated from *Cx. univittatus, Cx. antennatus* and *An. pharoensis*. The virus was also isolated from a hooded crow (*Corvus corone sardonius*), suggesting that birds acted as reservoir hosts (Taylor *et al.*, 1955).

Since then, SIN virus has frequently been isolated from ornithophilic mosquitoes (Table 2). Virus isolations and a high antibody prevalence in wild and domestic birds have confirmed that avian species are the principal vertebrate hosts (Niklasson, 1989).

Experimental data gained in South Africa shows that the majority of wild bird species tested are readily infected by SIN virus. The level of viraemia produced has the potential to infect mosquitoes (McIntosh *et al.*, 1969).

The isolation of SIN virus from bat organs in Zimbabwe (Blackburn *et al.*, 1982) and from a frog (*Rana ridibunda)* in western Slovakia (Kozuch *et al.*, 1978) implicates these species as further possible reservoirs. It is also of interest than a SIN virus strain was isolated from ticks *(Hyalomma marginatum)* in Sicily (Gresikova *et al.*, 1978).

TABLE 2. **SINDBIS VIRUS ISOLATES FROM DIFFERENT SPECIES AND REGIONS**

From mosquitoes	Country
Cx. pipiens/univittatus	Egypt
Cx. univittatus	Egypt
Cx. antennatus	Egypt
Anopheles pharoensis	Egypt
Cx. univittatus	South Africa
Cx. theileri	South Africa
Cx. pipiens	South Africa
Ma. africana	South Africa
Ma. fuscopennata	Uganda
Aedes spp.	U.S.S.R.
Culiseta spp.	Sweden
Cx. pipiens	Sweden
Cs. morsitans	Sweden
Cx. pipiens ('*molestus*')	Israel
Cx. univittatus	Israel
Cx. tritaeniorhynchus	Malaya
Cx. sinensis	Malaysia
Cx. gelidus	Malaysia
Cx. bitaeniorhynchus	Philippines
Cx. annulirostris	Australia
Ae. normanensis	Australia
Ae. vigilax	Australia
Cx. fatigans	Australia
Ma. septempunctata	Australia
Cx. pseudovishnui	Sarawak
Cx. tritaeniorhynchus	Sarawak
Cx. gelidus	Sarawak
Cx. tritaeniorhynchus	Taiwan
From birds	
Corvus corone sardonius	Egypt
Ploceus velatus	South Africa
Acrocephalus scirpaceous	Czechoslovakia
Nycticorax nycticorax	U.S.S.R.
Streptopelia turtur	Israel
Motacilla alba	India
Gracula religiosa	India

(Niklasson, 1989).

The vector for human infections is not well defined and may vary from region to region. Jupp (1973) reported on the feeding habits of mosquitoes on the highveld of South Africa, and found that *Cx. univittatus*, which is usually ornithophilic, might become anthropophilic at unusually high population densities and could thus infect man. *Cx. theileri* was found to be endophagic on man and might occasionally be responsible for infections.

Human infection with SIN virus is considered to be a dead end and humans are not thought to contribute to further virus transmission (McIntosh *et al.*, 1976).

Cx. univittatus has been implicated as the main enzootic and endemic vector, with the possibility of *Cx. theileri* as a link-vector, on the inland plateau of southern Africa (McIntosh *et al.*, 1967; Jupp, 1973).

2.2.3. DISEASE ASSOCIATIONS

2.2.3.1. ANIMALS

There is no evidence that SIN virus infection causes disease in animals. Several species of domestic and wild animals have been experimentally infected and, with the exception of young mice, induced infection is asymptomatic and transient (Taylor *et al.*, 1955).

Suckling white mice succumb to infection. Young adult rabbits, hamsters and monkeys resist intracerebral (i.c.) inoculation of SIN virus with no ill effects, and specific humoral neutralising antibodies can be demonstrated (Taylor *et al.*, 1955).

Antibody surveys conducted over a wide area in South Africa revealed that sheep and cattle had been infected with SIN virus, but no virus isolations were reported (Kokernot *et al.*, 1956; McIntosh *et al.*, 1962).

2.2.3.2. HUMANS

Relatively few human infections with SIN virus have been reported. The first recognised cases of SIN virus infection in man occurred in Uganda when the virus was isolated from acute phase blood (Niklasson, 1989; Clarence & Dalrymple, 1990).

Human cases have been reported from South Africa (Malherbe *et al.,* 1963; McIntosh *et al.*, 1964; Findlay & Whiting, 1968; McIntosh *et al.*, 1976; Jupp *et al.*, 1986), Zimbabwe (Maar, 1980), Central Africa (Clarence & Dalrymple, 1990), Australia (Doherty *et al.*, 1969; Guard *et al.*, 1982), Malaysia (Tesh *et al.*, 1975) and north-western Europe (Clarence & Dalrymple, 1990).

2.2.4. DIAGNOSIS

2.2.4.1. CLINICAL

The incubation period for SIN virus infection is usually less than one week (Niklasson, 1989). Disease begins typically with a rash and arthralgia, and most described patients have both. The rash may precede or follow joint manifestations by 1 or 2 days. Fever is usually not high and may even be absent (Espmark & Niklasson, 1984). Malaise, fatigue and headache are common occurrences. Anorexia, nausea, pharyngitis and paresthesias can occur in a minority of patients. Lymphadenopathy and conjunctivitis are not prominent (Malherbe *et al.*, 1963; McIntosh *et al.*, 1964; Maar, 1980).

The rash usually begins on the trunk as scattered macules and spreads to the palms and soles, but rarely to the head (Doherty *et al.*, 1969). Papules, with a tendency to vesiculate, can form on pressure points such as soles and palms. The colouration is typical: pink-red vesicles are surrounded by a white halo. The rash usually fades within a week leaving a brownish discolouration (McIntosh *et al.*, 1964). In some cases the rash can be haemorrhagic and recur in crops (Findlay & Whiting, 1968; Guard *et al.*, 1982).

Joint involvement is usually multiple. If the disease is acute, it most commonly affects the wrists and ankles, knees and elbows, and to a lesser extent the proximal articulations and axial skeleton (McIntosh *et al.*, 1964; Espmark & Niklasson, 1984). The persistence of rheumatic complaints has been a dominant feature and they can persist for 5 to 6 years (Niklasson *et al.*, 1988).

2.2.4.2. LABORATORY

SIN virus produces CPE in a wide variety of cell cultures, including continuous lines of Vero, HeLa and BHK-21 cells and primary chicken and Pekin duck embryo cells (Frothingham, 1955). SIN virus also grows in mosquito cell lines, although CPE is not evident (Stollar, 1987; Oelofsen *et al.*, 1990). However, CPE has been described in clones of *Ae. albopictus* cells (LT C-7) (Sarver & Stollar, 1977).

The isolation of SIN virus from arthropods or vertebrates is made by i.c. or i.p. inoculation into 3 to 5 day-old laboratory mice. Mice usually succumb within 2 to 5 days following a short period of paralysis (Taylor *et al.*, 1955).

Subsequently, cell cultures such as Vero cells have been shown to be sensitive for virus isolation and efficient for virus replication, and are usually used in preference to suckling mice (Niklasson, 1989).

SIN virus was isolated from acute phase blood from five patients in Uganda in 1961 and from a single case in Central Africa by inoculating suckling mice (Peters & Dalrymple, 1990). The virus has also been recovered from a skin lesion of a patient in South Africa but reisolation of the agent was not successful (Malherbe *et al.*, 1963).

Antibodies to SIN virus can be measured by HI (Doherty *et al.*, 1969), CF tests (McIntosh *et al.*, 1964), IF test, mixed haemadsorption and a neutralisation test (NT) (Skogh & Espmark, 1982; Espmark & Niklasson, 1984).

Antibodies initially appear during the first 7 to 10 days of illness, peak soon thereafter, and neutralisation or HI titers persist for years (Peters & Dalrymple, 1990).

IgM antibodies to SIN virus were demonstrated after serum specimens had been fractionated by sucrose density gradient ultracentrifugation (Guard *et al*., 1982). The IgM capture ELISA proved to be positive for acute phase sera (Calisher *et al.*, 1985; Niklasson *et al.*, 1988). The detection of IgM class antibodies to SIN virus may be of limited value as a diagnostic tool, as IgM antibodies were present after 3 to 4 years in almost a third of the patients tested in Sweden after a SIN infection (Niklasson *et al.*, 1988).

2.2.5. PREVENTION AND CONTROL

With increasing use of irrigation to satisfy world crop needs, habitats that attract birds and are suitable for mosquito breeding, will become increasingly common. This combination may well lead to an increase in human infections with SIN virus. Well organised and funded systematic mosquito abatement remains the most effective method of prevention of human cases of mosquito-borne virus disease, although emergency methods must be employed when outbreaks are imminent (Eldridge, 1987).

Vaccination is not available and the best option for prevention remains personal protection against mosquitoes.

2.3. WEST NILE VIRUS

2.3.1. CLASSIFICATION

WN virus is a member of the family *Flaviviridae* ('group B' arboviruses) and belongs to the genus *Flavivirus* (Murphy & Kingsbury, 1990).

2.3.2. EPIDEMIOLOGY

2.3.2.1. GEOGRAPHICAL DISTRIBUTION

The first isolation of WN virus was made from the blood of a woman who presented with fever in the West Nile province of Uganda in 1937 (Smithburn *et al.*, 1940).

No further isolations of WN virus were made until 1950, when WN virus was isolated from the blood of three apparently healthy children from the Sindbis sanitary district in Egypt, during a survey on poliomyelitis viruses (Taylor *et al.*, 1956).

Epidemics of WN fever were also recorded in Israel during the summer of 1950, when more than 500 patients were affected. Further epidemics occurred in areas south-east and to the north of Tel Aviv from 1951 to 1954 and in 1957 (Hayes, 1989).

An outbreak of WN fever was reported in the Rhone delta of the Camargue in France in 1962, with subsequent cases reported in 1963 and 1964 (Hayes, 1989).

The largest epidemic of WN virus yet reported occurred in South Africa in 1974. Following exceptionally heavy rains, this epidemic extended over an extensive area of the Karroo and northern Cape Province, involving thousands of humans. Field and laboratory investigations during this epidemic implicated simultaneous infections with SIN virus, although to a lesser extent (McIntosh *et al.*, 1976)

From mid-December 1983 until mid-April 1984 a smaller outbreak of WN fever took place in South Africa coincident with an epidemic of SIN virus infection in the Witwatersrand-Pretoria region (Jupp *et al.*, 1986).

WN virus has been isolated in at least 17 countries covering three different geographical regions, namely, the Palearctic, the Ethiopian and the Oriental. Serological surveys suggest that the geographic range of WN virus may be even more extensive (Hayes, 1989).

2.3.2.2. VECTORS AND HOSTS

Virus isolations and antibody surveys indicate that WN virus can infect a wide range of vertebrate species in nature. Virus has been isolated most commonly from man and wild birds (Taylor *et al.*, 1956; Kokernot & McIntosh, 1959; McIntosh *et al.*, 1969; Gaidamovich *et al.*, 1978). Domestic birds such as chickens, ducks and geese are also susceptible to the virus (Taylor *et al.*, 1956; Akov & Goldwasser, 1966).

WN virus antibodies have been detected in large domestic mammals such as horses, mules, donkeys, goats, water buffaloes, sheep, pigs, cows and camels (Taylor *et al.*, 1956; Akov & Goldwasser, 1966; Hayes *et al.*, 1982; Darwish *et al.*, 1983). Bovine species do not develop a viraemia after experimental inoculation, but antibodies are prevalent in cattle (Monath, 1990).

WN virus antibodies have been detected in the serum of several species of rodents and bats (Taylor *et al.*, 1956; Akov & Goldwasser, 1966; Darwish *et al.*, 1983). The susceptibility of laboratory mice to experimental WN virus infection, via different routes of inoculation, has been demonstrated. (Odelola & Oduye, 1977; Jupp *et al.*, 1986; George *et al.*, 1984).

Rabbits and guinea-pigs develop WN virus antibodies after experimental infection, but do not manifest any clinical symptoms of infection (Smithburn *et al.*, 1940). Rhesus and cynomolgus monkeys developed encephalitis after i.c. and intra-nasal inoculation with WN virus (Pogodina *et al.*, 1983).

Wild birds have consistently been implicated as the most important hosts in the transmission cycle of this virus. Migration of birds can be a mechanism for reintroducing the virus into temperate areas on an annual basis where the winters are too cold to support year-round transmission activity (Taylor *et al.*, 1956).

Man is frequently exposed to infection by WN virus. However, the low concentrations of virus found in peripheral blood following natural infections in humans, is probably below the threshold necessary to infect most mosquitoes (McIntosh *et al.*, 1976).

Mosquitoes and ticks are the only arthropods that WN virus has been isolated from in nature. Isolations of WN virus from various mosquito species implicate mosquitoes to be the most important vector of WN virus throughout its geographical range (Hayes, 1989).

The possibility that *Cx. univittatus* was the vector transmitting the virus to man in an endemic area of Egypt was strengthened by the isolation of WN virus from 23 febrile children during the same months that 9 isolations were obtained from *Cx. univittatus* (Taylor *et al.*, 1956).

The importance of *Cx. univittatus* as the epidemic vector of WN virus on the inland plateau of South Africa was shown by the very high infection rate found in these mosquitoes during the 1974 outbreak. *Cx. theileri* was the only other mosquito species found to be capable of transmitting virus, but to a minor extent (McIntosh *et al.*, 1976).

In south-western Asia, members of the *Cx. vishnui* complex have been implicated as vectors of WN virus, and in the Rhone Delta of France WN virus was isolated from *Cx. modestus*. *Cx. pipiens* has been implicated as a possible participant in the transmission cycle of WN virus in the USSR, Egypt and Israel. In Pakistan and India *Cx. fatigans* has been suspected as being a vector (Hayes, 1989).

2.3.3. DISEASE ASSOCIATIONS

2.3.3.1. ANIMALS

The isolation of WN virus from naturally infected domestic animals is a rare occurrence. Two isolated cases of naturally acquired WN encephalitis have been reported in horses (Hayes, 1989). However, low-level viraemia, antibody production and absence of clinical illness are the general rule.

WN virus was isolated from the blood, brain and spleen of a sick pigeon in Egypt (Taylor *et al.*, 1956). No other cases of disease in naturally infected wild animals have been reported.

2.3.3.2. HUMANS

Humans are certainly involved secondarily to avian infection with WN virus, and most human infections are largely 'blind alleys' in the virus transmission chain. Virus concentrations in human blood during acute illness are low and an insignificant number of mosquitoes would transmit virus after becoming infected from man (McIntosh *et al.*, 1976).

2.3.4. DIAGNOSIS

2.3.4.1. CLINICAL

Clinical disease caused by WN virus infection is usually mild. The incubation period of WN virus is approximately 3 to 6 days. Disease is characterised by an acute onset of fever ranging from 38 to 40 °C, lasting 5 to 6 days. Malaise, frontal headache, pain associated with eye movement and muscle pain are common symptoms. Gastro-intestinal disturbances, a sore or congested throat, enlarged lymph nodes and a maculopapular rash have been reported. Convalescence can be prolonged, lasting 1 to 2 weeks (Goldblum *et al.*, 1954; Weinbren, 1955; Marberg *et al.*, 1956; Taylor *et al.*, 1956).

Mild to severe meningoencephalitis characterised by impaired consciousness, including coma, has been reported in elderly as well as young patients (George *et al.*, 1984; Hayes, 1989).

Anterior myelitis, myocarditis and acute pancreatitis attributable to WN virus infection have also been reported from Israel (Perelman & Stern, 1974; Gadoth *et al.*, 1979).

Arthralgia has been described by McIntosh *et al.* (1964) in patients during an outbreak of SIN and WN virus infections in South Africa, but this does not appear to be a distinctive feature elsewhere.

2.3.4.2. LABORATORY

As with most arboviruses, the isolation of WN virus from arthropods or vertebrates is made in laboratory mice. Usually 1 to 4 day old mice are inoculated i.c. or i.p. with an infectious dose of WN virus. Death will occur within 2 to 5 days. Adult mice remain susceptible to infection, but the virus does not replicate to as high a titer as in infant mice (Taylor *et al.*, 1956).

Lethal infection in embryonated hens' eggs produced by WN virus was demonstrated by Taylor *et al.* (1956), but eggs are less susceptible than mice.

WN virus can infect and produce CPE in a variety of mammalian cell lines including Vero, BHK-21, chick embryo and pig kidney cells. The isolation of WN virus from the blood of a patient with encephalitis in phytohaemagglutinin-stimulated normal human mononuclear leucocytes, has also been reported (Hayes, 1989).

The mosquito cell lines *Ae. aegypti* and *Ae. malayensis* support WN virus replication and CPE has been reported in *Ae. albopictus* and *Ae. pseudoscutellaris* cell lines (Hayes, 1989; Oelofsen *et al.*, 1990).

The detection of WN virus in a naturally infected bird by an indirect haemagglutination test has been reported (Gaidamovich *et al.*, 1978). This technique provides a rapid way of detecting WN virus in a viraemic host compared to virus isolation, but the sensitivity is probably lower.

Virus can be isolated from the blood of patients during the first few days of illness. Studies conducted in Israel showed a high virus isolation rate on day 1 and day 2 of illness. No isolations were made on day 5 and day 6. Virus was also isolated 2 days prior to the onset of symptoms in two cases (Hayes, 1989).

As early as 1942, an antigenic relationship had been found between WN, Japanese encephalitis (JE) and St. Louis encephalitis (SLE) viruses (Smithburn, 1942). Besides JE and SLE, WN virus was shown to cross-react in a PRNT with Murray Valley encephalitis, Usutu, Kunjin, Kokobera, Stratford and Alfuy viruses (De Madrid & Porterfield, 1974).

Two distinct antigenic groups of WN virus, the African-Middle Eastern group and Indian strains, were demonstrated when WN virus isolates were tested in a HI test using different immune mouse sera (Price & O'Leary, 1967; Hayes, 1989). The antigenic differences between WN virus strains from different areas, as well as the existence of subtle antigenic variation within a specific strain, were demonstrated by means of monoclonal antibodies (Besselaar & Blackburn, 1988). These differences may not be revealed by serological methods employing polyclonal sera.

The CF and NT have been most commonly used for the serological diagnosis of WN virus infections in man. CF antibodies are first detectable during the second week of illness and are clearly demonstrable by the end of the second week. Peak titers are usually recorded 2 to 3 weeks after onset, and can stay at this level for 2 to 3 months. After 2 to 3 years, low CF titers can still be detected (Hayes, 1989).

Studies in Israel have also shown that neutralising antibodies in patients with WN virus infection developed slower than CF antibodies, but continued to persist at high levels after 2 to 3 years (Hayes, 1989).

The HI test has been used to diagnose WN virus infection in France, South Africa and Israel, but paired serum samples, demonstrating a fourfold rise in titer, are not always obtainable. In WN virus patients, previously exposed to another group B arbovirus by natural infection or vaccination, the CF, NT and HI tests are all broadly reactive, and heterologous antibody titers may be as high or higher than the WN antibody titers (McIntosh *et al.*, 1976; Feinstein *et al.*, 1985).

An indirect ELISA for the detection of IgG and IgM antibodies in WN virus-infected patients has been used successfully in Israel. Feinstein *et al.* (1985) found that IgM antibodies to WN virus were absent in the first serum sample taken 4 to 7 days after the onset of clinical signs, but could clearly be detected in more than 90 % of serum samples taken 23 to 28 days after the onset of illness. In the control group of randomly chosen blood bank sera, only antibodies of the IgG class to WN virus were detected.

