THE ISOLATION AND IMPORTANCE OF SIMBU GROUP VIRUSES IN SOUTH AFRICA

by

VASCO MANUEL DA COSTA MENDES

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF M.MED.VET.(VIR.) IN THE FACULTY OF VETERINARY SCIENCE UNIVERSITY OF PRETORIA, PRETORIA 0002

OCTOBER 1984

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ACKNOWLEDGMENTS

I am deeply grateful to my Promoter, Professor P. G. Howell, Head of the Department of Infectious Diseases of the Faculty of Veterinary Science for his encouragement, continuous support, guidance and tuition, and also for his patience and assistance during the preparation of this manuscript.

I am indebted to the Director of the Veterinary Research Institute, Dr. R. D. Bigalke for permission to carry out the studies without which this work would not have been possible.

I owe a debt of gratitude to the Head of the Virology Department, DR. B. J. Erasmus for his stimulating influence, advice and interest during the course of this work and for time given and permission to utilize the Departamental facilities.

I would like to thank the Head of the Arbovirus Unit of the National Institute for Virology, Dr. B. M. McIntosh as well as Dr. Izabel dos Santos, for kindly suppling the reference viruses and sera of the Simbu serogroup.

I wish to convey my sincere thanks to Mr R. A. Wixley of the National Research Institute for Mathematical Sciences (C.S.I.R.)

for his excellent help with the statistical analysis of the data, performed in the Institute's computer.

I want to record my gratitude to my colleagues Dr. G. H. Gerdes and Dr. Regina Alves for their help.

I wish to express my thanks to Miss S. M. Geyer and Mrs G. Gossen and Mr L. M. Pieterse for the preparation of innumerable tissue cultures, to Mr S. T. Boshoff for his help in the serological reactions and to Mrs E. Erasmus for her help with the egg work.

My thanks are due to Mr J. J. Paulsen and his Staff for the care devoted to photographing the specimens and preparing the prints.

I would like to express my sincere thanks to Miss Heline Creydt and Mrs Geraldine Burns for their technical assistance in the laboratory work.

CHAPTER I

REVIEW OF THE LITERATURE

Arboviruses are a group of viruses that are transmitted to the susceptible vertebrate host through the bite of a haematophagous insect.

The *Arboviruses* were initially classified into three defined serological groups A, B and C on the basis of serological cross-reactions in the haemagglutination inhibition, complement fixation and serum-virus neutralization tests (Casals, 1957). As more and more isolates from all over the world were characterized, the term *Arboviruses* could only be used to define all the different viruses that were transmitted by haematophagous arthropods; however, on the basis of other properties, they were classified into different families. Andrewes (1970) proposed the name Togaviridae to include both the "A" and "B" groups of the previously designated *Arboviruses*. The *Alphaviruses* included all members of the group A of Casals & Brown (1954); these viruses, sensitive to deoxycholate and lipid solvents, are enveloped virions 40-80 nm in diameter, with a single-stranded RNA genome, in which haemagglutinin is unaffected by trypsin. The *Flaviviruses* embrace the members of group B ; they are smaller enveloped viruses 20-50 nm in diameter and in contradistinction to the

above group their haemagglutinin and infectivity is inactivated by trypsin.

With the isolation of further viruses and their characterization, additional serological groups emerged, with the result that a few years later, Casals & Whitman (1960) proposed a separate *Bunyamwera* group to include four previously unclassified viruses: Bunyamwera virus, isolated in Uganda from *Aedes* mosquitoes, Wyeomyia virus isolated in Colombia from *Wyeomyia melanocephala*, Cache Valley virus isolated in Utah from *Culiseta inornata* and Kairi virus from *Aedes scapularis* caught in Trinidad. Even at that time the authors anticipated that the *Bunyamwera* group of viruses would rapidly expand.

Later, these antigenically unrelated *Arboviruses* were grouped under the generic name *Bunyamwera Supergroup* (Casals, 1963). Further studies on several viruses of this serological supergroup led to the creation of a new taxonomic group, the family Bunyaviridae (Murphy, Harrison & Whitefield, 1973). This, new family of spherical enveloped virions, 80-110 nm in diameter have a negative single-stranded RNA genome arranged in three distinct nucleocapsids. From serological reactions and based on molecular differences between the viruses, four genera were established - *Bunyavirus*, *Nairovirus*, *Phlebovirus* and *Uukuvirus*.

The *Bunyavirus* genus includes at least 124 viruses, mainly mosquito-transmitted, distributed into 13 serogroups plus three

unassigned viruses (Bishop, Calisher, Casals, Chumakov, Gaidamovich, Hannoun, Lvov, Marshall, Oker-Blom, Pettersson, Porterfield, Russell, Shope & Westaway, 1980).

The Simbu serogroup of the *Bunyavirus* includes 24 members, 19 registered and five unregistered . This large group of viruses shows extensive cross-reactions in the complement fixation test, making them indistinguishable from each other. As a result Kinney & Calisher (1981) proposed their allocation into five "complexes" - the Simbu "complex" with Simbu, Akabane, Yaba-7, Shamonda, Sabo, Tinaroo, Sango, Peaton, Aino and Sathuperi viruses; the Manzanilla "complex" including Manzanilla, Ingwavuma, Mermet, Inini and Buttonwillow viruses; the Oropouche "complex" which is formed by Oropouche, Utinga, Utive and Facey's Paddock viruses. Because Thimiri and Nola viruses are distinct in the complement fixation test, these two viruses are the sole members of the Thimiri "complex" and Nola "complex" respectively.

A review of the literature reveals that these viruses have a world-wide distribution. Manzanilla virus, the first of the Simbu serogroup to be mentioned in the literature was recovered in Trinidad from the blood of a howler monkey *Alouatta insularis* (Anderson, Spence, Downs & Aitken, 1960). Manzanilla virus forms the first member of the Manzanilla "complex".

Simbu virus was originally isolated in South Africa from pools of *Aedes circumluteolus* mosquitoes caught in 1955 and 1957 at Lake Simbu and in the Cameroons in 1966, from a pool of

Eretmapodites chrysogaster mosquitoes (Weinbren, Heymann, Kokernot & Paterson, 1957; Brooke Worth, Paterson & de Meillon, 1961; Salaün, Rickenbach, Bres, Brottes, Germain, Eouzan & Ferrara, 1969). Simbu, the second viral isolate, gave its name to the Simbu serogroup and "complex".

The third member of the Simbu serogroup, Oropouche virus, was first isolated in 1955 in Trinidad from a human serum and from a pool of *Coquillettidia venezuelensis* mosquitoes (Anderson, Spence, Downs & Aitken, 1969). In Brazil, Oropouche virus has been recovered from the blood of human patients, from the three-toed sloth *Bradypus tridactylus*, a pool of *Aedes serratus* and from separate pools of *Culex fatigans* and *Culex pipiens quinquefasciatus* mosquitoes. During an epidemic in 1975 the virus was recovered from the biting midge *Culicoides paraensis* (Pinheiro, Pinheiro, Bensabath, Causey & Shope, 1962; Pinheiro, Bensabath, Andrade & Woodal, 1968; Pinheiro, Rosa, Rosa & Bensabath, 1976; Pinheiro, Rosa, Rosa, Ishak, Freitas, Gomes, LeDuc & Oliva, 1981; Borborema, Pinheiro, Albuquerque, Rosa, Rosa & Dourado, 1982). From 1961 to 1978 several epidemics of Oropouche fever affecting more than 39 000 persons were recorded in the Amazon region, mainly in Para State. A more recent outbreak affecting more than 96 000 persons occured between 1980 and 1981 in Manaus. Epidemiological and entomological observations during these epidemics, together with the results of experimental transmission, suggest that because it is a more efficient vector than *Culex quinquefasciatus* mosquitoes, the biting midge *Culicoides paraensis* is the primary urban vector of Oropouche virus in Brazil. In Trinidad,

Colombia and in Brazil, neutralizing antibodies to Oropouche virus were found in humans, several species of primates and in wild and domestic birds (Dixon, Rosa, Rosa & Llewellyn, 1981; Roberts, Hoch, Dixon & Llewellyn, 1981; Pinheiro, Hoch, Gomes & Roberts, 1981). Oropouche virus gave its name to the Oropouche "complex" .

The fourth isolate, Sathuperi virus, was originally isolated in India from a pool of *Culex vishnui* mosquitoes and in Nigeria from dairy cattle and pools of *Culicoides spp.* (Dandawate, Rajagopalan, Pavri & Work, 1969; Causey, Kemp, Causey & Lee, 1972; Lee, 1979). A serological survey conducted on 4 to 9 year old Nigerian cattle showed that 17 out of 24 had neutralizing antibodies. Sathuperi virus is included in the Simbu "complex".

The fifth isolate, Ingwavuma virus, was originally recovered in South Africa from pooled organs of a spectacled weaver *Hyphanturgus ocularius* as well as from a pool of *Culex univittatus* mosquitoes. Later the virus was isolated in India from the blood of a paddy bird *Ardeola grayii* and in Cyprus from another bird *Muscicapa striata*. In Nigeria the virus was recovered from the pooled organs of another bird *Plesiositraga cucullatus*, while in Thailand the virus was isolated from a pool of *Culex vishnui* mosquitoes and from the blood of pigs. In Taiwan the virus was also recovered from pig blood. This species seems to be an important host, since in Thailand 16 out of 50 porcine sera had neutralizing antibodies (McIntosh, McGillivray & Dickinson, 1965; Pavri, Sheikh, Singh, Rajagopalan & Casals, 1969; Watson, Shope & Kaiser, 1969; Causey *et al.*,

1972; Gould, Edelman, Grossman, Nisalak & Sullivan, 1974; Top, Kraivapan, Grossman, Rozmiarek, Edelman & Gould, 1974). Ingwavuma virus has been placed in the Manzanilla "complex".

Akabane virus, of the Simbu "complex", was originally recovered in 1959 in the Gumma Prefecture, Japan, from separate pools of *Aedes vexans* and *Culex tritaeniorhynchus* mosquitoes. In 1968, the virus was isolated in Australia from pools of *Culicoides brevitarsis* and in 1972 in Kenya from a pool of *Anopheles funestus* mosquitoes (Matsuyama, Oya, Ogata, Kobayashi, Nakamura, Takahashi & Kitaoka, 1960; Doherty, Carley, Standfast, Dyce & Snowdon, 1972; Metselaar & Robin, 1976).

Buttonwillow virus was the first North American Simbu group isolate, later to be placed in the Manzanilla "complex". It was originally isolated from the blood of a desert cottontail *Sylvilagus auduboni*, from a blacktail jackrabbit *Lepus californicus* and from pools of parous *Culicoides variipennis* (Reeves, Scrivani, Hardy, Robberts & Nelson, 1970; Nelson & Scrivani, 1972). Haemagglutination inhibiting antibodies were found in *Lepus californicus* from California, Montana and Utah, in *Lepus americanus* from Alberta, in *Sylvilagus auduboni* from Alberta and Wisconsin and in one Canadian marmot.

The first Nigerian isolate belonging to the Simbu serogroup, Yaba-7 virus, was recovered from a pool of *Mansonia africana* mosquitoes in 1963. Yaba-7 virus was placed in the Simbu "complex".

Thimiri virus was originally recovered in India from the blood of a paddybird *Ardeola grayii*. In Egypt, in 1963 and in 1966, it was isolated from the blood of southward migrating birds, namely a whitethroat *Sylvia communis* and a lesser whitethroat *Sylvia curruca* . Later, the virus was isolated in Northern Australia from a pool of *Culicoides histrio* (Carey, Reuben, George, Shope & Myers, 1971; Darwish & Hoogstraal, 1981; Standfast & Dyce, 1982). Thimiri virus is to date the only member of the Thimiri "complex".

Aino virus, also of the Simbu "complex", was originally isolated in Japan from pools of *Culex tritaeniorhynchus* and from a mixed pool of *Culex pipiens* and *Culex pseudovishnui* mosquitoes and later in Australia from pools of *Culicoides brevitarsis* (Takahashi, Oya, Okada, Matsuo, Kuma & Nogushi, 1968; Doherty *et al.*, 1972).

In North America, Mermet virus was originally isolated from the blood of a purple Martin, a red winged blackbird, a Swayson's thrush and a cardinal, while another strain was obtained from the pooled organs of a Blue Jay and more recently, from separate pools of *Culex pipiens* and *Culex restuans* mosquitoes (Calisher, Kokernot, De Moore, Boyd, Hayes & Chappel, 1969; Jakob, Francy, Trimble & Calisher, 1979). According to the most recent work, Mermet virus is included in the Manzanilla "complex".

The next Brazilian Simbu serogroup isolate, Utinga virus, was first recovered in 1965 from whole blood and organs of a

three-toed sloth *Bradypus tridactylus*. Utinga virus was placed in the Oropouche "complex".

Sango virus was originally recovered from the blood of cattle at Ibadan, from calves in Northern Nigeria, from pools of *Culicoides spp.* caught at Ibadan and from pools of *Mansonia uniformis* in Kenya. Neutralizing antibodies were found in dairy cattle as well as in 13 out of 20 trade cattle and in 12 out of 20 goats (Causey, Kemp, Madbouly & Lee, 1969; Kemp, Causey & Causey, 1971; Causey *et al* ., 1972; Kemp, Causey, Moore & O'Connor, 1973; Metselaar, Henderson, Kirya, Tukei & De Geus, 1974; Lee, 1979). Sango virus was placed in the Simbu "complex".

Shamonda virus, a Simbu "complex" isolate , was recovered from cattle blood collected at Ibadan and in Northern Nigeria and from pools of *Culicoides spp.* and of *Culicoides imicola* caught at Ibadan. Neutralizing antibodies were found in a sentinel group of dairy cows and in 25 out of 28 market cattle (Causey *et al.*, 1969; Kemp *et al.*, 1971; Causey *et al.*, 1972; Kemp *et al.*, 1973; Lee, 1974).

Another Nigerian member of the Simbu "complex", Sabo virus, was isolated from the blood of a goat, a calf and from pools of *Culicoides spp.* and of *Culicoides imicola.* Neutralizing antibodies were found in 25 out of 28 market cattle, in four out of 20 sheep and in four out of 20 pigs (Causey *et al.*, 1969; Kemp *et al.*, 1971; Causey *et al.*, 1972; Kemp *et al.*, 1973; Lee, 1979).

Shuni virus was isolated from the blood of sheep from Ibadan, cattle from Northern Nigeria and from pools of *Culicoides spp.* . Neutralizing antibodies were found in dairy cattle, in 25 out of 28 trade cattle and in 23 out of 72 sheep (Causey *et al.*, 1969; Causey *et al.*, 1972; Kemp *et al.*, 1973; Lee, 1979). Of interest is the fact that Shuni virus was once recovered from the blood of a 18 month old Nigerian child (Moore, Causey, Carey, Cooke, Akinkugbe, David-West & Kemp, 1975). This virus was also recovered in South Africa from pools of *Culex theileri* mosquitoes caught near Johannesburg and from cattle and a goat in Empangeni, Natal (McIntosh, 1972; McIntosh, 1980). Because of its serological cross-reaction with Aino virus, Shuni is considered as a subtype of Aino virus.

Nola virus was recovered from a pool of *Culex perfuscus* mosquitoes caught in the Central African Republic. This virus shows a tenuous relationship with the Simbu serogroup through a one-way haemagglutination inhibition reaction with Oropouche virus, but no cross-reaction by complement fixation or neutralization with other members of the serogoup. It is thus the sole member of the Nola "complex".