The detection of IgM antibody to WN virus in the cerebrospinal fluid and serum of a patient with WN encephalitis on the fourth day after onset of illness, indicated that the IgM antibody capture ELISA method may be very useful in the early diagnosis of WN virus infection (Hayes, 1989).

2.3.5. PREVENTION AND CONTROL

No studies on the prevention and control of endemic or epidemic WN virus infections have been published. Epidemic activity in man will not be recognised unless the numbers of infected mosquito vectors are very high. The rapid reduction in the density of adult mosquitoes by using insecticides and avoidance of exposure to vectors, can control WN virus infections. In areas where irrigation is extensively practiced, environmental modifications to reduce mosquito breeding sites, should be effective (Hayes, 1989).

No vaccine is available and the development of a vaccine will probably remain a low priority, because the majority of cases are mild and epidemics have been infrequent.

2.4. WESSELSBRON VIRUS

2.4.1. CLASSIFICATION

WSL virus is a member of the family *Flaviviridae* ('group B' arboviruses) and belongs to the genus *Flavivirus* (Murphy & Kingsbury 1990).

2.4.2. EPIDEMIOLOGY

2.4.2.1. GEOGRAPHICAL DISTRIBUTION

The virus was first isolated from a lamb which died on the farm Magdalena in the Wesselsbron district of the Orange Free State during the late summer of 1954/55, following an illness thought to be RVF (Weiss *et al.*, 1956).

One month later, during a survey, WSL virus was isolated from the blood of a febrile field worker in the northern part of the Natal Province (Smithburn *et al.*, 1957).

WSL virus was also isolated from a lamb in the Kroonstad district, Orange Free State, during an outbreak of RVF in 1956 (Swanepoel, 1989).

During 1956 and 1957 an epidemic of an undiagnosed disease among sheep occurred in the Middelburg district of the Cape Province. Histological examinations made on specimens which were collected during the outbreak, suggested WSL virus involvement (Le Roux, 1959). Confirmed WSL virus infection in two field workers during that outbreak implied that infection was acquired either from mosquito bites or the handling of infectious post-post-mortem materials (Heymann *et al.*, 1958).

Although no further clinical WSL virus infections were described, the virus was isolated from cattle in 1974 and 1975 during a RVF epizootic in South Africa and Namibia (Coetzer *et al.*, 1978; Swanepoel, 1989). The virus was also isolated from the organs of a cow during a RVF epizootic in Zimbabwe in 1978 (Blackburn & Swanepoel, 1980).

To date WSL virus is known to occur in South Africa, Zimbabwe, Cameroon, Nigeria, Senegal, Ivory Coast, Central African Republic, Uganda, Kenya and Thailand (Swanepoel, 1989).

2.4.2.2. TRANSMISSION, VECTORS AND HOSTS

Wesselsbron fever is a mosquito-borne disease of sheep and, occasionally, human beings. WSL virus has been isolated from a variety of mosquito species and it seems likely that several species may act as vectors (Swanepoel, 1989).

During the WSL epizootic involving sheep in the Middelburg district of South Africa in 1957, virus was isolated from various *Aedes* species mosquitoes (Kokernot *et al.*, 1960), suggesting that these mosquitoes can be the main vectors of the virus for sheep.

Experimental transmission of WSL virus by *Culex* species has been demonstrated, but these species are not considered to be the main vectors on the plateau of southern Africa (McIntosh, 1980).

According to Swanepoel (1989), the isolation of WSL virus from *Rhipicephalus muhsamae* (ixodid tick) could be of epidemiological importance, but no infection and transmission studies were performed. Transstadial infection was demonstrated in *Ornithodorus savignyi* (argasid tick), but transmission tests were negative. This tick is confined to the northwestern part of the Cape Province, but it is unlikely to play a role in the transmission of WSL virus.

Transmission of WSL virus between vertebrate hosts, in the absence of arthropods, was demonstrated when transmission occurred between an inoculated and three uninoculated sheep (Coetzer *et al.*, 1978).

2.4.3. DISEASE ASSOCIATIONS

2.4.3.1. ANIMALS

During a routine survey in Nigeria, the virus was isolated from an asymptomatic camel. In newborn lambs, WSL fever is characterised by weakness, loss of appetite and often by mortality. Older lambs, goat kids and calves are less susceptible. Adult sheep, goats and cattle often develop a mild febrile infection and a mortality of 20 % has been reported in pregnant ewes. The outcome of infection in pregnant animals depends on the stage of pregnancy. Abortions were observed within a few days after exposure to the virus during late pregnancy (Swanepoel, 1989).

Coetzer & Barnard (1977) implicated WSL virus as one of the causal agents in an outbreak of *hydrops amnii* in ewes, following inoculation with attenuated RVF and/or WSL vaccines in South

Africa. Experimental work confirmed that wild and vaccine WSL viruses were responsible for the syndrome and severe foetal central nervous system abnormalities.

Macroscopic examination of lamb carcasses revealed mild to severe icterus with moderate hepatomegaly. Histopathological sections of the liver showed extensive necrosis of hepatocytes (Le Roux, 1959).

Apart from humans and several domestic livestock, the only other vertebrate from which WSL virus has been isolated is the Namaqua gerbil, *Desmodillus auricularis* (McIntosh, 1980).

2.4.3.2. HUMANS

The pathogenicity of WSL virus for humans was described by Weiss *et al.* (1956) when laboratory workers were infected with the virus during the course of experiments. Most of the laboratory workers became sick, but not critically ill, after an incubation period of 3 to 4 days. WSL virus was isolated from the blood of one individual during the febrile reaction. Serological evidence of WSL virus involvement was obtained from serum samples in the other workers.

The first naturally infected human from whom WSL virus was isolated was a member of the veterinary field staff who was infected and became ill 7 days after assuming his duties at Lake Simbu in Tongaland, northern Natal (Smithburn *et al.,* 1957).

A further two infections in man were described by Heymann *et al.* (1958) during field investigations of an outbreak of illness among sheep in the Middelburg district of the Cape Province. Both men had been frequently bitten by mosquitoes and one had performed post-mortems on infected animals. The second individual did not handle any living or dead infected animals.

From 1955 to 1983 a total of 23 isolations of WSL virus from humans were made. A further five human infections were diagnosed serologically. Most of these infections occurred in South Africa and laboratory infections were reported from Senegal, Nigeria, the Central African Republic, Uganda and the U.S. (Swanepoel, 1989).

2.4.4. DIAGNOSIS

2.4.4.1. CLINICAL

Incubation periods in human infections of WSL virus range from 2 to 7 days. The onset of disease is marked by acute fever, rigors, severe headache, tachycardia, anorexia, myalgia and arthralgia.

During the fever, which can last up to one week, patients often complain of hyperesthesia of the skin and an evanescent maculopapular rash can appear. Hepatomegaly or hepatosplenomegaly occurs in some patients and occasionally the serum alanine and transaminase levels are raised. In severely ill patients encephalitis, photophobia, blurred vision and mental impairment can occur. Fatal cases have not been reported (McIntosh & Gear, 1981; Swanepoel, 1989).

Most patients recovered from the acute illness in 1 to 3 days, but muscle pains can persist for several weeks (Swanepoel, 1989).

Seroconversion without any clinical evidence of illness was reported in a laboratory worker (Weiss *et al.,* 1956).

2.4.4.2. LABORATORY

The susceptibility of white mice for WSL virus was demonstrated by Weiss *et al.* (1956), during the isolation of a then unknown virus from a dead lamb.

Mice of all ages have been found to be susceptible to i.c. injection of WSL virus. The incubation period varies from 6 days in day-old mice to longer periods in adult mice. When virus is administered by the i.p. route, mice under the age of 10 days all succumb. Mice older than 3 weeks show no reaction. Mild febrile reactions were observed in guinea-pigs, rabbits, cattle, horses and pigs. Serum collected from these animals, in the convalescent phase, had high titers of specific neutralising antibodies to WSL virus (Weiss *et al.,* 1956).

Intracerebral inoculation of newborn mice probably remains the best method for the primary isolation of WSL virus, but since laboratory tests in mice are becoming increasingly expensive, cell cultures, used together with IF, could prove to be satisfactory for rapid isolation and identification of WSL virus.

Way *et al.* (1976) demonstrated the growth of WSL virus in several cell culture systems and no significant differences were found between NT done in mice and cell culture. Serodiagnosis of WSL virus infections has been based on the standard arthropod-borne virus techniques of HI, CF and NT (Shope & Sather, 1979).

Homologous antibodies were demonstrated by HI and neutralisation techniques on day 4 after experimental infection of WSL virus in sheep and cattle. Maximum titers were obtained from days 7 to 21. HI titers declined to undetectable levels in 25 months, while NT titers remained detectable in cell cultures. CF antibodies were demonstrated on day 10 and became undetectable by week 12 (Blackburn & Swanepoel, 1980).

Cross-reactivity of WSL virus with other flaviviruses occurs and is notably higher in HI than in CF and NT. Antibody responses, following a primary WSL infection, could be distinguished from cross-reacting flaviviruses by performing quantitative HI, CF or NT with a range of flaviviruses known to occur in the region concerned (Blackburn & Swanepoel, 1980).

The CF test proved to be specific in monitoring antibody levels in experimental or laboratory infections, but sera handled and forwarded to the laboratory under field conditions tended to have very low titers, making this test unsuitable for use (Blackburn & Swanepoel, 1980; Swanepoel, 1989).

The detection of WSL antibodies in sera of cows and ewes is not significant in an area where the virus is known to be prevalent. Since the investigation of teratogenic infection takes place retrospectively months after the infection, there is little prospect of making a diagnosis of WSL virus infection by demonstrating IgM antibody or rising antibody titers in paired serum samples of cattle and sheep (Coetzer & Barnard, 1977).

However, the development and use of an ELISA would facilitate automation of tests and the demonstration of specific IgM antibodies against WSL virus in human sera can be a diagnostic tool for the identification of a WSL virus infection in man.

2.4.5. PREVENTION AND CONTROL

An inactivated virus vaccine prepared in mouse brain was originally available for vaccination of livestock. Presently a freeze-dried veterinary vaccine, prepared from a strain of WSL virus (Weiss *et al.*, 1956) which has undergone 110 passages in primary lamb kidney cell cultures and 3 passages in BHK-21 cells is available (Swanepoel, 1989). This vaccine is recommended for use in non-pregnant sheep and cattle in winter or early spring, before mosquitoes become active. Immunity is considered to be durable and revaccination is not recommended.

General recommendations made to farmers concerning arthropod-borne infections of livestock include drainage or treatment of mosquito-breeding sites, housing of sheep at night and moving stock to high, wind-swept grazing areas during outbreaks of disease.

No human epidemics of WSL virus have been identified and it appears that human infections are sporadic. Human exposures from handling infected animals may account for many infections in the sheep-rearing areas of South Africa (McIntosh & Gear, 1981).

There is no human vaccine available and protective clothing should be worn by persons handling suspected infected organs of domestic animals.

2.5. DOT-ELISA

The dot-ELISA is a modification of the ELISA, which was described by Engvall and Perlmann (1972). This technique is based on two important biological phenomena: (i) the extraordinary discriminatory power of antibodies, based on the ability of the immune system to produce a virtually unlimited variety of proteins (antibodies), each with an affinity for a specific foreign compound (antigen or hapten); and (ii) the extremely high catalytic power and specificity of enzymes, which may quite often be detectable with great ease (Tijssen, 1985).

2.5.1. HISTORY

A method for transporting deoxyribonucleic acid (DNA) fragments from agarose gels to NC by capillary forces, was described by Southern (1975). This method has become known as 'Southern blotting'. An improvement to the Southern blot was published by Alwine *et al.* (1977). Ribonucleic acid (RNA) and small DNA fragments, which do not bind sufficiently to NC, were covalently coupled to diazobenzyloxymethyl paper by means of capillary blotting. This technique was adapted to proteins by Renart *et al.* (1979) by using polyacrylamide/agarose gels with special crosslinkers which could be cleaved easily, to facilitate the transfer. Towbin *et al.* (1979) transferred proteins electrophoretically from polyacrylamide gels on to NC membranes, which were probed with two layers of antibody, the second layer being labelled with ^{125}I, fluorescein or horseradish peroxidase.

Since these reports, many adaptations of immunoblotting techniques have been developed and applied in an enormous variety of studies.

The distinctive feature of immunoblotting is the ability to retain the original components of the proteins on a solid phase during the course of immunoassay procedures where the resolving power of electrophoresis is not needed (Towbin & Gordon, 1984). Thus, antigens can be applied in the format of dots or spots (Glenney *et al.,* 1982; Hawkes *et al.,* 1982; Herbrink *et al.,* 1982; Huet *et al.,* 1982; Bode *et al.,* 1984; Heberling & Kalter, 1986; Kalter & Heberling, 1988). This permits the use of any desired geometry, and has the further advantages of permitting multiple simultaneous assays, high sensitivity and simple operation (Hawkes *et al.,* 1982; Gouvea *et al.,* 1987; Leonardi *et al.,* 1990).

Several techniques have been developed to visualise protein binding onto different membranes.

The direct nonspecific protein staining is a simple method for the detection of all proteins electrophoresed in a gel and transferred to a membrane. Stains such as Coomassie Brilliant Blue,

Amido Black, Fast Green, etc. are used (Beisiegel, 1986).

The direct specific label staining identifies only selected proteins on the membrane. This specific label or antibody is labelled (radioactive, fluorescent or by an enzyme mediated colour reaction), and is therefore directly visible on the membrane (Beisiegel, 1986).

The specific detection of particular proteins on the membrane is most frequently achieved by an indirect immunolabel. This procedure uses an antibody as the first (direct) probe. This first marker binds directly to the protein/antigen on the matrix and is detected by the binding of a second (indirect) labelled antibody (Stott, 1989).

Alternatively to the use of antibodies as second probes, protein A, which specifically binds to the Fc part of many animal immunoglobulins, is often used (Heberling & Kalter, 1986).

Several detection mechanisms (radioisotopes, fluorochrome-labelled antibodies, gold-labelled antibodies, biotin/streptavidin and enzyme-conjugated probes) are used. Advantages of these mechanisms are the high sensitivity and easy quantification of antigen. Some disadvantages can be the exposure to radiation, rapid fading of results and uncontrolled background staining (Beisiegel, 1986; Stott, 1989).

2.5.2. MEMBRANES

A variety of membranes are available for immunoblotting, but NC is still the most widely used.

The use of diazobenzyloxymethyl paper (Alwine *et al.,* 1979), has the disadvantage of special pretreatment of the membrane, before each use, by converting aminobenzyloxymethyl paper to diazobenzyloxymethyl paper by diazotisation. This pretreatment of the matrix may result in significant non-reproducibility.

Nylon-based membranes, originally designed and manufactured for liquid and gas filtration, have been found to be advantageous in many respects, as they are as thin and as smooth surfaced as NC but much more durable. They also have a much higher protein binding capacity (up to 500 $\mu g/cm^2$), although this is highly concentration dependent (Gershoni & Palade, 1982). Proteins are not easily washed off as they bind with very high affinity to these membranes. A problem encountered is that non-specific proteins tend to bind to the membrane, resulting in very high backgrounds, even after prolonged blocking (Gershoni & Palade, 1983). Most of the commonly used protein stains (Coomassie Blue, Amido Black, Ponceau S, etc.) bind to nylon membranes, making the interpretation of results difficult (Stott, 1989).

NC filters were originally designed as 'surface filters' for microfiltration. In the blotting technique they serve as 'depth filters' in which the macromolecules concerned are absorbed throughout the filter matrix (Gershoni & Palade, 1983). The physico-chemical basis of binding of proteins to NC is believed to be largely due to hydrophobic interactions. Observations that non-ionic detergents such as Triton X-100 or Nonidet P-40 can cause up to 90% detachment of bound protein from the membrane, have given rise to this hypothesis (Gershoni & Palade, 1982; Lin & Kasamatsu, 1983). This should be taken into account if it is found to be necessary to use washing buffers containing detergents in order to reduce non-specific binding.

NC with a pore size of 0.45 μm is used for most purposes, but low molecular weight polypeptides can pass through without binding. Membranes with a pore size of 0.1 μm are recommended, since the higher matrix density results in more efficient trapping of low molecular weight proteins (Lin & Kasamatsu, 1983). Membranes containing mixtures of NC and cellulose acetate have been found to have a low binding capacity for proteins and it is preferable to use NC in the pure form (Towbin *et al.,* 1979).

2.5.3. BLOCKING

It is essential to block the protein binding sites on the membrane to prevent non-specific binding of the probe and to eliminate background staining. The blocking agents and conditions used in the dot-ELISA will depend on the type of membrane and sensitivity of the system under investigation (Stott, 1989).

Inactivation and blocking with tris (hydroxymethyl) aminomethane, ethanolamine, and gelatine have proven to be effective in reducing or eliminating non-specific reactions (Renart *et al.,* 1979; Herbrink *et al.,* 1982). Gelatine in phosphate buffered saline (PBS) was found to be an adequate blocking solution for NC membranes (Holmberg *et al.,* 1983; Zerbini *et al.,* 1987), but Towbin & Gordon (1984) reported that gelatine alone is not a satisfactory blocking agent.

Other blocking agents used include bovine serum albumin (BSA) (Towbin *et al.,* 1979; Gershoni & Palade, 1982; Bode *et al.,* 1984), animal serum (Hawkes *et al.,* 1982), and haemoglobin (Gershoni & Palade, 1982). Powdered nonfat milk gives good, low backgrounds (Heberling & Kalter, 1986; Gouvea *et al.,* 1987; Kalter & Heberling, 1988; Oprandy *et al.,* 1988). Milk is cheap and readily available but it may inhibit specific antibody binding to some antigens (Stott, 1989).

Non-ionic detergents have been used as blocking agents and have the advantage of being both cheap and readily available. Unfortunately they have the severe disadvantage of causing loss of

protein by detachment from the membrane (Lin & Kasamatsu, 1983). Tween 20 is claimed to give good results with some proteins (Batteiger *et al.,* 1982; Mohammad & Esen, 1989), but Hoffman & Jump (1986) found that some immunoglobulins and several other proteins were dissociated from NC by 0.05 % Tween 20 solution.

2.5.4. WASHING

Washing is an important step in the dot-ELISA. The membrane has to be washed thoroughly to remove any non-specifically bound proteins after incubation. The washing buffers may contain various detergents, in addition to BSA or other non-specific blocking proteins, to dissociate unwanted proteins from the membrane. However, detergents have to be applied carefully because of the possible loss of antigen from the membrane (Hoffman & Jump, 1986). The washing should be done with several changes of washing buffer for a sufficient length of time (Hawkes *et al.,* 1982).

2.5.5. ANTIGEN

Kafatos *et al.* (1979) used a dot-blot procedure in which the probe or antigen is applied directly to the NC as a dot, instead of being blotted from a gel.

The visibility of a dot, when using an enzyme-labelled indicator antibody, depends on the contrast of the colour generated against the background and is thus dependent on the density of antigen in a dot. It is more economical to apply antigens/antibodies in small volumes (0.5 to 1 μl) in concentrated form (Towbin & Gordon, 1984; Heberling & Kalter, 1986). Dry NC easily soaks up volumes of the above size range, and binds the material in a tight dot. Larger volumes can be applied under vacuum by means of multiple filtration manifolds or other apparatus (Zalis & Jaffe, 1987). If the antigen is very dilute, successive doses can be applied at the same site, taking care that the filter is allowed to dry before each application (Hawkes *et al.,* 1982).

Crude antigens have been used successfully in the dot-ELISA. Hawkes *et al.* (1982) described the screening of hybridoma clones by using media, as antigen, directly from culture wells. The quantitative determination of anti-adenovirus antibodies were demonstrated by Bode *et al.* (1984) by the application of crude adenovirus, propagated in monolayers of Vero cells, to a NC membrane in the dot-ELISA.

Commercially available antigens, normally used in the HI and CF test for different viruses, have successfully been applied as antigen source to NC in the dot-ELISA (Hawkes *et al.,* 1982; Heberling & Kalter, 1986).

2.5.6. ANTIBODIES

Immunology is recognised as the fundamental reaction evoked by an antigen, usually a protein, to form an antibody to react specifically with the antigen (Eisen, 1980).

Antibodies or immunoglobulins are multichain proteins with an equal number of heavy and light chains and have a dual function: (i) recognition of the antigen; and (ii) the activation, after antigen recognition, of a variety of biological functions to protect the host. The activation of these biological pathways depends on the different heavy chains, and the classification of immunoglobulins depends on the structure of these heavy chains (Tijssen, 1985).

Human immunoglobulins are classified into five classes of antibodies: IgG, IgM, IgA, IgD and IgE.

Immunoglobulin A occurs in a relative low concentration in the blood, and may represent a first line of defence against bacteria and viruses. Secretory IgA can act as a second line of defence against antigen invading the respiratory tract (Cooper, 1982).

Immunoglobulin D is present on the surface of some lymphocytes where it seems to operate as mutually interacting antigen receptors for the control of lymphocyte activation and suppression (Cooper, 1982).

Very low concentrations of immunoglobulin E are present in serum and a small proportion of the plasma cells in the body synthesise IgE. The main role of IgE would appear to be protection of external surfaces of the body by triggering an acute inflammatory reaction (Cooper, 1982).

Immunoglobulin G is the most common form of antibody in the body and can pass from mother to fetus before birth. In most individuals, it is present in high concentrations in the blood after exposure to antigen. The demonstration of specific IgG rarely gives any early diagnostic information. The presence of specific IgG may, however, be used for the rapid assessment of the immune status of a person, which may be valuable if it leads to adequate prophylaxis or epidemiological measures (Gardner & McQuillin, 1980).

Immunoglobulin M appears early after exposure to an antigen, but does not cross the placenta to an unborn child. IgM is a pentameric molecule, essentially intravascular, produced early in the immune response. It is an effective bacterial agglutinator and mediator of complement-dependent cytolysis and is therefore a powerful first-line defence against infections (Roitt, 1988). Specific IgM is only occasionally present on the first day of illness but the demonstration of its presence has a value for the early diagnosis of an acute virus infection (Gardner & McQuillin, 1980).

Rheumatoid factors (RF) can interfere in certain immunological test systems for antibody or antigen detection. These anti-IgG antibodies react preferably with modified, aggregated IgG or with antigen. This phenomenon leads to a false positive reaction, particularly in the indirect test method for detection of pathogen-specific IgM, where antigen is bound to a solid phase with the employment of μ-chain specific labelled antibodies (Tijssen, 1985).

If a serum sample contains specific IgG-antibodies which bind to the solid phase antigen, IgM-RF can also bind to the IgG. This results in the reaction of the labelled indicator antibody with IgM-RF, since it cannot distinguish between IgM-RF and antigen specific IgM (Figure 1).

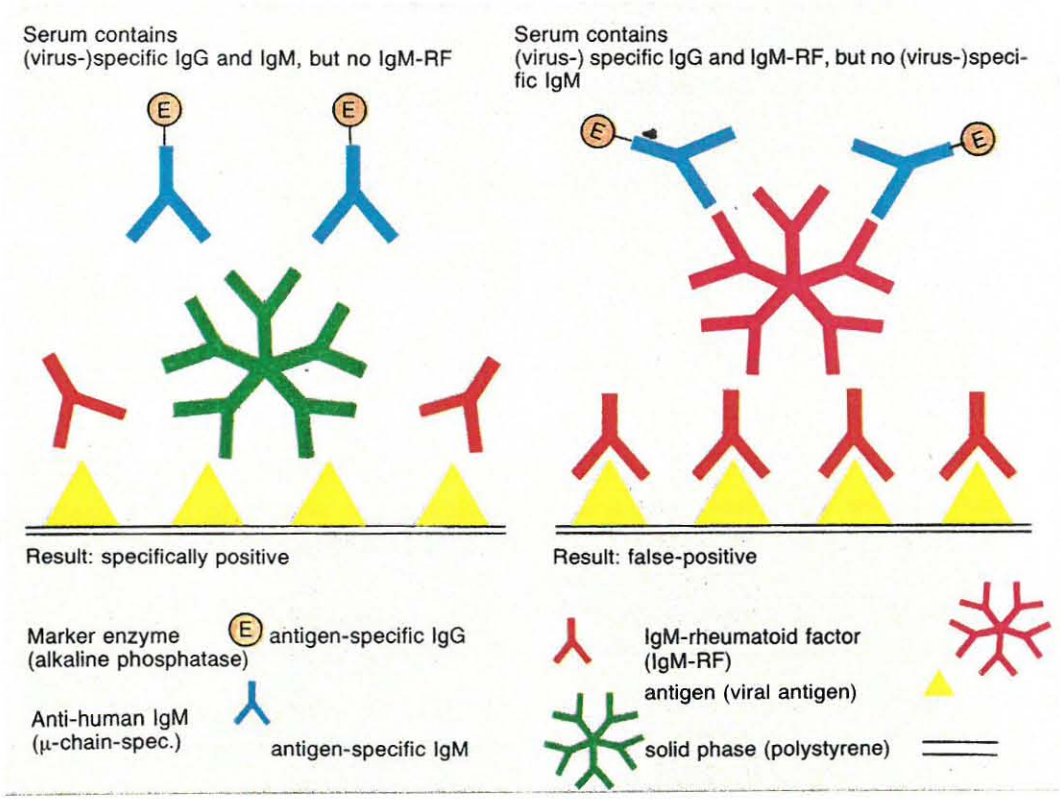


FIGURE 1. ELISA FOR VIRUS-SPECIFIC IgM (Ziegelmaier *et al.*,1985)

In order to prevent this undesirable reaction caused by IgM-RF, several methods for pretreatment of the serum sample with their disadvantages are described in table 3 (Ziegelmaier *et al.*, 1985).

Preparations of anti-human IgG (γ-chain) antiserum obtained from sheep by immunisation with immunosorbent-purified human IgG globulin are commercially available. The following are several advantages of using these preparations for specific IgM-diagnostics in the indirect dot-ELISA: the interference of RF is eliminated, independently of its concentration, specific IgG-antibodies are neutralised and a competition with specific IgM for antigen is rendered impossible and the

procedure is usually short and simple (Ziegelmaier *et al.*, 1985).

TABLE 3. **COMPARISON OF VARIOUS METHODS FOR AVOIDANCE OF RF INTERFERENCE**

Method	Mode of action	Disadvantages
Aggregated γ-globulin of human or animal origin	Binds RF	Specificity of antibodies used may not be directed against the test antigen. Limited capacity; human γ-globulin better than animal.
IgG-coated polystyrene particles	Binds RF	Limited capacity
Staphylococcal A-protein	Binds IgG	IgG is not bound (serum proportion of the total IgG =8%) Also binds IgM.
Anti-human IgG (γ-chain)	Binds IgG	Unknown

(Ziegelmaier *et al.* 1985)

2.5.7. ENZYME-ANTIBODY CONJUGATION

Several methods for the enzyme labeling of antisera have been described, but commercially prepared conjugates are available. These conjugated enzymes are available in pure form, conjugated to an antibody without loss of activity, and have a high specific activity. Horseradish peroxidase (HRP) or alkaline phosphatase (AP) conjugated antibodies are most widely used in ELISA and dot-ELISA (Lennette & Schmidt, 1979; Stott, 1989).

Peroxidases often occur as multiple isozymes and are widely distributed in plants. HRP is more sensitive to contaminants than AP. Water deionised with polystyrene resins is toxic to HRP and this enzyme can be inactivated by polystyrene plates, used as the solid-phase in ELISA, if Tween 20 is omitted. This enzyme is also very sensitive to the presence of bacteria or bacteriostatic agents and is inactivated by oxygen, hypochlorous acid and aromatic chlorocarbons often found in laboratory water (Tijssen, 1985).

The activity of HRP is also inhibited by azides and therefore sodium azide should not be included in wash buffers (Stott, 1989).

Alkaline phosphatases are found primarily in animal tissues and micro-organisms. These enzymes are produced from bovine intestinal mucosa and from *E. coli*. They have considerable differences in their properties (the bacterial enzyme has a lower activity than the bovine intestinal enzyme), and should not be comparatively assayed under similar conditions (Tijssen, 1985).

2.5.8. SUBSTRATES

Different substrates can be used for detection of enzyme-conjugated antibodies in the dot-ELISA (Table 4). HRP substrates become oxidised, usually to an insoluble polymer, when the peroxidase catalyses the release of oxygen from hydrogen peroxide. Most of these substrates are potentially carcinogenic and can give rise to a high background in the ELISA (Stott, 1989).

Alkaline phosphatases hydrolyse numerous phosphate esters, such as those of primary and secondary alcohols, phenols and amines. Substrates used with AP are more soluble than HRP substrates, therefore, an increase in sensitivity is obtained with AP (Young, 1989).

TABLE 4. **SUBSTRATES USED FOR DETECTION OF ENZYME-CONJUGATED ANTIBODIES**

Enzyme	Substrate	Color	Reference
Horseradish peroxidase	4-chloro-1-naphthol/H_2O_2	Blue	Hawkes *et al.* (1982) Dresel & Schettler (1984) Faye & Chrispeels (1985)
	Diaminobenzidine/H_2O_2	Brown/ Purple	de Blas & Cherwinski (1983) Young (1989)
	Aminoethylcarbazole/H_2O_2	Blue	Clegg (1982)
	o-dianisidine/H_2O_2	Orange/ Yeliow	Towbin *et al.* (1979)
Alkaline phosphatase	Nitro blue tetrazolium/ 5-bromo-4-chloro-idodyl phosphate	Blue	Leary *et al.* (1983) Blake *et al.* (1984)
	Naphthol AS-MX phosphate/fast red	Red	O'Conner & Ashman (1982)
	ß-naphthyl phosphate/fast blue B	Purple/ Brown	Turner (1983)

Different colour reactions can be obtained from different substrates, but in the dot-ELISA a definite insoluble spot can be observed at the site of the antigen/antibody complex (Table 4). The intensity of colour is directly related to the amount of conjugate fixed to the antigen-antibody complex (Heberling & Kalter, 1986; Kalter & Heberling, 1988).

2.6. IMMUNOFLUORESCENT STAINING

The IF technique was introduced by Coons *et al.* (1941), who employed the labile isocyanate derivative of fluorescein for conjugation of fluorescein to antibodies or antigens. The conjugation procedure was modified by Riggs *et al.* (1958), who replaced the labile isocyanate radical with stable isothiocyanate. Since then, numerous references for IF methods, most applying this modification to diagnostic virology, have appeared in the literature (Riggs, 1979).

The IF technique provides a means of observing an antigen-antibody reaction by chemically linking a fluorescent dye, such as fluorescein isothiocyanate (FITC), to specific antibody molecules. Such labeled antibodies retain the ability to react specifically with respective antigens, and the reaction site can be detected with a fluorescence microscope (Emmons & Riggs, 1977). Fluorescence microscopy is based on the principle that light of a short wavelength such as ultraviolet light will excite certain dyes to emit visible light of a longer wavelength which can be observed when the correct type of filter is placed between the objective and eyepiece of the microscope (Gardner & McQuillin, 1980).

There are three basic staining methods used for the fluorescent antibody technique:

Direct technique

In the direct technique, a fluorochrome labelled antibody is used to detect a selected agent (antigen) in a specimen or tissue culture.

Complement staining method

The complement staining method was devised to enhance the fluorescence of the antigen-antibody complex by the addition of complement to the system.

Indirect technique

The indirect staining technique can be used for detecting and identifying viral antigens as well as the serologic response of an individual to a specific viral antigen. Fluorochrome labelled antisera against the different classes of human immunoglobulins are commercially available but the specificity of such preparations should be thoroughly checked in the test procedure (Riggs, 1979;

Gardner & McQuillin, 1980). The indirect technique is more economical in that a single antiglobulin only needs to be labelled.

CHAPTER 3

MATERIALS AND METHODS

3.1. CELL CULTURE TECHNIQUES

3.1.1. MATERIALS

3.1.1.1. CELLS

Vero and BHK-21 cell lines were obtained from the National Institute for Virology, Sandringham. The passage levels were unknown.

3.1.1.2. MEDIA

Growth Medium (pH 7-7.2)

400 ml sterile Eagles minimum essential medium (MEM) with Earle's salts and L-Glutamine, without $NaHCO_3$ (Gibco, cat.no. 072-01100P)
40 ml Foetal calf serum (FCS) (Gibco, cat.no. 013-06290H)
6 ml of a 5 % $NaHCO_3$ solution in H_2O (Merck Chemicals, cat.no. 6329)
2 ml Penicillin, Streptomycin and Neomycin (PSN) stock solution (final concentration of 100 units, 100 and 50 μg/ml respectively).

The medium was again sterilised by filtration through a 0.22 μm membrane filter (Millipore, cat.no. SLGS 0250S) and was stored at 4 °C.

Maintenance Medium (pH 7.2-7.4)

400 ml MEM (as above)
8 ml FCS
8 ml of a 5 % $NaHCO_3$ solution
2 ml PSN stock solution
Sterilised by filtration and stored at 4 °C.

Phosphate Buffered Saline (pH 7.5)

9.7 g Dulbecco's PBS (Gibco, cat.no. 076-01300P)
1000 ml distilled water
Sterilised by filtration and stored at 4 °C.

Trypsin-EDTA

One vial of lyophilised trypsin-EDTA (Ethylenediaminetetra-acetic acid) (Gibco, cat.no. 061-05405D) was dissolved in 200 ml sterile, distilled water and sterilised by positive pressure filtration through a 0.22 μm membrane. The trypsin-EDTA was dispensed in 20 ml volumes (final concentration of 0.5 g Trypsin, 0.2 g EDTA and 0.85 g NaCl/l) into sterile McCartney bottles. The aliquots were stored at -20 °C. Each aliquot was thawed and opened once only.

3.1.1.3. ANTIGENS

The following freeze-dried stock viruses were obtained from the National Institute for Virology, Sandringham:

Strain AN 1830 of RVF virus (passage 5, of a 5 % mouse brain suspension in lactose-phosphate buffer (LPB)).

Strain H 442 of WN virus (passage 4, of a 5 % mouse brain suspension in LPB).

SIN virus (passage 7, of a 5 % mouse brain suspension in LPB).

WSL virus (passage 13, of a 5 % mouse brain suspension in LPB).

3.1.2. METHODS

3.1.2.1. TRYPSINISATION AND CULTIVATION OF MONOLAYER CELL CULTURES

The medium from a confluent monolayer of Vero and BHK-21 cells, in 83 cm^2 cell culture flasks (Nunc, cat.no. 15372), was removed aseptically. The monolayer of each cell line was then washed with PBS to remove any traces of medium, and 10 ml trypsin-EDTA solution, preheated to 37 °C, was added to the monolayer and left in contact with the cells for 15 to 30 seconds, after which the solution was removed. The culture was held at 37 °C until microscopic examination showed that the cells had separated. The cells were then suspended in 10 ml of growth medium and triturated until a homogeneous suspension was obtained. Cell counts were performed by counting the cells in a haemocytometer.

Cells obtained from trypsinisation were diluted in growth medium to a concentration of 2×10^5 cells/ml (Vero) and 5×10^4 cells/ml (BHK-21) respectively. Tissue culture flasks were seeded with 30 ml of cell suspension and incubated at 37 °C for 2 days after which the growth medium was changed. Confluent monolayers were obtained 3 days after initiation and the growth medium was replaced with maintenance medium. Cell cultures were maintained by a weekly schedule of subcultivation.

Cell culture tubes were prepared by seeding tissue culture tubes (Nunc, cat.no. 156758) with 1 ml of appropriate cell concentrations. Freshly set cultures were sloped and incubated at 37 °C. When the monolayers were confluent the growth medium was replaced by 2 ml of maintenance medium, which was removed and replaced by FCS free maintenance medium just before the tissue culture tubes were infected with virus.

3.1.2.2. ADAPTATION OF VIRUSES TO CELL CULTURES

Freeze-dried stock viruses (RVF, SIN, WN and WSL) were reconstituted with 1 ml of sterile water respectively. Each virus preparation was then further diluted with maintenance medium (containing no FCS) to a concentration of 1:10. A volume of 100 µl of each virus suspension was inoculated into each of 3 Vero cell culture tubes. The infected cell cultures were incubated at 37 °C on a roller-drum apparatus. Control cell culture tubes, containing no virus, were treated in the same manner. Each tube was monitored on a daily basis for CPE. After a CPE was observed, each virus was subcultured into a fresh set of cell culture tubes. After 3 passages, the virus suspensions were stored at -70 °C until further use.

3.1.2.3. TITRATION OF VIRUS IN MONOLAYER CELL CULTURE TUBES; $CCID_{50}$ ENDPOINTS

The amount of infectious virus present in cell culture fluids can be titrated by determining the highest dilution of the fluid which produces a CPE in 50 % of the cell cultures inoculated, resulting in the 50 % cell culture infectious dose endpoint ($CCID_{50}$) (Lennette & Schmidt, 1979).

To determine the $CCID_{50}$ of RVF, SIN, WN and WSL viruses, logarithmic dilutions of the viruses were prepared in FCS free maintenance medium. Pipettes were discarded between each dilution to avoid carrying virus particles on the pipette to the next dilution. Each virus dilution was added in a volume of 100 µl/tube to 4 cell culture tubes with a monolayer of cells. Vero cell cultures were used for RVF, SIN and WN viruses and BHK-21 cell cultures were used for WSL virus because the initial titration of WSL virus in Vero cells yielded a very low titer. Control cultures (inoculated with 100 µl serum free maintenance medium/tube) were included in each titration batch of cell culture tubes. The inoculated cell cultures were incubated at 37 °C and observed microscopically at daily intervals over a 7-day period. A specific viral CPE was considered positive, and the $CCID_{50}$ end point was calculated by the method of Reed and Muench (1938).

3.1.2.4. PREPARATION OF ANTIGEN IN CELL CULTURES

Confluent Vero and BHK-21 cell cultures in tissue culture flasks were used for virus propagation. The growth medium was removed from a representative culture of Vero and BHK-21 cells. The cells were dispersed with trypsin-EDTA and counted in a haemocytometer. Growth medium was removed from the remaining cultures, and they were infected with adapted seed virus at an

infectious dose of 10 viruses per cell. The total volume of fluid in each culture was brought up to 6 ml with maintenance medium without FCS if the volume of seed virus was less than 6 ml. Cultures were incubated at 37 °C for 60 min. Inocula were removed from each culture and a volume of 30 ml of serum free maintenance medium was added to each flask. Cultures were incubated until a total viral CPE was observed. The cells were then dislodged into the culture fluid by using a cell scraper (Nunc, cat.no. 179707). Control antigen (cell cultures without virus inoculation) was collected at the same time. Different cultures of the same virus were pooled and after several freeze-thawing cycles the antigen suspensions were clarified by centrifugation (7000 g for 30 min at 4 °C). The resulting supernatant fluids were then stored at -70 °C until further use.