Kaikalur virus was recovered from a pool of *Culex tritaeniorhynchus* mosquitoes collected in Khrisna district, India (Rodrigues, Singh, Dandawate, Soman & Bhatt, 1977). Due to a very close two-way cross-reaction, Kaikalur and Aino viruses are considered identical or varieties of a single virus.

The next member of the Simbu serogroup, Inini virus, was

recovered from the blood of a bird *Pteroglossus aracari* caught in French Guiana, South America (Digoute, 1973; Digoute, 1975). Inini virus has been placed in the Manzanilla "complex".

A member of the Oropouche "complex", Facey's Paddock virus, was originally isolated in Australia from separate pools of *Culex annulirostris* and *Aedes normanensis* mosquitoes and more recently from pools of *Culicoides spp.*. Neutralizing antibodies were found in cattle and wallabies, *Wallabia agilis* but not in man, dogs, dingoes, domestic fowls, buffalo or pigs (Doherty, Carley, Filippich, Barrow, Wilson & Brown, 1975; Doherty *et al.*, 1977; Doherty, Carley, Kay, Filippich, Marks & Frazier, 1979).

A new Australian member of the Simbu "complex", Peaton virus, was recovered from pools of *Culicoides brevitarsis* and from the blood of cattle (St George, Cybinski, Filippich & Carley, 1979; St George, Standfast, Cybinski, Filippich & Carley, 1980). Neutralizing antibodies were found in cattle, sheep, goats, buffalo and pigs, but not in camels, dogs, marsupials, reptiles, wild animals or in 76 human sera. After experimental infection, there is evidence of the pathogenicity of Peaton virus for the ovine foetus in which congenital defects were observed, while serum neutralizing antibodies were found in both the dam and foetus (Parsonson, McPhee & Della-Porta, 1982).

A brief communication from Australia reported two new candidates of the Simbu serogroup, Douglas virus and Tinaroo virus. Douglas virus was recovered from the blood of an apparently healthy cow

and later from pools of *Culicoides brevitarsis* . Tinaroo virus was recovered from pools of *Culicoides brevitarsis* and later from cattle blood. In contrast to other Australian Simbu serogroup isolates, neutralizing antibodies to Douglas virus were only found in bovine sera obtained from New Guinea. In Australia, neutralizing antibodies to both viruses were found in cattle, sheep, goats, buffalo, deer, while human, pig, kangaroo and wallabies were negative. There is no evidence that both viruses are pathogenic for adult animals, but a lamb with arthrogryposis had neutralizing antibodies to Tinaroo virus. Both these viruses are included in the Simbu "complex", and Douglas virus, because of its serological cross-reactions, is considered to be a subtype of Sathuperi virus (St George *et al.*, 1979; Cybinski, 1984).

The most recent candidate of the Simbu serogroup recovered in Panama, from the blood of a three-toed sloth *Bradypus variegatus* was named Utive virus, after a local village of that name (Seymour, Peralta & Montgomery, 1983). In cross-complement fixation tests, Utive is indistinguishable from Utinga and Pintupo viruses. This latter virus was isolated in Panama from the biting midge *Culicoides diabolicus* . By plaque reduction neutralization tests, these three viruses are nevertheless distinct from each other and Utive virus has been placed in the Oropouche "complex" . In Panama, neutralizing antibodies were only observed in sloths, but no antibodies were found in bird or mammal plasma. Utive virus is less pathogenic for mice than the related Utinga and Pintupo viruses and is furthermore not pathogenic for suckling hamsters when injected intracerebrally.

From this review it would appear that the viruses within the Simbu serogroup are not only geographically widely distributed, but are also present in a wide range of invertebrate and vertebrate hosts. It is also apparent that their pathogenicity varies from one member to another. The identification of natural hosts and vectors, within particular ecological niches, from dry deserts to tropical forests, only serves to emphasize the importance of establishing the potential pathogenicity of these viruses when man and his domesticated animals intrude and are accidentally involved in the cycle of transmission.

CHAPTER II

PRELIMINARY IDENTIFICATION OF VIRUSES

INTRODUCTION

In March/April 1967 in the Vryburg District, an unusually high incidence of a hitherto unrecognised disease amongst cattle was reported. The affected animals exibited conjunctival and periorbital oedema, swollen lips and tongue, but no febrile reaction was observed. During the outbreak both sexes and all age groups were affected. Specimens sent to the Virology Section of the Veterinary Research Institute, Onderstepoort, were subinoculated into cattle. From these recipient cattle three viruses were recovered, one of them, subsequently called "Lambrecht virus", was studied by Dr.I.M. Solberg for the purpose of identification and characterization.

Solberg (1970) demonstrated that Lambrecht virus was very sensitive to chloroform, sodium deoxycholate and low pH. Furthermore, BHK infected cells stained with acridine-orange showed an intense intracytoplasmic orange-red fluorescence, while the multiplication of Lambrecht virus in this cell system was not affected by the presence of BUDR or of Actinomycin D.

To establish the pathogenicity of Lambrecht virus for domestic ruminants, 27 cattle and 25 sheep were inoculated with a fresh mouse brain suspension of the sixth to the ninth suckling mouse

brain passage. After inoculation, the animals were kept in an open shelter at the Institute. The 12 Merino sheep, 10 pregnant Dorper ewes and three newborn lambs remained clinically normal and afebrile. Later, the pregnant ewes gave birth to normal lambs. A viraemia of very short duration occured in the first 4 days post-inoculation and Lambrecht virus was reisolated from 13 of the 25 inoculated sheep. Only two cattle showed a mild febrile reaction, one on day eleven (7507) and the other on day eighteen (7519). From blood samples collected daily, viruses were recovered from seven of the animals. Four of the viruses which were identified according to the donor (7507, 7519, 8912, 1262) were not neutralized by Lambrecht hyperimmune ascitic fluid.

From light traps placed in several locations at the Institute wild caught midges (*Culicoides spp.*) yielded several additional viruses identified as CUL 1/69, 2/69, 3/69, 4/69, 1/70.

In cross-complement fixation tests, Solberg demonstrated that the four bovine isolates and one of the Culicoides isolates (CUL 1/70) as well as Lambrecht virus all shared a common group specific complement fixing antigen. Although Solberg was unable to classify Lambrecht virus, he concluded that it had characteristics of a typical Arbovirus. Since these viruses had not been identified and classified, it was considered important to characterize the bovine and Culicoides isolates and this investigation constitutes the primary objective of this study.

MATERIALS AND METHODS

VIRUSES- the virus isolates used in this study are listed in Table 1 .

TABLE 1 - Viruses from cattle and biting midges (*Culicoides spp.*) isolated during 1969/1970.

NUMBER OR NAME	SAMPLE RECEIVED FOR CHARACTERIZATION FROM: CATTLE	CULICOIDES SPP.
LAMBRECHT	*#6SMB	
7507	#5SMB	
7519	#5SMB	
8912	#4SMB	
1262	Blood Sample	
CUL 1/70		#3SMB

* # SMB = passage level in suckling mouse brain.

Virus 1262- The original blood sample, diluted 1:2 in BLP (M/50 phosphate buffer pH 7,5 with 5% lactose and 1% neutral peptone) was inoculated intracerebrally into 10 families of suckling mice. On the fourth day post-inoculation, a mouse showing signs of encephalitis was sacrificed and the brain removed aseptically. A 10% suspension was prepared in BLP and cleared by centrifugation at 4 000 rpm/30 minutes. The clear supernatant was diluted 1:2 in BLP and reinoculated into 10 families of

suckling mice. On the fourth day post-inoculation, some mice were showing signs of encephalitis. The brains were removed aseptically, pooled and a 10% suspension in BLP was prepared and centrifuged as before. The clear supernatant was distributed into glass ampoules in 0,5 ml aliquots and lyophilized. The sealed ampoules were stored at -20°C.