3.2. DOT-ELISA

3.2.1. MATERIALS

3.2.1.1. NITROCELLULOSE

Fully supported, 100 % NC sheets (Hybond C-Super, Amersham, cat.no. RPN 1210G) with a pore size of 0.45 μm were used as the solid phase.

3.2.1.2. ANTIGENS

Supernatant fluids obtained from the cell cultures (3.1.2.4), and containing RVF, SIN, WN and WSL viruses were used.

3.2.1.3. ANTISERA

Human serum samples, which tested positive for HI antibodies (National Institute for Virology, Sandringham) for RVF (titer; 80 and 160), SIN (titer; 1280), WN (titer; 1024) and WSL (titer; 640), were used for the standardisation of the dot-ELISA for detecting IgG antibodies. These serum samples were also used as positive controls.

Human serum samples, known to be positive for the IgM class antibodies against RVF, SIN, WN and WSL viruses respectively, were kindly supplied by Dr. N.K. Blackburn (Arbovirus Unit, National Institute for Virology, Sandringham). These samples were used for the standardisation of the IgM dot-ELISA, and served as positive control sera in the test procedure .

Patients' sera, accumulated over several years, stored at -20 °C, and previously tested at the National Institute for Virology, Sandringham, by the HI test for antibodies against several

arboviruses, as well as randomly collected sera from farm workers from several farms in the Orange Free State, were included for evaluation of the test.

3.2.1.4. BLOCKING REAGENTS

The following blocking reagents (Table 5) were tested to determine the effectiveness of these reagents in the dot-ELISA.

TABLE 5. **BLOCKING REAGENTS**

Blocking agent	Reagents	Cat. No.
PBS-Tween (0.1 %)	1 ml Tween 20 999 ml PBS	Technikon Chemicals:T21-030915
Bovine Albumin Fraction V (5 %)	5 g Bovine albumin 100 ml PBS	Gibco: 066-01018H
FCS (3 %)	3 ml FCS 97 ml PBS	Gibco: 013-06290H
Skimmed Milk (5 %)	50 g Skimmed milk powder 100 µl Anti-foam A 1 µl Thiomersal 100 ml PBS	Oxoid: L31 Sigma: A5758 BDH: 30416 4 H
Gelatine (1%)	1 g Gelatine 100 ml PBS	Difco: 0143-01

3.2.1.5. CONJUGATES

Goat anti-human IgG, (heavy and light chain specific), conjugated to alkaline phosphatase (Calbiochem®, cat.no. 401453) was standardised in a block titration before use.

Purified anti-human IgM, (µ-chain specific), prepared in goat, and conjugated to alkaline phosphatase (Calbiochem®, cat.no. 401902) was used in a block titration to determine the optimal working strength.

3.2.1.6. SUBSTRATE

The alkaline phosphatase substrate, 5-Bromo-4-Chloro-3-Indolyl Phosphate p-Toluidine salt (BCIP), in conjunction with Nitro Blue Tetrazolium (NBT) as amplifier, were used in the dot-ELISA procedure. This substrate system (Table 6) produces an insoluble end product that is blue in color and can be observed visually.

TABLE 6. **SUBSTRATE SYSTEM**

Stock Solution	**Reagents**	**Cat. No.**
BCIP	25 mg BCIP tablet 500 µl Dimethylformamide	Sigma: B0274 Sigma: D4254
NBT	10 mg NBT tablet 1 ml Deionised H_2O	Sigma: N5514
Substrate Buffer (pH 9.5)	12.11 g Tris Base 5.84 g NaCl 1 g $MgCl_2$ 1000 ml deionised water HCl to adjust pH	Boehringer Mannheim: 708968 Merck: 6404 Merck: 5831 Merck: 9970.0001
Working Substrate Solution	330 µl NBT stock 10 ml Substrate buffer 33 µl BCIP stock	

3.2.1.7. WASHING SOLUTION

A solution of PBS and Tween 20 (concentration of 0.1 %) was used as washing solution in the dot-ELISA.

3.2.1.8. RF ABSORBENT

RF Absorbent (Behring, cat.no. OUCG 15) was dissolved in 2 ml of sterile distilled water, and was frozen in 200 µl quantities at -20 °C until further use.

3.2.2. METHODS

3.2.2.1. ANTIGEN PREPARATION

Approximately 60 ml of each antigen obtained from the supernatant fluids in cell cultures (3.1.2.4.) for RVF, WN, SIN and WSL were concentrated by means of centrifugation (95000 g for 120 min at 4 °C). The pellet of each 10 ml tube was suspended in 500 µl PBS. Each concentrated virus suspension was pooled and then aliquoted into 50 µl volumes which were stored at -70 °C. Control antigen was also prepared in the same manner.

RVF antigen was inactivated before use in the dot-ELISA by incubating 50 µl of concentrated antigen suspension at 56 °C for 60 min. SIN, WN and WSL antigens were not inactivated, but all

manipulations were carried out in a biohazard laminar flow cabinet.

3.2.2.2. BLOCKING

A schematic explanation of the blocking procedure is given in figure 2.

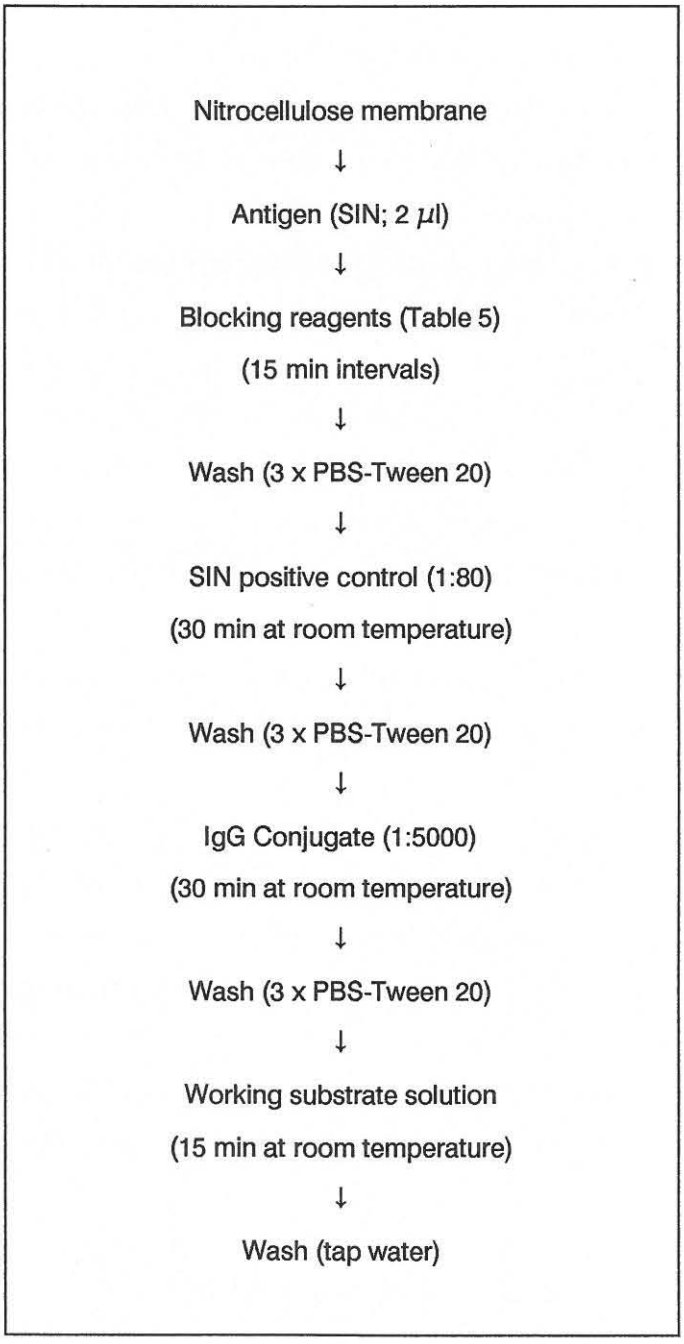


FIGURE 2. BLOCKING PROCEDURE

3.2.2.3. STANDARDISATION

Block titration of conjugate

IgG

A nitrocellulose sheet (5 x 3 cm) was prepared by drawing a grid pattern (1 cm^2) with a pencil. Logarithmic dilutions of concentrated SIN antigen were prepared in PBS (10^{-1} to 10^{-3}). One μl of each antigen dilution was spotted onto 5 grids in a row with a pipette (Labsystems Finnpipette, cat.no. 4027000), starting with the concentrated antigen. The antigen spots were allowed to dry. The NC sheet was submerged in blocking reagent and allowed to react for 30 min. after which it was washed with 3 consecutive changes of PBS-Tween 20 (washing solution) by flooding a Petri dish with washing solution and rocking it gently.

A reference human serum with a HI titer of 1024 for SIN antibodies was diluted to a concentration of 1:40 in PBS. Approximately 4 ml of serum solution was layered onto the NC sheet and allowed to react at room temperature for 30 min. The NC sheet was washed as previously described.

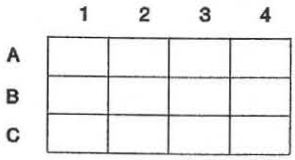
The NC sheet was then cut into strips containing different dilution factors of antigen and each strip was placed in a Petri dish. The NC membrane was handled throughout with forceps.

The goat, anti-human IgG conjugate was diluted in PBS (1:500, 1:1000, 1:2000, 1:3000 and 1:4000) and approximately 1 ml of each concentration was placed on each NC strip with the antigen and antibody complex and incubated for 30 min at room temperature. The excess conjugate was removed by submitting the strips to another wash cycle.

Working substrate solution (Table 6) was added to each strip and the substrate solution was allowed to react for 15 min. The reaction was stopped by washing the strips in tap water.

IgM

The same test procedures as for IgG were followed and a schematic representation of the concentration of the goat, anti-human IgM is listed in figure 3. Serum samples used for IgM standardisation, as well as for evaluations, were first treated with an equal volume of RF absorbent for 15 min at room temperature before application.

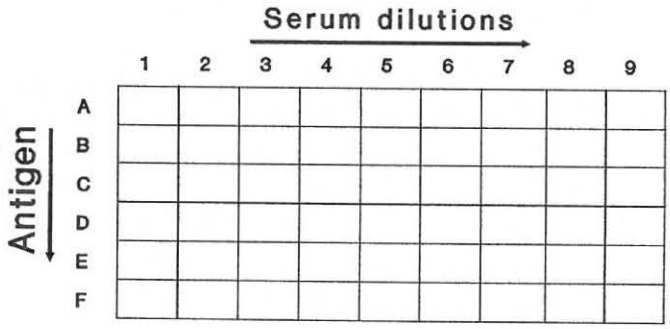


Row A,B,C = SIN concentrated antigen (1 µl)
Row A = SIN IgM positive serum (1:40)
Row B = SIN IgM positive serum (not treated 1:40)
Row C = Control serum (Anti-Rubella IgM positive 1:40)
Column 1 = Conjugate 1:1000 Column 3 = Conjugate 1:3000
Column 2 = Conjugate 1:2000 Column 4 = Conjugate 1:4000

FIGURE 3. BLOCK TITRATION OF CONJUGATE FOR IgM DETERMINATIONS

Block titration of antigen and serum

The concentration of the antigen and sera for each virus was determined by block titrations as schematically illustrated in figure 4. The volume of antigen spotted on the NC was standardised at 1 µl/grid. Approximately 1 ml of serum dilution was added to each column.



A = Concentrated antigen (1 µl)
B = Diluted antigen (10^{-1})
C and D = Alternative antigen (concentrated)
E = Vero control cells
F = Negative serum control
Column 1 to 8 = Serum dilutions (1:20; 1:40 etc.)
Column 9 = Negative serum (1:20)

FIGURE 4. BLOCK TITRATION OF ANTIGEN AND ANTISERUM.

3.2.2.4. DOT-ELISA

The following method was used in the test procedure:

One µl of the concentrated antigen of RVF, SIN, WN, WSL and control was spotted respectively on blocks of 5 cm^2 NC strips. The NC membrane was allowed to dry. To block the unreacted protein-binding sites, the strips were submerged in PBS-Tween 20 (0.1 %) solution in a Petri dish and left at room temperature for 30 min. The strips were then washed with washing solution (3 cycles).

Serum samples were diluted in PBS at a concentration of 1:80 (IgG) and 1:40 (IgM) respectively. For IgM determinations the diluted serum samples were treated with equal volumes of RF Absorbent (final concentration of serum: 1:80).

The strips were then flooded with approximately 1 ml of serum dilution and allowed to react for 30 min. Positive control sera were included in each batch to be tested. After the strips had been washed, conjugate (diluted to a concentration of 1:4000 for IgG and 1:3000 for IgM) was added. Following an incubation of 30 min the strips were washed and then submerged in working substrate solution. The substrate solution was allowed to react for 15 min after which the strips were washed with tap water. The NC was allowed to dry before visual interpretation of results.

Positive reactions were determined by a visual cut-off point. A clearly visible blue spot was regarded as a positive result. Reactions lighter in intensity than that of the positive control were interpreted as negative.

3.3 IMMUNOFLUORESCENCE

A modification of the indirect IF test as described by Shope & Sather (1979) was used.

3.3.1. MATERIALS

3.3.1.1. REAGENTS

PBS.
Acetone at 4 °C.
Goat anti-human IgM (µ-chain) FITC conjugate (Sigma, cat.no. F5384).
Goat anti-human IgG (whole molecule) FITC conjugate (Sigma, cat.no. F3512).
RF Absorbent

Evans blue stain (stock solution)
1 g Evans blue powder (Merck, cat.no. 3169)
100 ml Distilled water
Dissolve the powder in the distilled water and store the stain solution at 4 °C.

3.3.1.2. ANTIGENS

RVF, SIN, WN and WSL virus (passage 3 in Vero cells, as prepared in 3.1.2.2)

3.3.1.3. ANTISERA

Human serum samples (3.2.1.3).

3.3.1.4. MISCELLANEOUS

Teflon-coated 8 well multitest microscope slides (Flow Laboratories, cat.no. 60-408-05).
Nikon Optiphot microscope, equipped with epi-fluorescence accessories, ultraviolet filter cassettes and a high pressure mercury lamp.

3.3.2. METHODS

Infected cell cultures were prepared as described in 3.1.2.4. Once a CPE was observed, the cells were dislodged from the cell culture flask and centrifuged at 7000 g for 30 min at 4 °C. The pellet was reconstituted into 1 ml of serum free maintenance medium. To each virus-infected suspension, the same volume of control cells (non-infected Vero cells) was added. After thorough mixing, 25 µl of cell suspension was spotted onto each well of the teflon-coated, 8 well multitest microscope slides. The slides were dried in air and then fixed in acetone at 4 °C for 10 min. The slides were stored at -70 °C until use.

3.3.2.1. STANDARDISATION

The optimal working dilution of the goat, anti-human IgG and IgM FITC conjugate and patient's sera were determined.

Double dilutions (starting at 1:4) of positive, as well as negative control sera were diluted in PBS to a final dilution of 1:32. Each serum dilution was added in a volume of 25 µl to 3 wells of a previously prepared antigen-coated multitest microscope slide. The slides were incubated in a humid chamber at 37 °C for 30 min. The slides were then washed with 3 cycles of PBS for 10 min.

The FITC conjugate for IgG was diluted in PBS in concentrations of 1:8; 1:16 and 1:32. Each concentration of conjugate was added in a volume of 25 µl to a well of the microscope slide containing a different dilution of control serum. The slides were incubated at 37 °C for 30 min, and then washed in PBS for 10 min. Counterstaining was done with Evans blue (final concentration,

1:10000 in H_2O) for 3 min at room temperature. The slides were washed with PBS and allowed to dry before examination under the fluorescence microscope.

To determine the optimal working dilution of the IgM FITC conjugate, positive, negative and a RF positive serum were diluted at double dilutions starting at 1:4. Each dilution of serum was treated with an equal volume of RF Absorbent (reconstituted to a volume of 1.5 ml with distilled water), and incubated at room temperature for 15 min. A volume of 25 µl of the different dilutions of control samples were added to three wells of previously prepared IF slides. The slides were incubated at 37 °C for 120 min after which they were washed with 3 changes of PBS for 10 min. The conjugate (concentrations of 1:50, 1:100 and 1:200) was added to each well of different serum dilutions. The test was further performed as described for IgG standardisation.

3.3.2.2. TEST PROCEDURE

In the test procedure, serum samples were diluted to 1:16 with PBS and each serum sample was tested for RVF, SIN, WN and WSL antibodies of the IgG class. The working dilution of the goat, anti-human IgG FITC conjugate was determined to be 1:16.

Volumes, incubation times, temperatures and wash cycles were followed as stated in 3.3.2.1.

For IgM determinations the serum samples were tested at a final dilution of 1:10, and the goat, anti-human IgM FITC conjugate gave a satisfactory result at a concentration of 1:50.

The prepared IF slides were allowed to dry in the dark before examination by fluorescence microscopy by two independent observers.

3.4. STATISTICAL ANALYSIS OF DATA

3.4.1. *KAPPA* STATISTICS

Kappa statistics (κ) (Fisher and Van Belle, 1993) were applied to the results obtained in the dot-ELISA and IF test to measure the amount of agreement between the methods, where

$$\kappa = \frac{p_o - p_e}{1 - p_e}$$

p_o = observed probability of concordance between two methods

p_e = expected probability of concordance between two methods

Evaluation of *Kappa* statistics:

$\kappa > 0.75$ denotes an *excellent* amount of agreement

$0.75 \leq \kappa \geq 0.4$ denotes a *good* amount of agreement

$0.4 \leq \kappa \geq 0.0$ denotes a *marginal* amount of agreement.

3.4.2. TWO BY TWO CONTINGENCY TABLE

Data obtained from the dot-ELISA, IF, and HI test were submitted to the 2 x 2 contingency table to analyse the sensitivity and specificity of the dot-ELISA versus the IF and HI test.

		Dependent variable +	Dependent variable -	
Independent variable	+	a	b	a+b
	-	c	d	c+d
		a+c	b+d	n

a = true positive

b = false positive

c = false negative

d = true negative

n = total specimens

Sensitivity = a / a+c

Specificity = d / b+d

Accuracy = a+d / n

CHAPTER 4

RESULTS

4.1. STANDARDISATION

4.1.1. BLOCKING REAGENTS

The different blocking solutions tested gave significantly different background staining (Table 7). The use of PBS-Tween 20 (0.1%) and FCS (3%) as blocking reagents gave the lowest background staining at the time interval of 30 min, while Gelatine (1 %) and skimmed milk (5 %) gave a high background staining at the same time interval.

TABLE 7. **BACKGROUND STAINING WITH DIFFERENT BLOCKING REAGENTS**

Blocking reagent	**Time**			
	15 min	**30 min**	**45 min**	**60 min**
Gelatine (1%)	2	2	1	1
Skimmed milk (5%)	2	2	2	2
PBS-Tween 20 (0.1%)	1	0	0	0
FCS (3%)	1	0	0	0
Bovine albumin Fraction V (5%)	2	1	0	0

0 = No background staining
1 = Background staining
2 = High background staining

4.1.2. CONJUGATES

The working dilutions of the goat, anti-human immunoglobulin G and M for the dot-ELISA were determined to be 1:3000 and 1:4000 respectively.

4.1.3. ANTIGENS

The optimal antigen concentration of each virus was determined in the block titration. Figure 5 clearly shows that a positive reaction was obtained with concentrated antigen in each case of the titration of RVF, SIN and WN viruses. Only concentrated antigen was spotted in the case of WSL virus. No or minimal cross-reaction was observed between RVF, SIN and WN viruses (Figure 5 a,b,d). In the case of WSL antigen, cross-reaction was observed between RVF, WN and SIN antigen (Figure 5 c).

4.1.4. ANTISERA

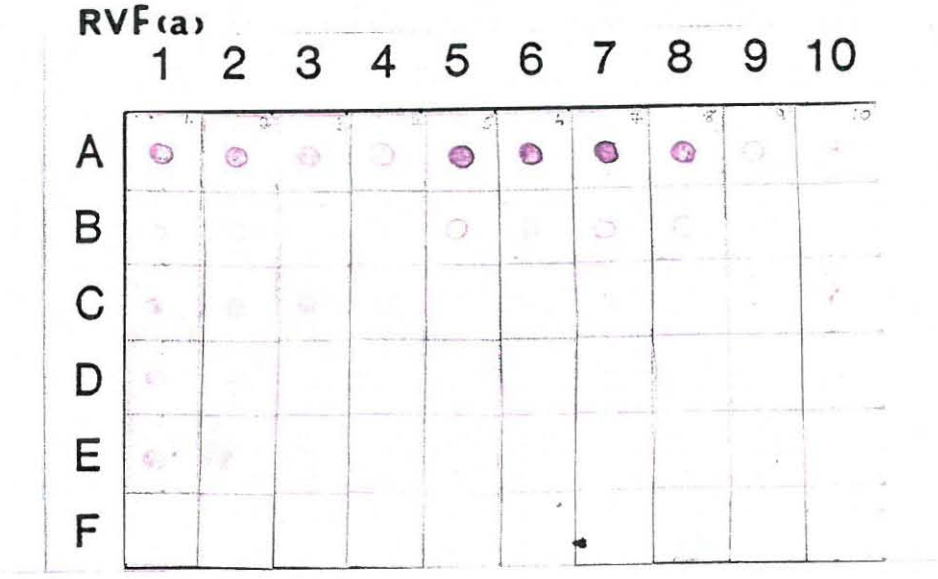
As the positive control sera yielded reactions up to dilutions of 1:80 or higher, it was decided to use a serum dilution of 1:80 in the test assay for detecting IgG (Figure 5), as well as IgM antibodies (Figure 6) against RVF, SIN, WN and WSL viruses. In the block titration for RVF virus (Figure 5a) two different positive serum samples were used, the second sample giving a higher titer for RVF IgG antibodies and was subsequently used as control in the test assay.

4.1.5. IMMUNOFLUORESCENCE

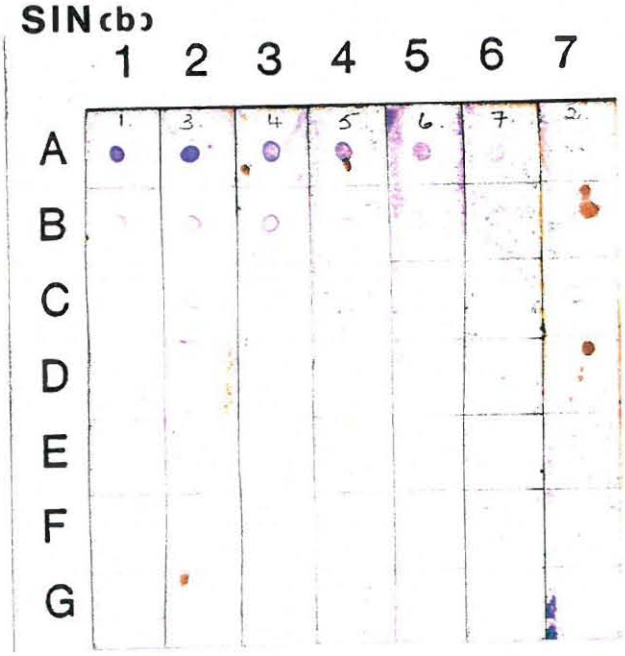
In the IF test for IgG and IgM evaluations, the working dilution of patient's sera was determined to be 1:16 and 1:10 respectively. It was necessary to dilute the conjugate 1:16 (IgG) and 1:50 (IgM) for satisfactory results. Photographic examples of IF for RVF, SIN, WN and WSL (IgG antibodies) are listed in the appendix.

4.2. SEROLOGICAL RESULTS

For the evaluation and validation of the dot-ELISA, 100 serum samples were tested for IgG and IgM antibodies to RVF, SIN, WN, and WSL viruses.

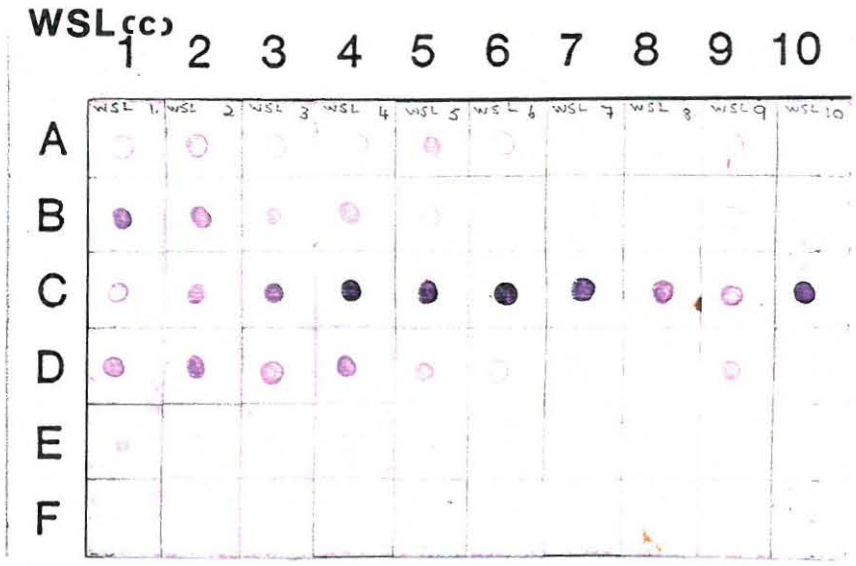


A = RVF antigen concentrated
B = RVF antigen (10^{-1})
C = SIN antigen
D = WN antigen
E = Control cells
F = Serum control
1 = RVF positive serum 1 (1:20)
2 = RVF positive serum 1 (1:40)
3 = RVF positive serum 1 (1:80)
4 = RVF positive serum 1 (1:160)
5 = RVF positive serum 2 (1:20)
6 = RVF positive serum 2 (1:40)
7 = RVF positive serum 2 (1:80)
8 = RVF positive serum 2 (1:160)
9 = Negative serum (1:20)
10 = Antigen control



A = SIN antigen concentrated
B = SIN antigen (10^{-1})
C = RVF antigen
D = WN antigen
E = Control cells
F = Serum control
G = Antigen control
1 = SIN positive serum (1:20)
2 = SIN positive serum (1:40)
3 = SIN positive serum (1:80)
4 = SIN positive serum (1:160)
5 = SIN positive serum (1:320)
6 = SIN positive serum (1:640)
7 = Negative control serum (1:20)

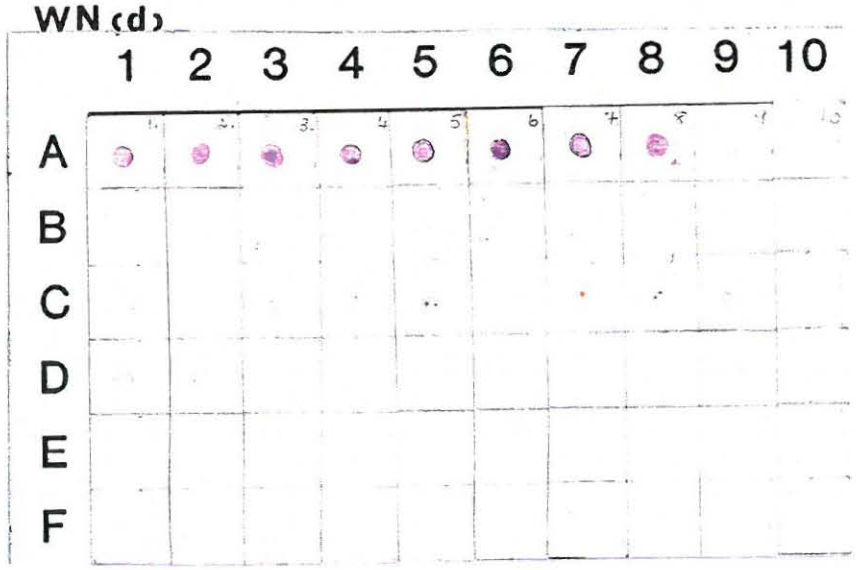
FIGURE 5. BLOCK TITRATIONS OF ANTIGENS AND ANTISERA.



A = WSL antigen concentrated
B = RVF antigen
C = WN antigen
D = SIN antigen
E = Control cells

F = Serum control
1 = WSL positive serum (1:20)
2 = WSL positive serum (1:40)
3 = WSL positive serum (1:80)
4 = WSL positive serum (1:160)

5 = WSL positive serum (1:320)
6 = WSL positive serum (1:640)
7 = WSL positive serum (1:1280)
8 = WSL positive serum (1:2560)
9 = Negative serum (1:20)
10 = Antigen control

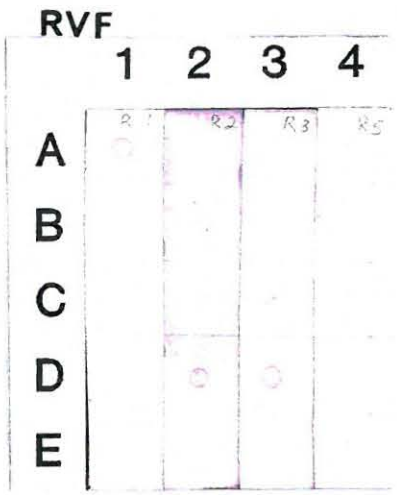


A = WN antigen concentrated
B = WN antigen (10^{-1})
C = SIN antigen
D = RVF antigen
E = Control cells

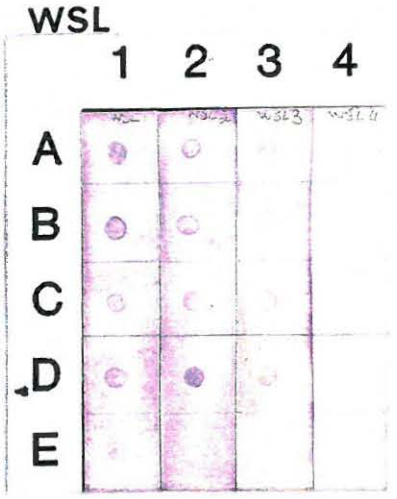
F = Serum control
1 = WN positive serum (1:20)
2 = WN positive serum (1:40)
3 = WN positive serum (1:80)
4 = WN positive serum (1:160)

5 = WN positive serum (1:320)
6 = WN positive serum (1:640)
7 = WN positive serum (1:1280)
8 = WN positive serum (1:2560)
9 = Negative control serum (1:20)
10 = Antigen control

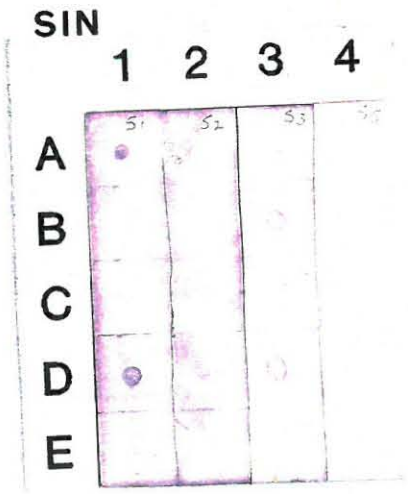
FIGURE 5 (continue). BLOCK TITRATION OF ANTIGENS AND ANTISERA.



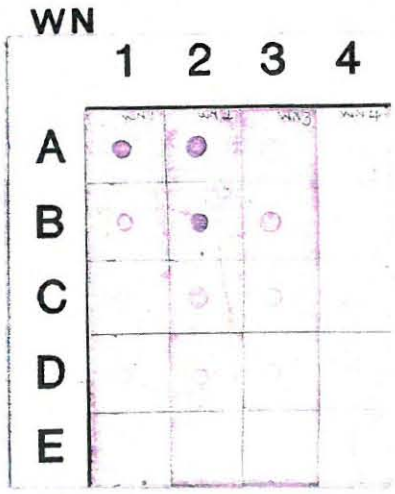
A = RVF antigen concentrated
B = SIN antigen
C = WN antigen
D = WSL antigen
E = Vero control cells
1 = RVF IgM positive serum (1:80)
2 = RVF IgG positive serum (1:80)
3 = RF positive serum (1:80)
4 = Antigen control



A = WSL antigen concentrated
B = WN antigen
C = SIN antigen
D = RVF antigen
E = Vero control cells
1 = WSL IgM positive serum (1:80)
2 = WSL IgG positive serum (1:80)
3 = RF positive serum (1:80)
4 = Antigen control



A = SIN antigen concentrated
B = RVF antigen
C = WN antigen
D = WSL antigen
E = Vero control cells
1 = SIN IgM positive serum (1:80)
2 = SIN IgG positive serum (1:80)
3 = RF positive serum (1:80)
4 = Antigen control



A = WN antigen concentrated
B = WSL antigen
C = SIN antigen
D = RVF antigen
E = Vero control cells
1 = WN IgM positive serum (1:80)
2 = WN IgG positive serum (1:80)
3 = RF positive serum (1:80)
4 = Antigen control

FIGURE 6. ANTIGEN AND ANTISERUM STANDARDISATION FOR IgM.

4.2.1. IgG ANTIBODY DETERMINATIONS

The antibody results obtained for each serum sample for the different viruses in the dot-ELISA and IF test are listed in the appendix.

A summary of the statistical results obtained in the dot-ELISA and IF test, for each virus, is supplied in table 8.

4.2.1.1. RIFT VALLEY FEVER VIRUS

All the sera which tested IgG antibody positive in the IF test (Table 8) for RVF antigen, were positive in the dot-ELISA (sensitivity, 100%). Ten sera tested positive in the dot-ELISA but negative in the IF test for RVF antibodies (specificity, 87%). The amount of agreement between the two surveys was good (κ = 0.7259).

4.2.1.2. WEST NILE VIRUS

The comparison of antibody testing (IgG) for WN virus in the dot-ELISA with the IF test as standard, showed a sensitivity of 100% (Table 8). One serum sample tested positive with the dot-ELISA but negative with the IF test (specificity, 98%). The *Kappa* value (0.9605) denoted an excellent amount of agreement, and the statistical calculation of the accuracy of the test was 99 %.

4.2.1.3. SINDBIS VIRUS

Results obtained for SIN virus IgG antibodies revealed a sensitivity of 88% and a specificity of 86% (Table 8). Thirteen sera tested positive with the dot-ELISA and negative with the IF test. One specimen tested positive with IF but negative with the dot-ELISA. The amount of agreement between the two surveys was good.

4.2.1.4. WESSELSBRON VIRUS

Results for WSL antibody (IgG) testing showed a sensitivity of only 54%. Five serum samples tested positive with the dot-ELISA, while 6 serum samples tested positive for IF antibodies (specificity, 94%). The *Kappa* value of 0.490 denoted a good amount of agreement and the accuracy was calculated to be 89 %.

TABLE 8. **RESULTS OF DOT-ELISA IgG VERSUS IF TEST AS STANDARD** (n =100)

Virus	True Positive	False Positive	True Negative	False Negative	Sensitivity %	Specificity %	Kappa	Accuracy of test (%)
RVF	21	10	69	0	100	87	0.7259	90
WN	47	1	52	0	100	98	0.9605	99
SIN	7	13	79	1	88	86	0.6022	86
WSL	7	5	82	6	54	94	0.4900	89

The HI titers of 33 serum samples were known and by using these results as the standard test, the results obtained in the dot-ELISA were compared in table 9. The reproducibility of each test varied from excellent (WN virus: κ=0.8412), to marginal (WSL virus: κ=0.2303). For RVF virus (κ=0.6731) and SIN virus (κ=0.7390) a good amount of agreement was obtained.

TABLE 9. **RESULTS OF DOT-ELISA IgG VERSUS HI AS STANDARD** (n = 33)

Virus	True Positive	False Positive	True Negative	False Negative	Sensitivity %	Specificity %	Kappa	Accuracy of test (%)
RVF	6	2	23	2	75	92	0.6731	88
WN	19	2	11	1	95	85	0.8412	91
SIN	10	1	19	3	77	95	0.7390	88
WSL	3	2	19	9	25	90	0.2303	67

4.2.2. IgM ANTIBODY DETERMINATIONS

Results obtained for RVF, WN, SIN and WSL determinations of IgM antibodies in 100 serum samples by the dot-ELISA and IF tests are listed in the appendix.

The calculated statistical results for the IgM determinations are summarised in table 10.

In the case of RVF, SIN and WN virus, for IgM antibodies, a 100% correlation between the dot-ELISA and IF test was observed. No positive results were obtained for WSL virus IgM antibodies in both the dot-ELISA and IF test.

The serum samples which tested positive for IgM antibodies for the different viruses, yielded negative results in the latex test for RF.

TABLE 10. **RESULTS OF DOT-ELISA IgM VERSUS IF TEST AS STANDARD** (n = 100)

Virus	True Positive	False Positive	True Negative	False Negative	Sensitivity %	Specificity %	Kappa	Accuracy of test (%)
RVF	1	0	99	0	100	100	>0.75	100
WN	4	0	96	0	100	100	>0.75	100
SIN	1	0	99	0	100	100	>0.75	100
WSL	0	0	100	0	100*	100	>0.75	100

* Mathematical manipulation of a 0 value by adding 0.5 to all values of formula

CHAPTER 5

DISCUSSION

The diagnosis of viral infections requires the use of rapid and sensitive assays, therefore the need to develop easy and inexpensive assays which are not commercially available. Among the important viral infections occurring in the Orange Free State are mosquito borne arbovirus infections. According to McIntosh (1980), the prevalent arboviruses found in the Orange Free State are RVF, SIN, WN and WSL viruses. It was thus decided to develop a combined rapid test for detecting antibodies against these viruses in human blood specimens as no commercial tests are available.

In the development of the dot-ELISA for the detection of IgG and IgM antibodies to RVF, SIN, WN and WSL viruses, the antigens used were crude virus preparations. They consisted of clarified culture supernatant media, concentrated by centrifugation and stored at - 70 °C for several months before use. Although crude preparations of viruses were used successfully in the dot-ELISA (Bode *et al.*, 1984; Gouvea *et al.*, 1987; Boctor *et al.*, 1989; Leonardi *et al.*, 1990), concentrated antigens enhanced the sensitivity of results (Heberling & Kalter, 1986).

It was found that 1 μl of crude concentrated antigen gave a sufficient size dot to distinguish between a positive and negative result, thus allowing for a minimal volume of antigen in this test. This contributed in making this technique particularly valuable when dealing with antigens that are expensive and difficult to obtain or to purify.