Lambrecht, 7507, 7519, 8912, 1262 and CUL 1/70 viruses- As these isolates had been previously passaged in suckling mouse brain, a 10^{-3} dilution of the lyophilized sample was prepared in BLP and inoculated into suckling mice. By the third day all mice were showing signs of encephalitis. The brains were removed aseptically, pooled and a 10% suspension in BLP was prepared. After centrifugation at 4 000 rpm/30 minutes, the clear supernatant was distributed into glass ampoules in 0,5 ml aliquots and lyophilized. The sealed ampoules were kept at -20°C until required.

PRELIMINARY ATTEMPTS TO ESTABLISH A RELATIONSHIP BETWEEN THESE VIRUS ISOLATES

In his studies Solberg showed by the complement fixation test, that Lambrecht virus was not related to Rift Valley Fever, Wesselsbron, Ephemeral Fever and Bluetongue viruses. In order to establish the antigenic relationship between these six virus isolates, cross-complement fixation and cross serum-virus neutralization tests were conducted.

ANTIGENS - limiting dilution passages were carried out in

suckling mice with all the isolates. Tenfold dilutions of each antigen were prepared in BLP and each dilution was inoculated intracerebrally into one family of suckling mice. The first mice inoculated with the highest dilution and showing symptoms of encephalitis were collected. The brains were removed aseptically and a 10% suspension was made. After clearing by centrifugation at 4 000 rpm/30 minutes, the supernatant was diluted tenfold in BLP and reinoculated intracerebrally into suckling mice. This procedure was repeated three times. The last limiting dilution passage of each antigen was diluted 10^{-3} in BLP and inoculated intracerebrally into 30 families of suckling mice, which were sacrificed as soon as they showed signs of encephalitis. The brain tissue was subdivided into two aliquots. One was used to prepare mouse ascitic fluid (MAF) containing homologous specific antibody while the other was used to prepare the antigen by the sucrose-acetone method of Casals (1967) for the complement fixation test.

PREPARATION OF MOUSE ASCITIC FLUID CONTAINING HOMOLOGOUS SPECIFIC ANTIBODY - According to the method of Sommerville (1967) 50 adult female albino mice per antigen were inoculated intraperitoneally with 0,4 ml of the virus, consisting of a cleared 10% brain suspension in BLP. As outlined in Table 2, equal volumes of PBS or 10% virus suspension were mixed with complete or incomplete Freund's adjuvant. The ascitic fluid was withdrawn by paracentesis using a 14 Gauge needle attached to a 10 ml syringe. Fluid was collected 4 days after the fifth inoculation, 3 days after the sixth and 3 days after the seventh. The fluid was pooled and cleared by centrifugation at

10 000 rpm/30 minutes. The supernatant was collected and dispensed in ampoules in 0,5 ml aliquots, lyophilized and stored at -20°C.

A control ascitic fluid was similarly produced using normal brain as antigen. Before use in all tests, the lyophilized MAF was reconstituted with sterile distilled water and immediately inactivated at 56°C for 30 minutes.

TABLE 2 - Schedule of injections for the preparation of mouse ascitic fluid.

INJECTION NUMBER	DAY OF INOCULATION	I N O C U L U M
1	1	* Adjuvant 1 + P B S
2	14	** Adjuvant 2 + Virus suspension
3	21	Adjuvant 2 + Virus suspension
4	25	Adjuvant 2 + Virus suspension
5	30	Adjuvant 1 + Virus suspension
6	37	Adjuvant 1 + P B S
7	42	Adjuvant 1 + P B S

* Adjuvant 1: consisted of Freund's complete adjuvant (DIFCO) plus 5 mgm % of live *Mycobacterium smegmatis*

** Adjuvant 2: consisted of Freund's incomplete adjuvant (DIFCO)

PREPARATION OF ANTIGEN FOR THE COMPLEMENT FIXATION TEST- Each antigen was extracted by the sucrose-acetone method of Casals (1967). Each gram of brain tissue was homogenized with 4 volumes (4 ml) of chilled 8,5% aqueous solution of sucrose. This homogenate was added dropwise to 20 volumes of chilled acetone, stirring continuously and then centrifuged at 1 500 rpm/5 minutes. After carefully decanting the supernatant acetone, the sediment was resuspended in fresh chilled acetone to the original volume. The sediment was stirred and the bottle held at 4°C in an ice bath for 1 hour, shaking vigourously to avoid sedimentation. After centrifugation at 1 500 rpm/5 minutes, the supernatant acetone was decanted and the sediment dried under vacuum. The dried sediment was then resuspended in 0,9% NaCl solution using 4/10 the volume of the original homogenate. The rehydrated extract was held overnight at 4°C and centrifuged at 10 000 rpm/60 minutes. The supernatant, which constituted the antigen, was dispensed in 0,5 ml aliquots, lyophilized and the sealed ampoules stored at -20°C.

DILUENT - Veronal buffer was prepared according to the formula of Mayer, Osler, Bier & Heildelberger (1946).

COMPLEMENT - guinea-pig serum lyophilized and standardized in preliminary tests was used as a source of complement. The highest dilution showing complete lysis was taken as one unit of complement. In the tests two units were used.

SHEEP ERYTHROCYTES - the blood cells were collected in Alsever's solution and kept for no longer than 1 week at 4°C. The cells

were used as a 2% suspension in the test.

HAEMOLYSIN - was obtained from adult rabbits immunized intravenously with a 20% washed sheep erytrocyte suspension. Seven injections were given at 3 day intervals and 10 days after the last injection the rabbits were bled, the serum separated and inactivated at 56°C for 30 minutes. Therefater it was mixed with an equal volume of glycerol and stored at -20°C in sealed ampoules. Haemolysin was titrated in the presence of two units of complement and the highest dilution giving complete haemolysis was taken as one unit. In all subsequent tests two units were used.

PROCEDURE - the test was performed according to a standard technique. In the primary reaction, unit volume of antigen and of a serum dilution, together with two units of complement were allowed to interact at 37°C for 90 minutes. In the secondary reaction, one unit volume of sensitized erytrocytes was added and the test reincubated at 37°C for 30 minutes.

CROSS SERUM-VIRUS NEUTRALIZATION TESTS - fivefold dilutions of each of the six antigens prepared in BLP were mixed with an equal volume of undiluted specific M A F and held at 37°C for 1 hour. A similar series of virus dilutions were mixed with an equal volume of normal MAF. Suckling Swiss albino mice were inoculated intracerebrally with 0,03 ml of the serum-virus mixtures. Specific mortality was recorded after 11 days and endpoints were calculated by the method of Reed & Muench (1938).

RESULTS

In Table 3 the endpoint dilutions of the fixation of complement by each mouse ascitic fluid is presented.

TABLE 3 - Cross-complement fixation between viral isolates

ANTIGEN NUMBER / NAME	*DILUTION	IMMUME MOUSE ASCITIC FLUID 7507	7519	8912	1262	CUL 1/70	LAMBRECHT
7507	1:64	**1024	768	384	171	171	214
7519	1:32	1024	638	384	106	85	214
8912	1:32	1024	384	384	85	54	106
1262	1:32	192	26	26	1336	1336	214
CUL 1/70	1:64	64	26	26	1336	1336	106
LAMBRECHT	1:16	106	96	106	106	106	1706

* Represents two units

** Reciprocal of the dilution fixing 50% of the complement.

As all the viruses shared a common group antigen, they were considered to belong to the same serogroup. It was possible, however, to distinguish three "subgroups". The first incorporates the three cattle isolates 7507, 7519 and 8912, of which the latter strain was used as the prototype for all future investigations. The second "subgroup" is formed by the cattle

virus isolate 1262 as well as an isolate from *Culicoides spp.* (CUL 1/70). This latter strain was also used as the prototype in subsequent studies. Lambrecht virus is the only member of "subgroup" three. These results also show that the mouse ascitic fluids contained very high concentrations of antibody.