Hawkes *et al.* (1982) found that binding of the antigen to NC appears to be instantaneous and in experiments with the application of various volumes of antigen spotted, it was found that a spot with the application of 0.1 μl antigen was still clearly visible. This was the smallest volume applied to NC with commonly available equipment.

The instantaneous binding of antigen to NC was confirmed by the size of the antigen dot on the NC which never exceeded the limit of the grid. Although a 1 µl drop of antigen wetted beyond the edge of the block, no mixing of antigen occurred and the colour intensity and the dot size of the positive control sera were satisfactory.

Antigens associated with viruses considered to be highly pathogenic for humans, could be inactivated with a psoralen derivate and long-wavelength ultraviolet light irradiation for use in the dot-ELISA (Heberling & Kalter 1986). This procedure is not readily available and a method for heat inactivation (Saluzzo *et al.* 1988) was modified for use in the dot-ELISA for RVF antigen. SIN and WN antigens yielded no reactions in the dot-ELISA after heat inactivation and it was thus decided to use infectious antigen in the assay, the test procedure being performed in a biohazard laminar flow cabinet.

The stability of the viral antigens on dried NC membranes was not determined as the storage facilities for antigen were readily available and the dotting procedure could easily be performed. However, it was found that antigen stored at 4 °C for 3 months still performed adequately in the block titration (Figure 5d), except that in the WSL block titration (Figure 5c) reactions occurred in row C. This indicated probable contamination of the WN antigen as a reaction was observed with the negative control serum as well as the Vero control cells.

While testing for different blocking reagents in the dot-ELISA, it was noted that gelatine and skimmed milk gave a blue background staining, even after 60 min adsorption, making it impossible to recognise the antigen/antibody complex on the NC membrane. This was in contrast to Heberling & Kalter (1986) and Lavanchy *et al.* (1990) who found a solution of 5 % nonfat dry milk in PBS containing 0.01 % Anti-foam A and 0.0001 % merthiolate and a solution of 1 % dry powder milk in Tris-buffered saline to be excellent blocking reagents. However, the incubation period in both cases was overnight at room temperature.

With PBS-Tween 20 (0.1 %) and FCS (3 %) as blocking reagents, no background staining occurred after 30 min incubation (Table 7). Hoffman and Jump (1986) reported that several immunoglobulins and proteins dissociated from NC using PBS-Tween 20, but excellent results were obtained in this study. Batteiger *et al.* (1982) and Leonardi *et al.* (1990) also claim that PBS-Tween 20 as blocking reagent gives good results in the dot-ELISA and because of the advantage of being both cheap and readily available it was decided to use PBS-Tween 20 in the dot-ELISA.

Data supplied in table 8 show that the sensitivity of the dot-ELISA was comparable to that of the conventional IF test. The dot-ELISA recognised all the positive sera that tested positive with the IF test for IgG determinations for RVF and WN. 'False positive' results as reported in table 8 can be interpreted as greater sensitivity of the dot-ELISA. Greater sensitivity of the dot-ELISA detection of antibodies to several viruses as compared to the IF test were reported by Heberling and Kalter (1986) and Kalter and Heberling (1988). Two serum samples which tested positive with the dot-ELISA and negative with the IF test for RVF IgG antibodies were tested by a NT in Vero cell cultures, and neutralised RVF virus at a dilution of 1:20 (data not supplied). Therefore, the dot-ELISA is probably a more sensitive test. The single negative result obtained by the dot-ELISA which was positive in the IF test for SIN IgG antibodies cannot be explained. The test was not repeated due to insufficient serum sample and the result may thus be due to technique.

The low sensitivity obtained with the dot-ELISA for WSL IgG antibodies compared to the IF test may be due to cross-reaction with the other antigens. This phenomenon was observed in the block titration of WSL antigen (figure 5c) where positive WSL serum reacted with RVF, WN and SIN antigens. The interpretation of WSL IgG results in the dot-ELISA was also difficult. A colour reaction, with nearly the same intensity as the positive control, was observed in the test, making the decision for a positive or negative result nearly impossible. WSL is known to cross-react serologically with other flaviviruses, but homologous WSL titers can be distinguished from heterologous flavivirus titers after a primary WSL infection by performing serological tests with a range of flaviviruses known to occur in the region concerned (Blackburn and Swanepoel, 1980).

In the acute phase of an infection, or shortly thereafter, the detection of specific IgM antibodies is diagnostically useful. Rheumatoid factors of the IgM class can cause a false-positive result in indirect serological methods for the detection of specific IgM antibodies, if the sample also contains specific IgG antibodies (Tijssen, 1985; Ziegelmaier *et al.*, 1985). Besselaar *et al.*(1989) found non-specific staining in the IF test when testing untreated sera for SIN and WN IgM antibodies. In this study both the dot-ELISA and IF test sera were pretreated with RF Absorbent (anti-human IgG) to neutralise IgG and to eliminate the considerable skill and experience required to distinguish between true positives and non-specific staining in the assays.

The correlation of IgM results in the dot-ELISA and IF test was 100%, making it difficult to identify the better test. A definite advantage of the dot-ELISA is the relative crude antigen needed on the solid phase, as well as one dilution of serum to be tested for multiple antigens at the same time. The dot-ELISA can be performed in a short time and visual evaluation of the colour reaction eliminates expensive laboratory instrumentation. The used membranes can also be kept as a visual result for some time without any noticeable change in colour. These characteristics enable the use of the dot-ELISA for the rapid screening of large numbers of sera under field trial conditions, since a single dilution of serum under test reacts with strips dotted with the various antigens of RVF, SIN, WN and WSL.

Although interpretation of the dot-ELISA was visual and, therefore, subjective, positive and negative reactions could easily be distinguished as concurrence in reading of results by two independent observers were reached in the dot-ELISA as well as the IF test. The test could be automated using a recording densitometer as shown by Furuya *et al.* (1984), but the utility of the dot-ELISA for routine diagnosis lies in its simplicity and rapidity in obtaining a positive or negative result.

In conclusion, the dot-ELISA is a simple, rapid and sensitive assay for detecting both IgG and IgM antibodies to RVF, WN, SIN and WSL viruses in human sera. The test can be completed in 2 to 3

hours and can be used in the smallest laboratory or under field conditions, where sophisticated equipment and highly trained technical staff may not be available. It should prove to be useful in human seroprevalence and seroepidemiological studies on arboviruses.

SUMMARY

Arboviruses are maintained in nature through transmission between susceptible vertebrate hosts by haematophageous arthropods. Mosquitoes are the most important arbovirus vectors and are responsible for the most prevalent arbovirus infections in the Orange Free State namely, RVF, WN, SIN and WSL.

With generalised 'flu-like' clinical symptoms, a conclusive diagnosis for arbovirus infections can only be made by the laboratory, but commercial diagnostic assays testing for virus specific IgG and IgM for RVF, WN, SIN and WSL viruses are not available.

A dot-ELISA was developed by using a NC membrane as solid phase on which crude concentrated antigen preparations of RVF, WN, SIN and WSL viruses were spotted. The use of enzyme-conjugated anti-human immunoglobulins of the IgG or IgM class, with an insoluble substrate, produced visual results to distinguish between the IgG and IgM class antibodies of the previously mentioned viruses.

Serological data obtained in the dot-ELISA were compared with results obtained in the IF and HI test to determine the sensitivity and specificity of the dot-ELISA for each virus.

The sensitivity of the dot-ELISA versus the IF test for IgG antibodies varied from: RVF 100 % (specificity, 87 %); WN, 100 % (specificity, 98 %); SIN, 88 % (specificity, 86 %) and WSL, 54 % (specificity, 94 %).

A 100 % correlation was observed between the dot-ELISA and IF test for RVF, WN, SIN and WSL antibodies of the IgM class, providing 100 % sensitivity and specificity for each virus.

The simplicity, low costs, and demonstration of IgM and IgG antibodies in the dot-ELISA should prove to be of diagnostic value in the early detection of arbovirus infection as well as in seroepidemiological studies on arboviruses.

SAMEVATTING

Die arbovirusse word in die natuur deur virus oordraging tussen geskikte werweldier gashere en bloedsuiende artropode onderhou. Muskiete is die belangrikste arbovirus vektore en is in die Vrystaat vir Slenkdalkoors-, Sindbis-, Wesnyl- en Wesselsbronvirusinfeksies verantwoordelik.

Die simptome van arbovirusinfeksies kan verwar word met die van 'n "griep-beeld", daarom is dit belangrik om 'n diagnose deur laboratoriumtoetse te bevestig. Kommersiële toetse om te toets vir spesifieke IgG en IgM antiliggame teen Slenkdalkoorsvirus, Sindbisvirus, Wesnylvirus en Wesselsbronvirus is nie beskikbaar nie.

'n Dot-ensiemgebonde immuunsorbantessai (dot-ELISA) is ontwikkel deur gebruik te maak van nitrosellulose membrane waarop ongesuiwerde, gekonsentreerde antigeen van Slenkdalkoorsvirus, Wesnylvirus, Sindbisvirus en Wesselsbronvirus in klein hoeveelhede gebind word. Die gebruik van ensiem-gekoppelde anti-mens immuunglobulien verskaf 'n sigbare resultaat aangesien onoplosbare substraat in die sisteem gebruik word om antiliggame van die IgG en IgM klas van die bg. virusse aan te dui.

Die sensitiwiteit en spesifisiteit van die dot-ELISA vir elke virus is bepaal deur die serologiese resultate van die immunofluoressensie en hemagglutinasie-inhibisietoetse te vergelyk met die van die dot-ELISA.

Die sensitiwiteit en spesifisiteit van die dot-ELISA vir IgG bepalings van elke virus was soos volg: Slenkdalkoorsvirus, 100 % sensitiwiteit en 87 % spesifisiteit; Wesnylvirus, 100 % sensitiwiteit en 98 % spesifisiteit; Sindbisvirus, 88 % sensitiwiteit en 86 % spesifisiteit en Wesselsbronvirus, 54 % sensitiwiteit en 94 % spesifisiteit.

Resultate van die dot-ELISA en immunofluoressensietoets vir die bepaling van IgM klas antiliggame van bg. virusse het 'n uitstekende korrelasie getoon. In hierdie geval is die sensitiwiteit en spesifisiteit as 100 % vir elke virus bepaal.

Die eenvoudige prosedures, lae kostes en die feit dat IgG sowel as IgM klas antiliggame teen die verskillende arbovirusse bepaal kan word, maak die dot-ELISA 'n bruikbare toets vir vroeë diagnose van arbovirusinfeksies, sowel as vir sero-epidemiologiese studies.

APPENDIX

PATIENT	LOCATION	RVF IgG					SIN IgG					WN IgG					WSL IgG				
		D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI
1	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
2	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
3	Petrus Steyn	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
4	Petrus Steyn	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
5	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
6	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
7	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
8	Hospital	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+
9	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+	+	+
10	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
11	Hospital	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
12	Hospital	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+
13	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
14	Hospital	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	-	-	-	-	-
15	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+
16	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+	+	+
17	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
18	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+
19	Hospital	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
20	Hospital	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+
21	Hospital	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-
22	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-

D1=dot-ELISA observer 1 D2=dot-ELISA observer 2 F1=IF test observer 1 F2=IF test observer 2 HI=Haemagglutination Inhibition

PATIENT	LOCATION	RVF IgG					SIN IgG					WN IgG					WSL IgG				
		D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI
23	Hospital	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
24	Hospital	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
25	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
26	Hospital	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
27	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+
28	Hospital	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	-
29	Hospital	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	-	-	-
30	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+	+	+
31	Hospital	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-
32	Hospital	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
33	Hospital	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
34	Hospital	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
35	Hospital	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	Hospital	-	-	-	-		+	+	+	+		-	-	-	-		-	-	-	-	
37	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
38	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
39	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
40	Valspan	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
41	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
42	Valspan	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
43	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
44	Valspan	+	+	-	-		-	-	-	-		-	-	-	-		-	-	-	-	

D1=dot-ELISA observer 1 D2=dot-ELISA observer 2 F1=IF test observer 1 F2=IF test observer 2 HI=Haemagglutination Inhibition

PATIENT	LOCATION	RVF IgG					SIN IgG					WN IgG					WSL IgG				
		D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI
45	Valspan	-	-	-	-		+	+	-	-		+	+	+	+		-	-	-	-	
46	Valspan	+	+	+	+		+	+	-	-		-	-	-	-		-	-	-	-	
47	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-
48	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
49	Valspan	+	+	+	+		+	+	+	+		-	-	-	-		+	+	+	+	
50	Valspan	+	+	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
51	Valspan	+	+	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
52	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
53	Preezfontein Noord	+	+	-	-		+	+	+	+		+	+	+	+		-	-	-	-	
54	Preezfontein Noord	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
55	Hospital	-	-	-	-		+	+	+	+		-	-	-	-		-	-	-	-	-
56	Hospital	+	+	-	-	+	+	+	-	-		-	-	-	-		-	-	+	+	
57	Hospital	-	-	-	-	+	-	-	-	-		-	-	-	-		-	-	-	-	
58	Hospital	+	+	-	-	+	-	-	-	-		-	-	-	-		-	-	-	-	
59	Hospital	+	+	+	+		-	-	-	-		-	-	-	-		-	-	-	-	
60	Hospital	+	+	+	+		-	-	-	-		-	-	-	-		-	-	-	-	
61	Hospital	+	+	+	+	+	-	-	-	-		+	+	+	+		+	+	+	+	+
62	Hospital	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
63	Hospital	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
64	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
65	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
66	Hospital	-	-	-	-		-	-	-	-		+	+	-	-		-	-	-	-	

D1=dot-ELISA observer 1 D2=dot-ELISA observer 2 F1=IF test observer 1 F2=IF test observer 2 HI=Haemagglutination Inhibition

III

PATIENT	LOCATION	RVF IgG					SIN IgG					WN IgG					WSL IgG				
		D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI
67	Kareelaagte	+	+	+	+		-	-	-	-		+	+	+	+		-	-	-	-	
68	Kareelaagte	+	+	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
69	Kareelaagte	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
70	Kareelaagte	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
71	Kareelaagte	+	+	+	+		-	-	-	-		+	+	+	+		+	+	-	-	
72	Kareelaagte	+	+	+	+		-	-	-	-		+	+	+	+		+	+	-	-	
73	Kareelaagte	+	+	+	+		-	-	-	-		+	+	+	+		+	+	-	-	
74	Kareelaagte	+	+	+	+		-	-	-	-		+	+	+	+		-	-	-	-	
75	Kareelaagte	-	-	-	-		+	+	-	-		+	+	+	+		-	-	-	-	
76	Kareelaagte	+	+	+	+		-	-	-	-		+	+	+	+		-	-	-	-	
77	Kareelaagte	+	+	-	-		+	+	-	-		+	+	+	+		+	+	+	+	
78	Kareelaagte	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
79	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
80	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
81	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
82	Kareelaagte	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
83	Kareelaagte	+	+	+	+		-	-	-	-		-	-	-	-		-	-	-	-	
84	Kareelaagte	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
85	Kareelaagte	+	+	+	+		-	-	-	-		-	-	-	-		-	-	-	-	
86	Kareelaagte	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
87	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
88	Kareelaagte	+	+	+	+		+	+	-	-		-	-	-	-		-	-	-	-	

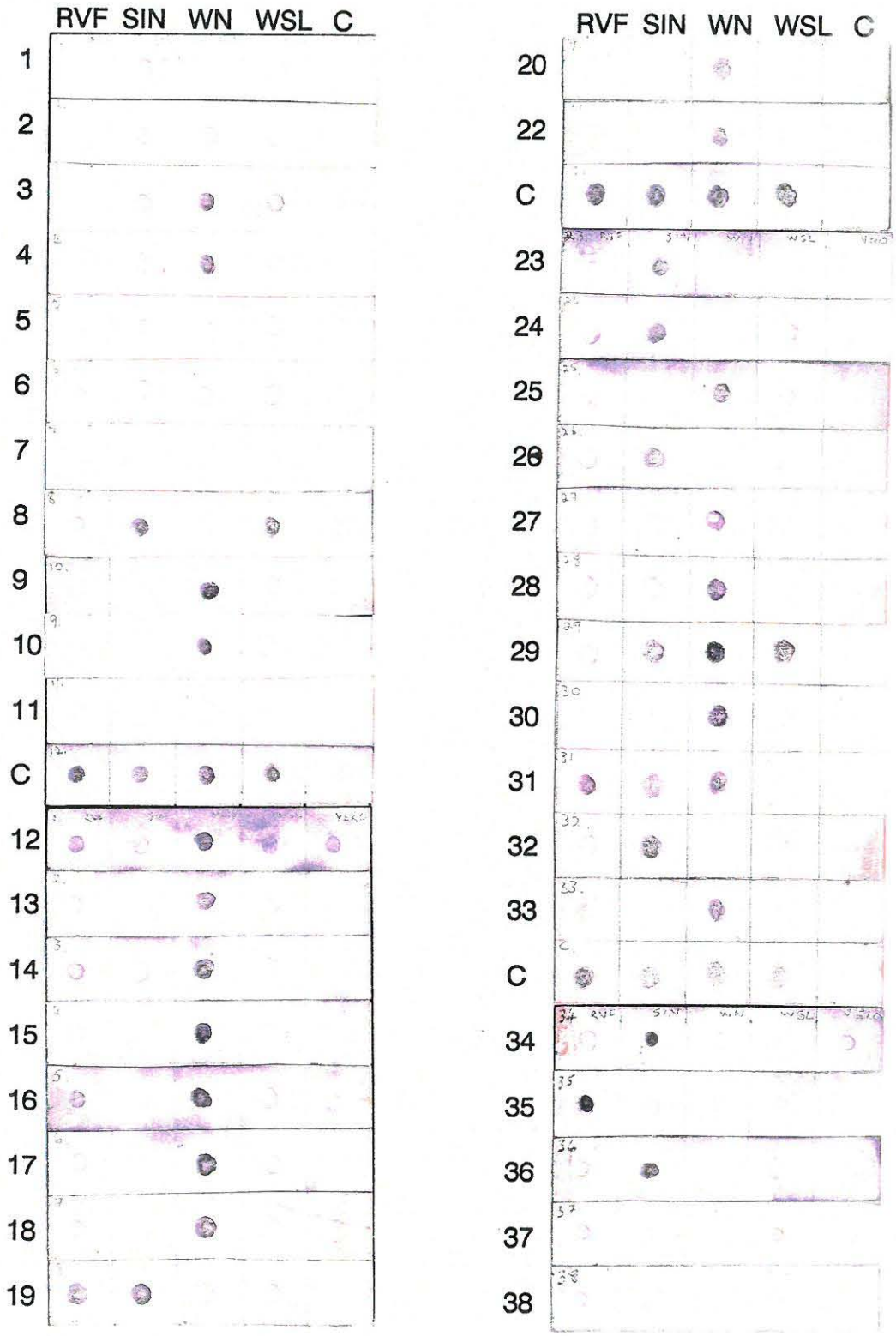
D1=dot-ELISA observer 1 D2=dot-ELISA observer 2 F1=IF test observer 1 F2=IF test observer 2 HI=Haemagglutination Inhibition