In Table 4 the results of the cross serum-virus neutralization tests of the six isolates are presented.

TABLE 4 - Cross serum-virus neutralization tests with the viral isolates from cattle and from *Culicoides spp.*

	TITER IN NORMAL ASCITIC FLUID	HYPERIMMUME MOUSE ASCITIC FLUID					
VIRUS		7507	7519	8912	1262	CUL 1/70	LAMBRECHT
7507	*7,2	**7,2	7,2	7,2	0,1	0,6	1,2
7519	6,7	6,7	6,7	6,7	0,8	1,6	1,6
8912	5,6	5,6	5,6	5,6	0,0	0,0	0,3
1262	8,0	0,8	0,8	0,8	8,0	3,0	2,8
CUL 1/70	7,2	0,0	0,4	0,1	7,2	7,2	1,8
LAMBRECHT	7,0	0,8	1,4	1,5	0,3	2,0	7,0

* Reciprocal of endpoint dilution giving 50% mortality/0,03 ml

** Log Neutralization Index

In contradistinction to the results obtained with the complement fixation test, these results demonstrate the antigenic specificity of the homologous antibody-virus interaction and there would appear to be only negligible cross-reactions between the strains of each serogroup. This test would therefore appear to be most suitable for serological surveys and the detection of infection in various species.

With these results all the isolates selected for this primary investigation can now be allocated into one of three "subgroups":

SUBGROUP		
1	2	3
7507	1262	LAMBRECHT VIRUS
7519	CUL 1/70	
8912		

DISCUSSION

In March-April 1967 after a good summer rainfall, an unusual disease in cattle was reported in the Vryburg District, the high incidence of which was related to an increase in the insect population.

From specimens sent to the Virology Section of the Veterinary Research Institute at Onderstepoort, Lambrecht virus was recovered and although not fully characterized, Solberg demonstrated that Lambrecht virus had properties typical of an "Arbovirus".

Unwittingly the study of the pathogenicity of the Lambrecht virus in cattle also provided a group of sentinels from which the other viruses were isolated, due to the fact that these animals were not accomodated in insect-proof stables. Because of the high level of insect activity shown by the light trap catches near the cattle, it was not therefore unexpected that viruses would be recovered from both vector and exposed hosts. It is all the more significant that the viruses obtained from the two sources appeared to belong to the same "group".

Results of the serological tests have confirmed their identity. The original observations of Solberg on the short duration of viraemia without obvious signs of clinical disease were confirmed in the sentinel cattle and sheep.

CHAPTER III

IDENTIFICATION OF VIRUSES

INTRODUCTION

A number of unavoidable factors led to the temporary suspension of this work and during this intervening period, as indicated in the review, it was shown that the Simbu serogroup of viruses was widespread and had been isolated all over the world.

One of these viruses, transmitted by *Culicoides paraensis* was responsible for extensive outbreaks of Oropouche fever among the human population of Brazil, South America, while serious outbreaks of abortion, premature births, stillbirths and congenital malformations (arthrogryposis and hydranencephaly) caused by Akabane virus, also of this serogroup, were reported in domestic ruminants in Japan, Australia and Israel (Markusfeld & Mayer, 1971; Inaba, Kurogi & Omori, 1975; Hartley, Wanner, Della-Porta & Snowdon, 1975). In Australia, the main vector of Akabane virus is the biting midge *Culicoides brevitarsis*.. Other Australian Simbu serogroup viruses, Aino (previously Samford virus), Tinaroo and Peaton were also suspected of being implicated in congenital abnormalities. In the light of these findings it was, therefore, considered necessary to establish the identity of the local viral isolates obtained from cattle and biting midges, in view of the fact that a preliminary test had indicated that one of them might belong to the serogroup.

MATERIALS AND METHODS

VIRUSES - In addition to the viruses listed in Table 5, the strains used in the previous experiments were also included in this investigation. Eight reference strains of the Simbu serogroup were kindly supplied by DR. B. McINTOSH, Head of the ARBOVIRUS UNIT of the NATIONAL INSTITUTE FOR VIROLOGY.

TABLE 5 - List of Simbu serogroup viruses used in the serum-virus microneutralization tests.

NAME OR NUMBER	REFERENCE	MOUSE PASSAGE LEVEL
AKABANE VIRUS	7648	# 2
INGWAVUMA VIRUS	AN 4165	# 23 (1057)
SABO VIRUS	ex 87234	# 8
SANGO VIRUS	ex 87237	# 4
SATUPERI VIRUS	ex 87491	# 22
SHAMONDA VIRUS	ex 87240	# 2
SHUNI VIRUS	ex 87244	# 3
SIMBU VIRUS	AR 53	# 5 (48109/A)

All these viruses were adapted to a cell line derived from the African green monkey (Vero cells) and two additional passages were made. The supernatant of the tissue culture was mixed with an equal volume of BLP, distributed in 0,5 ml volumes in glass ampoules, lyophilized and stored at -20°C.

TISSUE CULTURE- Vero cells were grown in Roux flasks, in Hank's-Eagles medium supplemented with 10% tryptose phosphate broth and 10 % bovine serum free of antibodies for these viruses. Before use, 200 IU/ml of Penicillin and 200 micrograms/ml of Streptomycin were added.

MOUSE ASCITIC FLUID - reference antisera to the known Simbu serogroup viruses were kindly supplied by Dr. B. McINTOSH. The ascitic fluid against the unknown viruses was prepared as previously described. The lyophilized material was reconstituted with sterile distilled water and immediately inactivated for 30 minutes at 56°C.

INFECTIVITY ASSAY - 0,05 ml serial tenfold dilutions of the viruses were delivered into wells each containing 0,1 ml of a cell suspension of $5x10^5$ cells/ml and 0,1 ml of Hank's-Eagles medium with bovine serum to give a final concentration of 2%. Five wells were used per dilution. The microplates were sealed with cellotape and incubated at 37°C for 5 days. The $TCID_{50}$ endpoint titer was calculated from the cytopathogenic effect recorded after 5 days.

For the neutralization test, 0,025 ml serial twofold dilutions of the hyperimmune ascitic fluid were mixed in wells with 0,025 ml medium containing 200 $TCID_{50}$/0,050 ml of virus. Two wells were used for each dilution of the ascitic fluid. The virus-ascitic fluid mixtures were incubated at room temperature for 1 hour. To each well, 0,1 ml of the Vero cell suspension and 0,1 ml of Hank's-Eagles medium with 2% bovine serum free of

antibody to Simbu group viruses was added. The microplates were sealed with cellotape and incubated for 5 days at 37°C. The serum antibody titer was expressed as the reciprocal of the highest ascitic fluid dilution completely inhibiting cytopathogenic effect.

RESULTS

Table 6 shows the results obtained in the cross serum-virus microneutralization tests between the Simbu serogroup viruses and the cattle and *Culicoides spp.* isolates.

TABLE 6 - Cross serum-virus microneutralization tests between Shuni, Sabo and Shamonda viruses and the cattle and *Culicoides spp.* isolates

HYPERIMMUNE	A N T I G E N S				
ASCITIC FLUIDS	SHUNI	8912	SABO	CUL 1/70	LAMBRECHT
SHUNI	*256	1024	0	0	0
8912	128	128	0	0	0
SABO	0	0	512	64	0
CUL 1/70	0	0	32	64	0
SHAMONDA	0	0	0	0	64
LAMBRECHT	0	0	0	0	128

* Reciprocal of the ascitic fluid dilution inhibiting CPE.

From these results it can be concluded that Lambrecht virus is a local strain of Shamonda virus and that the isolate 1262 from cattle and isolate CUL 1/70 from *Culicoides spp.* are local strains of Sabo virus. The three cattle virus isolates, 7507, 7519 and 8912 are local strains of Shuni virus.

DISCUSSION

The first report of the recovery of Simbu serogroup viruses from domestic ruminants in Africa, was in Nigeria in 1965/1966. The Shamonda prototype virus was recovered from cattle, the Sabo prototype from a goat and the Shuni virus from cattle. Subsequent Simbu serogroup viral isolates were recovered from cattle, sheep and *Culicoides spp.* (Shamonda, Sabo and Shuni) and also from *Culicoides imicola* (Shamonda and Sabo) (Causey *et al.*, 1969; Kemp *et al.*, 1971; Causey *et al* ., 1972; Kemp *et al.*, 1973; Lee, 1979).

It is of interest to note that one of the three viral isolates from the cattle specimens collected at Vryburg in the 1967 "epizootic" which was ascribed to the increased number of insects after the good summer rainfall was Lambrecht virus, the South African representative of Shamonda virus. In 1967, another Shamonda virus was also isolated in South Africa from *Culicoides imicola* caught at Kaalplaas, near Onderstepoort (McIntosh, 1980).

In sheep, Solberg found that Lambrecht virus produced an early viraemia of very short duration and a similar viraemia has been

produced by Akabane virus in cattle in Australia (St George, Standfast & Cybinski, 1978). It should be noted that the viraemia observed by Solberg in bovine 7507 on day 11 post-inoculation as well as that observed in bovine 7519 on day 18 post-inoculation, were in fact due to Shuni viruses, which are also transmitted by biting midges, as previously established. Other Shuni viruses were recovered after 1970 in South Africa from the mosquito *Culex theileri* caught at Olifantsvlei, as well as from cattle and from a goat in Natal (McIntosh, 1972; McIntosh, 1980).

The investigation of Solberg was made more complex by the fact that bovine 1262 was circulating Sabo virus at the commencement of the experiment, in addition to being experimentally inoculated with Shamonda virus. Simultaneously, yet another strain of Sabo virus was recovered from biting midges caught in the adjoining light traps.

There is thus conclusive evidence that viruses of the Simbu serogroup were active over a wide area of South Africa from as early as 1967, and it is unfortunate that predated serum samples are not available to establish the extent to which these viruses may have been present during earlier years.

CHAPTER IV

PATHOGENICITY

INTRODUCTION

The first reports of congenital malformations of the central nervous system of domestic animals were related to the vaccination of the dam in early pregnancy.

Pregnant sows immunized at 20 to 90 days of gestation with a rabbit-modified Hog cholera virus vaccine and tissue culture attenuated vaccine, gave birth to deformed piglets showing cerebellar hypoplasia, hypomyelinogenesis, small cerebral gyri, cerebellar agenesis and congenital tremors (Sauter, Young, Luedke & Kitchell, 1953; Young, Kitchell, Luedke & Sauter, 1955; Emerson & Delez, 1965; Done & Harding, 1966; Johnson, Ferguson, Byington & Redman, 1974).

In the field, more remarkable effects were seen associated with a chicken embryo attenuated live Bluetongue virus vaccine used to immunize pregnant ewes, during the critical fifth to seventh week of gestation. This resulted in increased stillbirths, neonatal deaths and deformed lambs which revealed various degrees of brain lesions from hypoplasia to hydranencephaly at necropsy. Similar congenital abnormalities can be produced experimentally in lambs, with lesions varying from hydranencephaly to subcortical cysts, depending on the

gestational age at which the dams are innoculated.

The severity of the lesions are directly related to the age of the foetus (Shultz & Delay, 1955; Cordy & Shultz, 1961; Young & Cordy, 1964; Richards & Cordi, 1967; Osburn, Silverstein, Prendergast, Johnson & Parshall, 1971; Osburn, Johnson, Silverstein, Prendergast, Jochim & Levi, 1971).

Bovine viral diarrhoea-mucosal disease virus produces abortions and stillbirths in cattle. In North America congenital malformations are most frequently seen when the dam is infected during the critical period of 100 to 170 days of gestation. Calves are born ataxic and blind, with cerebellar degeneration, severe cavitation and folial atrophy. Elsewere in the United Kingdom and Australia workers emphasized the severe hydranencephaly with cerebellar hypoplasia in some calves, or a marked hydrocephalus with cerebellar hypoplasia in others.

In sheep, the outcome of infection with this virus is also variable. In North America, ewes gave birth to dead, mummified foetuses or stillbirths and neonatal deaths occured. A dead lamb with malformed hind limbs showing hydrocephaly and cerebellar hypoplasia at necropsy was also found. In Australia a marked difference in the pathogenicity of three different strains of mucosal disease virus of bovine origin was noted . The most virulent strain produced death and mummification during the first third of pregnancy, but during the second trimester a high percentage of abortions or deaths just before birth occured . Again in Australia, ewes experimentally inoculated at 49 to 70

days of gestation with mucosal disease virus of ovine origin, besides producing abortions and mummified foetuses, gave birth to smaller "hairy shaker lambs". One "hairy" lamb was born ataxic and died 48 hours latter. At necropsy there was a mild microencephaly, but a severe cerebellar hypoplasia was also present (Ward, Roberts, McEntee & Gillespie, 1969; Ward, 1969; Kahrs, Scott & de Lahunta, 1970a; Kahrs, Scott & de Lahunta, 1970b; Ward, 1971; Casaro, Kendrick & Kennedy, 1971; Kahrs, 1973; Scott, Kahrs, de Lahunta, Brown, McEntee & Gillespie, 1973 ; Brown, de Lahunta, Scott, Kahrs, McEntee & Gillespie, 1973; French, Hore, Snowdon, Parsonson & Uren, 1974; Brown, de Lahunta , Bistner, Scott & McEntee, 1974; Snowdon, Parsonson & Brown, 1975; Plant, Acland & Gard, 1976; Plant, Gard & Acland, 1976; Markson, Winkler & Tulecki, 1976; Plant, Gard & Acland, 1977; Parsonson, O'Halloran, Zee & Snowdon, 1979; Done, Tulecki, Richardson, Harknen, Sands, Patterson, Swesey, Shaw, Winkler & Duffell, 1980 ; Badman, Mitchell, Jones & Westbury, 1981).

In South Africa it was reported that pregnant ewes vaccinated simoultaneously at 30 to 105 days of gestation with both an attenuated Rift Valley Fever and Wesselsbron disease vaccine, not only had early abortions and *hydrops amnii* but the foetuses showed hydranencephaly and arthrogryposis. Experimentaly, ewes 42 to 74 days pregnant, inoculated with either wild-type Wesselsbron disease virus, attenuated Rift Valley fever vaccine or attenuated Wesselsbron disease vaccine, produced hydranencephalic and arthrogrypotic foetuses together with *hydrops amnii* of the dam.

Experimentally, wild-type Wesselsbron virus inoculated *in utero* into cattle at 113 to 115 days gestation resulted in abortion, with one of the foetuses showing porencephaly and cerebellar hypoplasia (Coetzer & Barnard, 1977; Coetzer, Theodoridis, Herr & Kritzinger, 1979).

The natural silent infection of the pregnant female by certain viruses and the teratogenic consequences observed months later in their offspring, was first reported a decade ago in Japan, Australia and Israel.