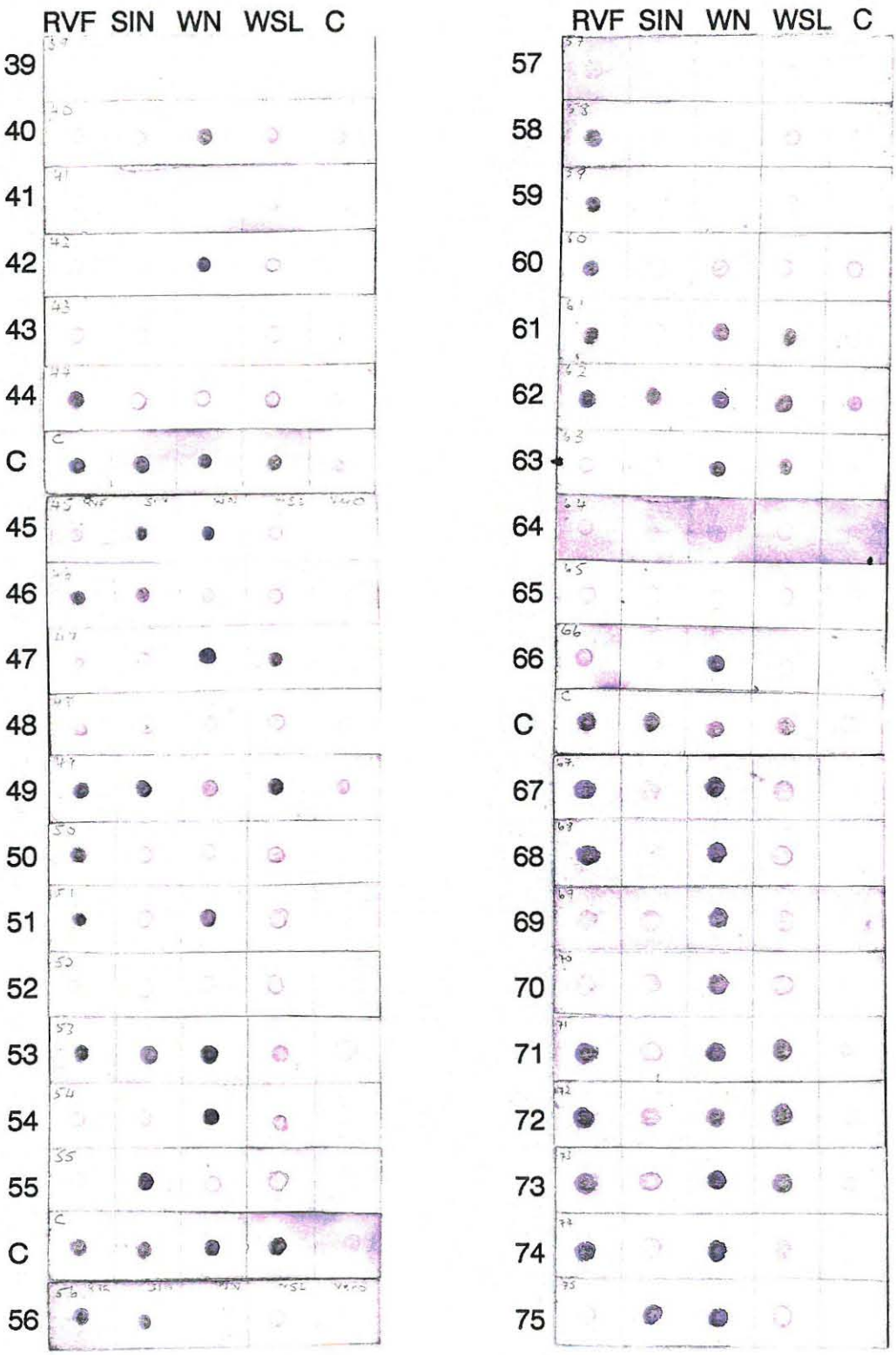
IV

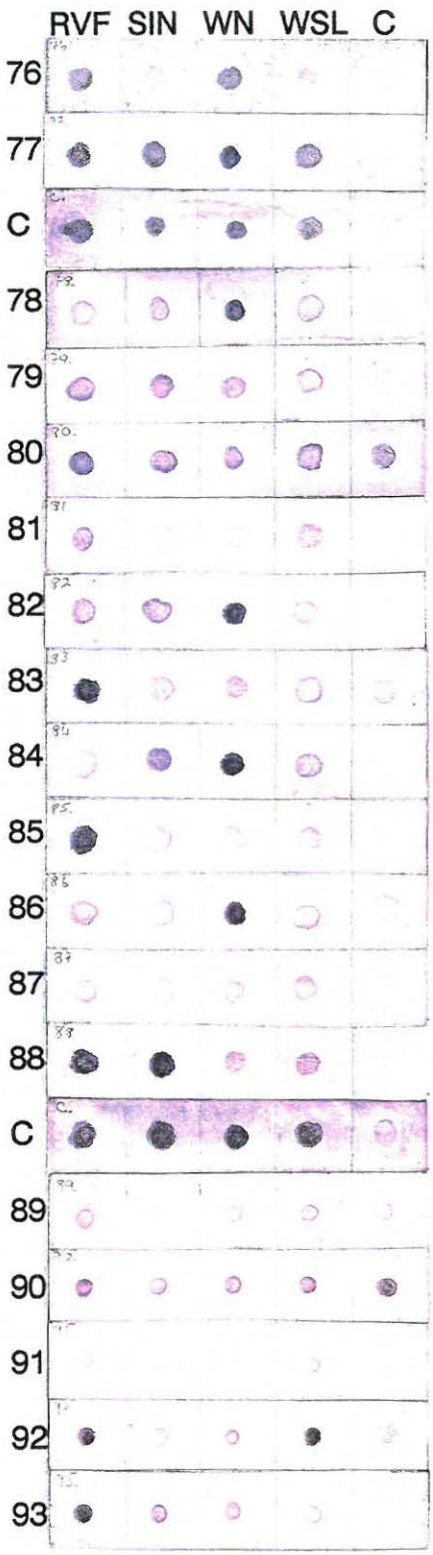
PATIENT	LOCATION	RVF IgG					SIN IgG					WN IgG					WSL IgG				
		D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI
89	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
90	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
91	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
92	Kareelaagte	+	+	-	-		-	-	-	-		-	-	-	-		+	+	-	-	
93	Kareelaagte	+	+	+	+		-	-	-	-		-	-	-	-		-	-	-	-	
94	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
95	Kareelaagte	+	+	+	+		-	-	-	-		-	-	-	-		-	-	-	-	
96	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
97	Kareelaagte	+	+	+	+		-	-	-	-		-	-	-	-		-	-	-	-	
98	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
99	Kareelaagte	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
100	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	

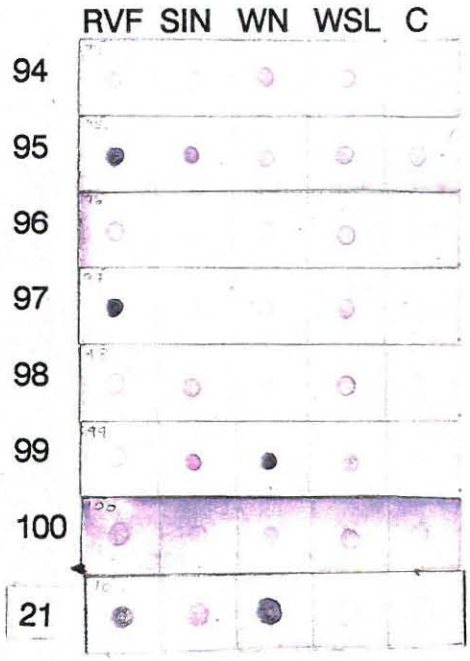
D1=dot-ELISA observer 1 D2=dot-ELISA observer 2 F1=IF test observer 1 F2=IF test observer 2 HI=Haemagglutination Inhibition

1.2. DOT-ELISA RESULTS

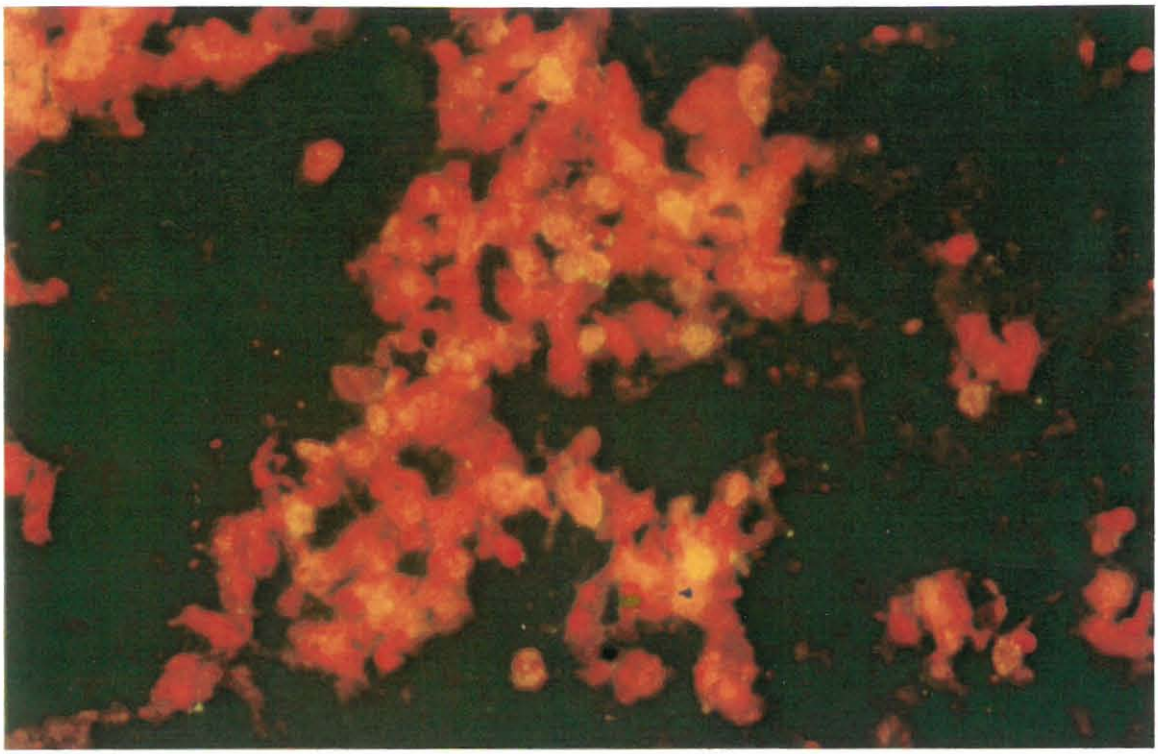




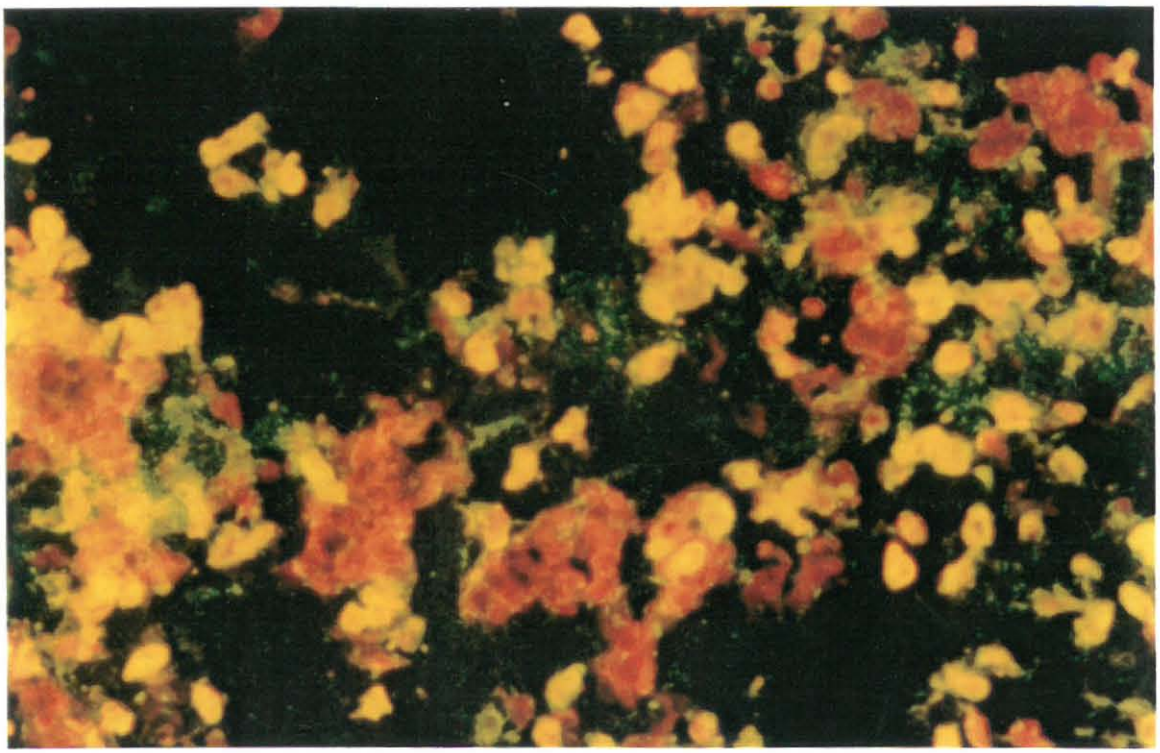




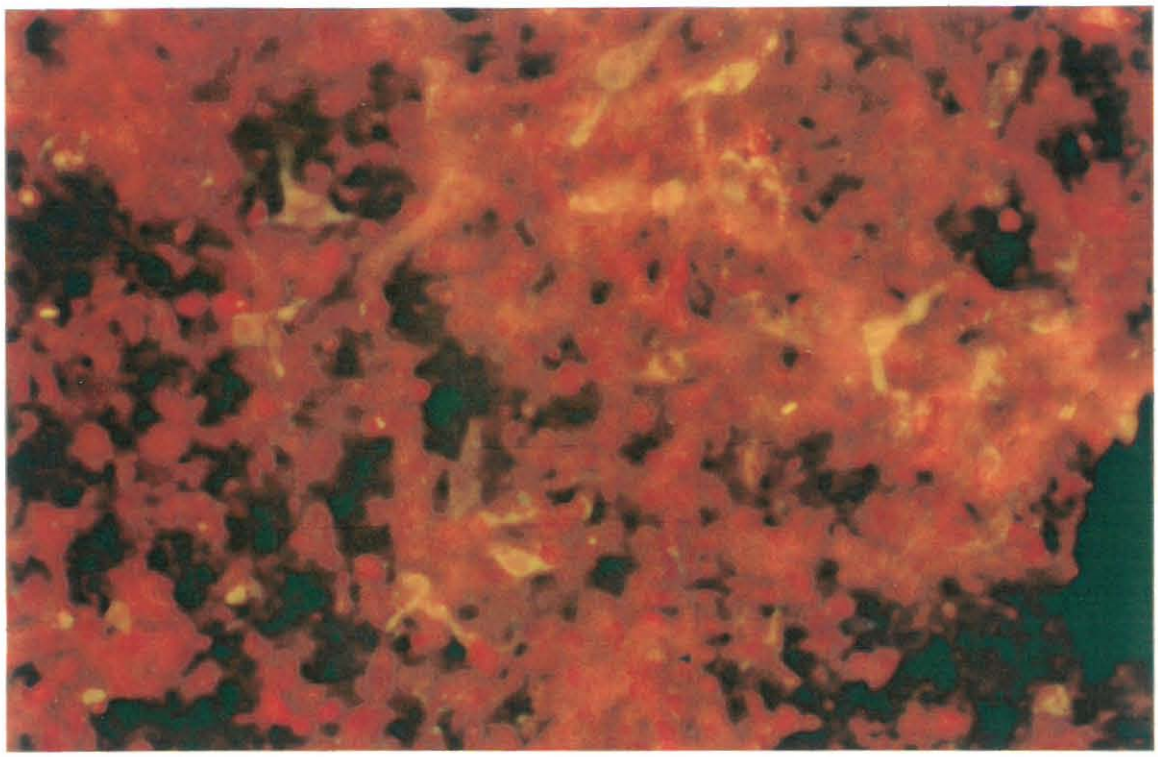
1.3. IMMUNOFLUORESCENCE EXAMPLES



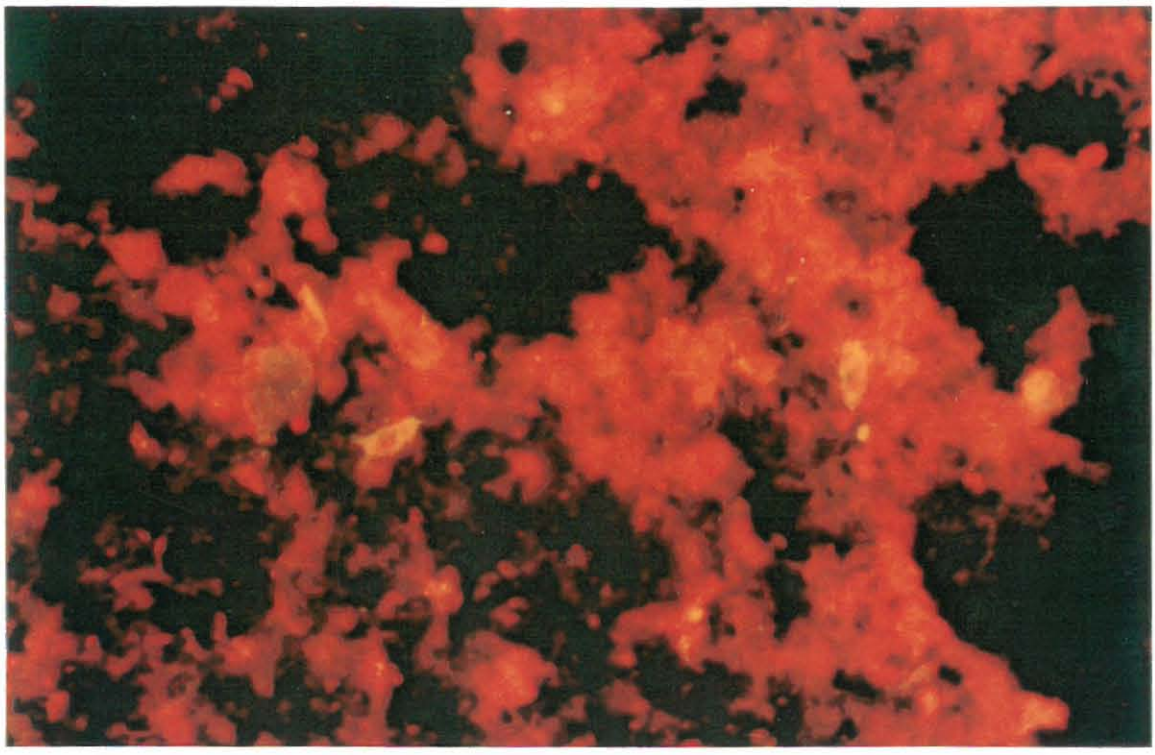
RVF virus infected Vero cells stained by the indirect fluorescent antibody technique with positive RVF human serum and anti-human IgG conjugate. (Magnification x 200)



SIN virus infected Vero cells stained by the indirect fluorescent antibody technique with positive SIN human serum and anti-human IgG conjugate. (Magnification x 200)



WN virus infected Vero cells stained by the indirect fluorescent antibody technique with positive WN human serum and anti-human IgG conjugate. (Magnification x 200)



WSL virus infected Vero cells stained by the indirect fluorescent antibody technique with positive WSL human serum and anti-human IgG conjugate. (Magnification x 200)

1.4. STATISTICAL ANALYSIS OF SEROLOGICAL RESULTS

RVF virus

D1:F1; D1:F2; D2:F1; D2:F2.