In cattle, sheep and goats severe epizootics of foetal death, abortion, stillbirth, premature birth, congenital arthrogryposis and hydranencephaly were reported to be linked to two viruses of the Simbu serogroup, namely Akabane and Aino virus. These congenital malformations have been experimentally reproduced in pregnant cows, ewes and goats (Markusfeld *et al.*, 1971; Miura, Hayahsi, Ishiara, Inaba, Omori & Matumoto, 1974; Kalmar, Peleg & Savir, 1975; Kurogi, Inaba, Goto, Miura, Takahashi, Sato, Omori & Matumoto, 1975; Hartley *et al.*, 1975; Inaba *et al.*, 1975; Parsonson, Della-Porta, Snowdon & Murray, 1975; Kurogi, Inaba, Takahashi, Sato, Omori, Miura, Goto, Fujiwara, Hatano, Kodama, Fukuyama, Sasaki & Matumoto, 1976; Della-Porta, O'Halloran, Parsonson, Snowdon, Murray, Hartley & Haughey, 1977; Kurogi, Inaba, Takahashi, Sato, Goto & Omori, 1977; Parsonson, Della-Porta & Snowdon, 1977; Kurogi, Inaba, Takahashi, Sato, Satoda, Goto, Omori & Matumoto, 1977; Coverdale, Cybinski & St George, 1978; St George, Cybinski & Coverdale,1978; Hashiguchi, Nanba & Kumagai, 1979; Coverdale, Cybinski &

St George, 1979; Miura, Inaba & Hayashi, 1980; Parsonson, Della-Porta, O'Halloran, Snowdon, Fahey & Standfast, 1981; Parsonson, Della-Porta & Snowdon, 1981; Parsonson *et al.*, 1982).

In their first approach to the study of the pathogenic potential of the several strains of Akabane virus, the Japanese used suckling mice as their laboratory model. These strains of Akabane virus showed different peripheral infectivity for suckling mice. In the field the infection of the domestic ruminant occurs via the peripheral route, so the Japanese scientists tried to correlate the pathogenicity of the different strains of Akabane virus for the ruminant, with the periperal infectivity for suckling mice (Kurogi, Inaba, Takahashi, Sato, Akashi, Satoda & Omori, 1978).

An alternative approach to the study of the relative pathogenicity of Akabane virus and other Bunyaviruses was made by the Australian and Japanese workers, who used the chicken embryo as a laboratory model to assess the teratogenic potential of the virus. The chicken embryo model is now used to study this effect in a wide range of other viruses, since it has the advantage of being economical and shows a rapid response. The only disadvantage is the lack of a placental barrier (Ikeda & Yonaiyama, 1978; Miah & Spradbrow, 1978; McPhee, Parsonson & Della-Porta,1982; McPhee, Parsonson, Della-Porta & Jarret, 1984).

In the Republic of South Africa, Nigeria and Kenya, several

other Simbu serogroup viruses including Shuni, Sabo and Shamonda have been isolated from apparently healthy cattle, sheep, goats and from several species of *Culicoides*.

In addition, several outbreaks of abortion and congenital malformation where other viruses may be implicated have been reported in the Republic. It was, therefore, considered necessary to survey the indigenous Simbu serogroup isolates in order to establish their pathogenicity as well as their teratogenic potential.

MATERIALS AND METHODS

VIRUSES - Aliquots of the local strains of Shuni, Sabo and Shamonda viruses were prepared as follows :

Suckling Swiss albino mice were inoculated intracerebrally with 0,02 ml of a 10^{-3} dilution of the virus. Sick mice were collected and sacrificed. The brain tissue was harvested and suspended to 10% (w/v) in phosphate buffer (pH 7,4) containing 10% neutral peptone and 2% lactose (BLP).

The brain tissue suspension was clarified by centrifugation at 4 000 rpm/30 minutes in a refrigerated centrifuge. The supernatant fluid was dispensed in 5 ml aliquots in glass ampoules and stored sealed in liquid nitrogen.

TABLE 7 - Index of Strains Used in Pathogenicity Studies.

VIRUS	ISOLATES FROM CATTLE	ISOLATES FROM CULICOIDES SPP.
SHUNI	19827 # 4smb*	78/61 # 3smb
	7507 # 6smb	80/11 # 4smb
	7519 # 6smb	
	8912 # 7smb	
SABO	1262 # 7smb	1/70 # 4smb
SHAMONDA	LAMBRECHT # 6 smb	

*smb= suckling mouse brain passage.

ASSAY OF PERIPHERAL INFECTIVITY

This technique with minor modifications, was used as described by Kurogi *et al.* (1978).

a) EXPERIMENTAL HOSTS

MICE - Swiss albino mice of 2 and 20 to 22 days of age were used exclusively. All mice which died within 24 hours of inoculation were disregarded in the calculation of the results.

EGGS - were obtained from a flock of breeding hens of high fertility. All embryos found dead within 24 hours of inoculation

were also disregarded.

b) DETERMINATION OF PERIPHERAL INFECTIVITY

Each virus was diluted tenfold in Hank's-Eagles medium and each dilution was inoculated into seven mice. The dose of inoculum was 0,02 ml by the intracerebral route in suckling mice and in three-week-old mice, 0,05 ml by subcutaneous route in suckling mice and 0,1 ml in three-week-old mice.

The MLD_{50} of the viruses was estimated from the mortality recorded at the end of the 2nd week of observation and the infectivity titer (LD_{50}/1 ml) for all age groups and routes of inoculation was determined by the method of Reed & Muench (1938).

The difference between the infective titer as determined by the intracerebral route and by the subcutaneous route in suckling was used to express the *peripheral infectivity* of the respective viruses. Similarly, the difference in the infectivity titer (LD_{50}/1 ml) determined by the intracerebral route in suckling mice and three-week-old mice expressed the *intracerebral infectivity* for three-week-old mice. The pathogenicity of the various strains for mice was then compared.

c) PATHOGENICITY FOR SUCKLING MICE OF SHUNI VIRUS STRAIN 78/61 AT INCREASING PASSAGE LEVELS

Intracerebral passage of Shuni virus strain 78/61 was carried

out in suckling mice. Several families were inoculated intracerebrally with 0,02 ml of a 10^{-3} dilution of the previous passage level. When the mice were sick, the brains were harvested and a 10% suspension (w/v) in BLP was prepared. It was clarified by centrifugation at 4 000 rpm/30 minutes and 5 ml aliquots were distributed in glass ampoules and stored in liquid nitrogen.

The LD_{50} for suckling mice and three-week-old mice inoculated by the intracerebral and subcutaneous routes of Shuni virus strain 78/61 was determined at the 3rd, 5th, 10th, 15th, 20th, 25th and 30th passage level.

PATHOGENICITY OF THE LOCAL SIMBU SEROGROUP VIRUSES USING THE CHICKEN EMBRYO MODEL

The technique was carried out as described by Ikeda *et al.* (1978) and Miah *et al.* (1978) with minor modifications.

a) CHICKEN EMBRYO INOCULATION

Each virus tested was rapidly thawed and tenfold dilutions prepared in Hank's-Eagles medium containing 0,75% of inactivated horse serum (devoid of antibodies for these viruses) ; in addition, a 7,5% stock solution of sodium bicarbonate was added at a rate of 2%. The serial tenfold dilutions were kept at 4°C during manipulations.

After candling the eggs, four-day-old chicken embryos were inoculated by the yolk sac route with 0,2 ml of dilutions of

each virus to be tested.

Only 25 eggs were inoculated with 10^{-4} and 10^{-5} dilutions, whereas all the other dilutions were used to infect 84 eggs. As controls, 42 eggs were inoculated with the diluent only.

The inoculated eggs were then returned to an incubator with automatic humidity control and turning device.

All embryos that died within 24 hours of inoculation were discarded .The remaining eggs were candled daily for 14 days. After this period, the surviving embryos were placed in a refrigerator, the eggs opened, the chickens observed and the abnormalities recorded for each dilution.

With the same virus dilutions used in the eggs, suckling mice and three-week-old mice were inoculated both intracerebrally and subcutaneously.

RESULTS

PERIPHERAL INFECTIVITY FOR MICE

The results of inoculation of the indigenous Simbu serogroup viruses are presented in Table 8.

TABLE 8 - Peripheral infectivity for mice of different strains of Shuni, Sabo and Shamonda viruses.