		IF TEST +	-	TOTAL
dot-ELISA	+	21	10	31
	-	0	69	69
	TOTAL	21	79	100

SENSITIVITY	:	100%
SPECIFICITY	:	87%
KAPPA	:	0.7259
ACCURACY	:	90%

D1:HI; D2:HI.

		HI +	-	TOTAL
dot-ELISA	+	6	2	8
	-	2	23	25
	TOTAL	8	25	33

SENSITIVITY	:	75 %
SPECIFICITY	:	92 %
KAPPA	:	0.6731
ACCURACY	:	88%

WN virus

D1:F1 ; D1:F2 ; D2:F1 ; D2:F2.

		IF TEST +	-	TOTAL
dot-ELISA	+	47	1	48
	-	0	52	52
	TOTAL	47	53	100

SENSITIVITY	:	100%
SPECIFICITY	:	98%
KAPPA	:	0.9605
ACCURACY	:	99%

D1:HI; D2:HI.

		HI +	HI -	TOTAL
dot-ELISA	+	19	2	21
	-	1	11	12
	TOTAL	20	13	33

SENSITIVITY	:	95 %
SPECIFICITY	:	85%
KAPPA	:	0.8412
ACCURACY	:	91%

SIN virus

D1:F1 ; D1:F2 ; D2:F1 ; D2:F2.

		IF TEST +	IF TEST -	TOTAL
dot-ELISA	+	7	13	20
	-	1	79	80
	TOTAL	8	92	100

SENSITIVITY	:	88 %
SPECIFICITY	:	86 %
KAPPA	:	0.6022
ACCURACY	:	86%

D1:HI; D2:HI

		HI +	HI -	TOTAL
dot-ELISA	+	10	1	11
	-	3	19	22
	TOTAL	13	20	33

SENSITIVITY	:	77%
SPECIFICITY	:	95%
KAPPA	:	0.7390
ACCURACY	:	88%

D1:HI; D2:HI.

		HI +	HI -	TOTAL
dot-ELISA	+	19	2	21
	-	1	11	12
	TOTAL	20	13	33

SENSITIVITY : 95 %

SPECIFICITY : 85%

KAPPA : 0.8412

ACCURACY : 91%

SIN virus

D1:F1 ; D1:F2 ; D2:F1 ; D2:F2.

		IF TEST +	IF TEST -	TOTAL
dot-ELISA	+	7	13	20
	-	1	79	80
	TOTAL	8	92	100

SENSITIVITY : 88 %

SPECIFICITY : 86 %

KAPPA : 0.6022

ACCURACY : 86%

D1:HI; D2:HI

		HI +	HI -	TOTAL
dot-ELISA	+	10	1	11
	-	3	19	22
	TOTAL	13	20	33

SENSITIVITY : 77%

SPECIFICITY : 95%

KAPPA : 0.7390

ACCURACY : 88%

WSL virus

D1:F1 ; D2:F1 ; D1:F2 ; D2:F2.

		IF TEST +	-	TOTAL
dot-ELISA	+	7	5	12
	-	6	82	88
	TOTAL	13	87	100

SENSITIVITY	:	54%
SPECIFICITY	:	94%
KAPPA	:	0.4900
ACCURACY	:	89%

D1:HI; D2:HI.

		HI +	-	TOTAL
dot-ELISA	+	3	2	5
	-	9	19	28
	TOTAL	12	21	33

SENSITIVITY	:	25%
SPECIFICITY	:	90%
KAPPA	:	0.2303
ACCURACY	:	67%

PATIENT	LOCATION	RVF IgM					SIN IgM					WN IgM					WSL IgM				
		D1	D2	F1	F2		D1	D2	F1	F2		D1	D2	F1	F2	NIV	D1	D2	F1	F2	
1	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
2	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
3	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
4	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
5	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
6	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
7	Petrus Steyn	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
8	Hospital	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
9	Hospital	-	-	-	-		-	-	-	-		+	+	+	+	+	-	-	-	-	
10	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
11	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
12	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
13	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
14	Hospital	+	+	+	+		-	-	-	-		-	-	-	-		-	-	-	-	
15	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
16	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
17	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
18	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
19	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
20	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
21	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
22	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	

D1=dot-ELISA observer 1 D2=dot-ELISA observer 2 F1=IF test observer 1 F2=IF test observer 2 NIV=National Institute for Virology

PATIENT	LOCATION	RVF IgM					SIN IgM					WN IgM					WSL IgM				
		D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI
23	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
24	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
25	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
26	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
27	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
28	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
29	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
30	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
31	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
32	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
33	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
34	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
35	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
36	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
37	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
38	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
39	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
40	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
41	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
42	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
43	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
44	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	

D1=dot-ELISA observer 1 D2=dot-ELISA observer 2 F1=IF test observer 1 F2=IF test observer 2 HI=Haemagglutination Inhibition

PATIENT	LOCATION	RVF IgM					SIN IgM					WN IgM					WSL IgM				
		D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI
45	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
46	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
47	Hospital	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
48	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
49	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
50	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
51	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
52	Valspan	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
53	Preezfontein Noord	-	-	-	-		+	+	+	+		-	-	-	-		-	-	-	-	
54	Preezfontein Noord	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
55	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
56	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
57	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
58	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
59	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
60	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
61	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
62	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
63	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
64	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
65	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
66	Hospital	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	

D1=dot-ELISA observer 1 D2=dot-ELISA observer 2 F1=IF test observer 1 F2=IF test observer 2 HI=Haemagglutination Inhibition

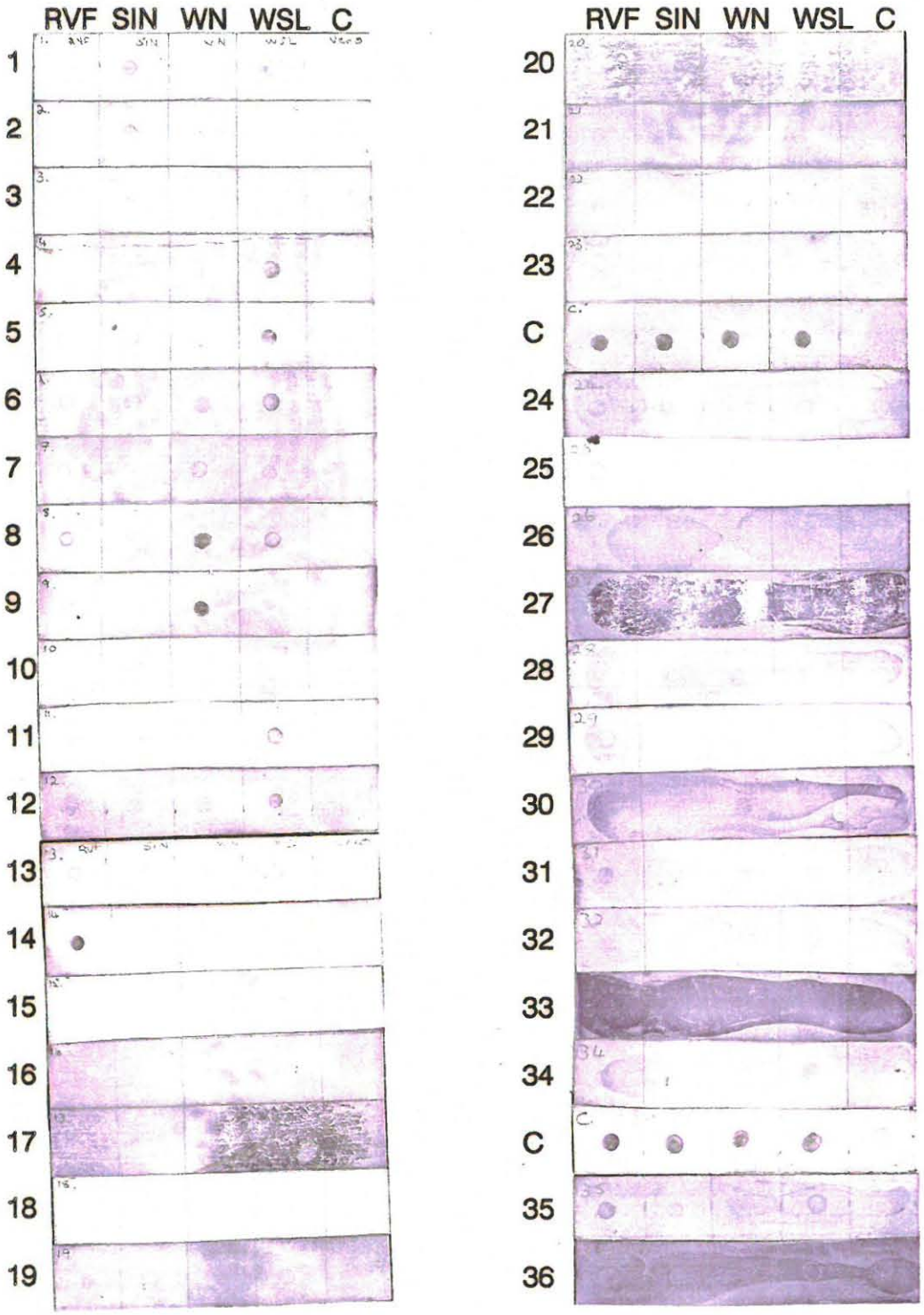
PATIENT	LOCATION	RVF IgM					SIN IgM					WN IgM					WSL IgM				
		D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI
67	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
68	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
69	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
70	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
71	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
72	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
73	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
74	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
75	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
76	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
77	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
78	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
79	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
80	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
81	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
82	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
83	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
84	Kareelaagte	-	-	-	-		-	-	-	-		+	+	+	+		-	-	-	-	
85	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
86	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
87	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
88	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	

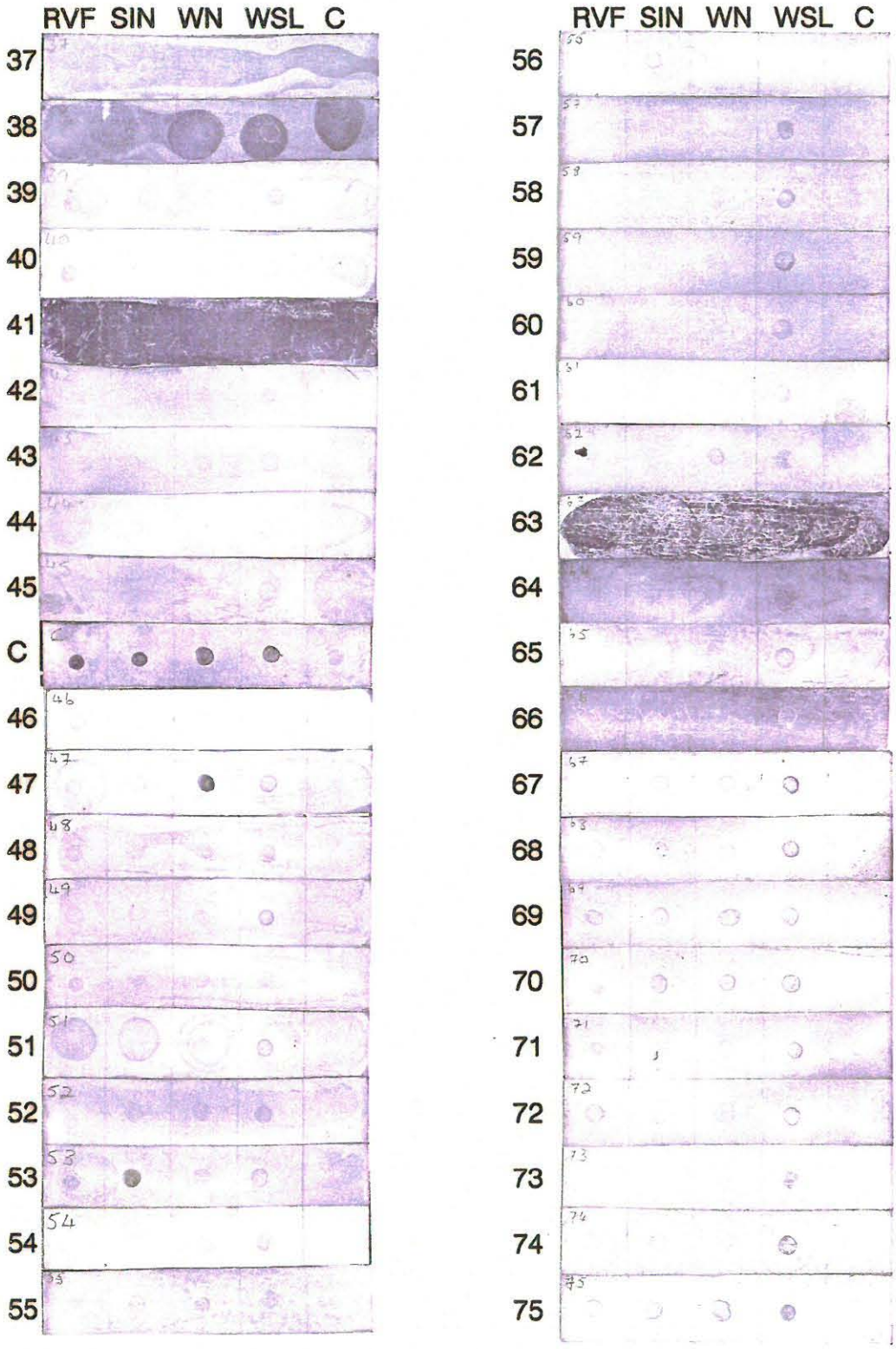
D1=dot-ELISA observer 1 D2= dot-ELISA observer 2 F1=IF test observer 1 F2=IF test observer 2 HI=Haemagglutination Inhibition

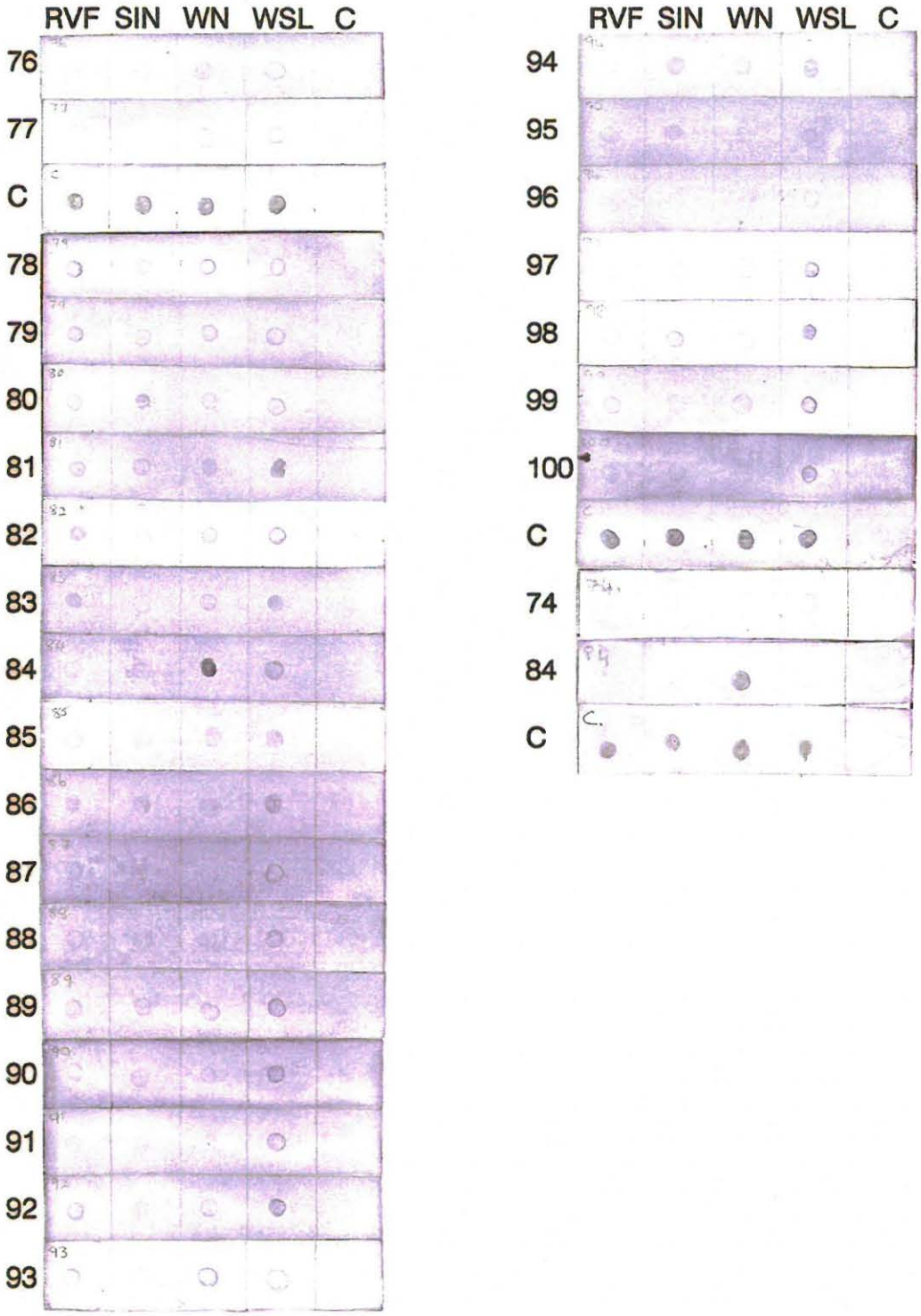
PATIENT	LOCATION	RVF IgM					SIN IgM					WN IgM					WSL IgM				
		D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI	D1	D2	F1	F2	HI
89	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
90	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
91	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
92	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
93	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
94	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
95	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
96	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
97	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
98	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
99	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
100	Kareelaagte	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	

D1=dot-ELISA observer 1 D2=dot_ELISA observer 2 F1=IF test observer 1 F2=IF test observer 2 HI=Haemagglutination Inhibition

2.2. DOT-ELISA RESULTS







2.3. STATISTICAL ANALYSIS OF SEROLOGICAL RESULTS

RVF virus

D1:F1; D1:F2; D2:F1; D2:F2.

		IF TEST +	IF TEST -	TOTAL
dot-ELISA	+	1	0	1
	-	0	99	99
	TOTAL	1	99	100

SENSITIVITY : 100%

SPECIFICITY : 100%

KAPPA : >0.75

ACCURACY : 100%

WN virus

D1:F1; D1:F2; D2:F1; D2:F2.

		IF TEST +	IF TEST -	TOTAL
dot-ELISA	+	4	0	4
	-	0	96	96
	TOTAL	4	96	100

SENSITIVITY : 100%

SPECIFICITY : 100%

KAPPA : >0.75

ACCURACY : 100%

SIN virus

D1:F1; D1:F2; D2:F1; D2:F2.

		IF TEST +	IF TEST -	TOTAL
dot-ELISA	+	1	0	1
	-	0	99	99
	TOTAL	1	99	100

SENSITIVITY : 100%

SPECIFICITY : 100%

KAPPA : >0.75

ACCURACY : 100%

WSL virus

D1:F1; D1:F2; D2:F1; D2:F2.

		IF TEST +	-	TOTAL
dot-ELISA	+	0.05*	0	0.05
	-	0	100	100
	TOTAL	0.05	100	100.05

SENSITIVITY	:	100%
SPECIFICITY	:	100%
KAPPA	:	>0.75
ACCURACY	:	100%

*Mathematical manipulation

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