VIRUS	PASSAGE LEVEL IN BABY-MICE	VIRUS TITER IN BABY-MICE IC (A)	VIRUS TITER IN BABY-MICE SC (B)	PERIPHERAL INFECTIVITY TO BABY-MICE (A-B)	VIRUS TITER IN 3-WEEK-OLD MICE IC	VIRUS TITER IN 3-WEEK-OLD MICE SC
SHUNI STRAINS						
7507	7	*8,46	*4,30	4,16	*8,2	0
7519	7	8,45	5,8	2,65	8,4	0
8912	7	8,39	3,84	5,55	8,2	0
78/61	3	8,39	6,89	1,5	8,4	0
80/11	4	8,27	6,88	1,39	8,19	0
19827	4	8,28	5,54	2,47	7,94	0
19911	3	8,0	5,61	2,39	8,1	0
SABO STRAINS						
1262	7	8,2	3,35	4,85	8,4	0
1/70	4	8,77	6,40	2,37	7,9	0
SHAMONDA STRAIN						
LAMBRECHT						
	7	6,82	3,89	2,93	6,7	0

* Log base 10.

From the results obtained it can be seen that the peripheral infectivity for suckling mice is variable and is possibly related to the passage level of the strain. It is interesting to

note that *Culicoides* isolates showed the highest peripheral infectivity. It appears that the peripheral infectivity decreases as the passage level in mouse brain increases.

None of the virus strains were lethal for three-week-old mice by the subcutaneous route. Suckling mice appear to be slightly more sensitive by intracerebral route than three-week-old mice.

PERIPHERAL INFECTIVITY FOR SUCKLING MICE OF SHUNI VIRUS STRAIN (78/61) AT VARYING PASSAGE LEVEL

This experiment was conducted in order to determine the effect of the intracerebral passage level on the peripheral infectivity of an indigenous Shuni virus. The results are presented in Table 9.

As the intracerebral passage level in suckling mice increases the peripheral infectivty of the Shuni virus for suckling mice decreases. This is a valid conclusion because the susceptibility of suckling-mice to the intracerebral route of infection does not appear to change.

TABLE 9 - Peripheral Infectivity for suckling mice of Shuni virus strain 78/61 at various passage levels in suckling mouse brain

PASSAGE LEVEL IN BABY-MICE BRAIN	VIRUS TITER IN BABY-MICE IC (A)	SC (B)	PERIPHERAL INFECTIVITY TO BABY-MICE (A - B)
3	*8,39	*6,89	1,5
5	8,55	5,03	3,52
10	8,6	3,7	4,9
15	7,6	2,86	4,84
20	7,2	2,75	4,45
25	8,29	3,13	5,16
30	8,1	2,3	5,8

* log base 10

PATHOGENICITY OF THE SOUTH AFRICAN SIMBU SEROGROUP ISOLATES FOR THE CHICKEN EMBRYO

It was seen that high doses of the strains kill the embryos leaving no survivors. To facilitate statistical calculations a larger number of embryos were inoculated in those dilutions where survivors could be expected, in order to record the incidence of congenital abnormalities. Fourteen days

post-inoculation, the survivors were killed by chilling in the refrigerator, the embryos were removed, observed and the results tabulated in Table 10.

Among the deformities observed, there was stunted growth compared to the controls. They were not only smaller in size but showed a pronounced hydranencephaly, "hairy feathers" and shortened limbs with arthrogryposis. On more detailed examination of the hydranencephaly in some embryos, an imperfectly closed cranium, exopthalmus and unequal development of the beak with lack of coaptation of the beak was observed. No abnormalities were seen in the wings. In some cases the abdominal cavity was not completely closed, exposing the viscera which were frequently protruding from the abdomen.

In the most severe cases, the limbs were only vestigial and the legs and toes could hardly be distinguished. Without exception the embryos were smaller, with various degrees of arthrogryposis.

During the last few days of incubation some of the eggs, when candled, showed a green colour which, when opened, could be associated with a deformed chick.

The results of the inoculation of the chick embryos with the South African strains of Simbu serogroup viruses are presented in Table 10.

Deformities in 18-day-old chicken embryos inoculated with South African Simbu serogroup viruses at day 4 : (A) Uninoculated control; (B) Induced by Shamonda virus; (C) Induced by Shuni virus; (D) Induced by Sabo virus.All the same magnification. Stunted growth, variable degrees of arthrogryposis, nonoclusion of abdomen or encephalic cavity, exopthalmus, unequal development of the beak.

TABLE 10 - Deaths and Deformities in chicken embryos associated with virus inoculation.

VIRUS	TITER MLD_{50}/ml	% Deaths (log dilution)					% Deformities (log dilution)				
		-4	-5	-6	-7	C	-4	-5	-6	-7	C
SHUNI ISOLATES											
80/11	8,27	-	100	87	42	0	-	0	8,7	16,6	0
19827	8,28	-	100	72	38	0	-	0	0	2,7	0
7507	8,46	-	100	93	52	0	-	0	0	2,4	0
7519	8,45	-	100	79	44	0	-	0	0	2,6	0
8912	8,39	100	93	88	37	0	0	4,8	5,7	4,8	0
78/61	8,05	-	100	80	28	0	-	0	0	12	0
SABO ISOLATES											
1/70	7,2	-	100	86	49	0	-	0	0	1,2	0
1262	8,2		75	27	17	0		0	4,8	0	0
SHAMONDA ISOLATES											
Lambrecht											
	6,8		100	63	30	0		0	0	1,5	0
Shamonda											
	7,94	79	55	25	21	0	2,4	14,2	6,3	3,6	0

Without exception these strains produced antigens of consistently high infectivity titers, therefore, any comparative evaluations would be valid. From these results it would appear that mortality, rather than deformity, is the predominant effect

of these viruses on the chick embryo. This is reflected in the low percentage of deformities obtained when a large number of eggs are inoculated with the highest dilutions, where more survivors would be expected.

Although a *polychotomous* response was observed that is, dead, alive but deformed and alive but not deformed, it was unfortunately not possible to computer analyse the results taking this into account because of the absence of the appropriate statistical software.

However, using the Probit analysis program in SAS (Statistical Analysis System) the dichotomous responses "deaths" and "deaths plus deformities" were analysed separately. This procedure results in probit regression lines for the two responses which intersect, but this objection is more of theoretical than of pratical importance. The results of this statistical analysis are presented in Table 11 where the values of the 50% effective dose (ED_{50}) calculated for both deaths and for deaths plus deformities are presented, as well as the 95% confidence limits.

In the previous experiment designed to determine peripheral infectivity in mice, it was found that the passage level of the strain in suckling mice brain influenced this property. In order to observe the effect which passage level would have on the pathogenicity for embryonated eggs, an experiment similar to the one above was undertaken, using the same mouse brain preparations.

TABLE 11 - Comparative pathogenic potential of the South African Simbu serogoup members in the chicken embryo.

VIRUS	TITER MLD_{50} /EGG	NUMBER OF EGGS INOCULATED	DEAD	ALIVE ABNORMAL	ALIVE NORMAL	ED_{50} DEATHS:	ED_{50} DEATHS + ABNORMALS
SHUNI ISOLATES							
80/11	3,72	24	10	4	10	4,92*	2,54*
#4smb	37,24	23	20	2	1	(1,6/9,3)	(0,3/5,0)
	372,42	21	21	0	0		
19827	3,81	74	28	2	44	5,67	5,0
#4smb	38,10	74	53	0	21	(2,7/9,3)	(2,2/8,5)
	381,09	23	23	0	0		
7507	5,76	42	22	1	19	5,29	4,79
#6smb	57,68	42	39	0	3	(2,0/8,9)	(1,6/8,3)
	576,80	25	25	0	0		
7519	5,63	39	17	1	21	8,85	7,99
#6smb	56,36	42	33	0	9	(3,8/15,4)	(3,2/14,3)
	563,67	42	42	0	0		
8912	4,90	84	31	4	49	15,45	10,54
#7smb	49,09	88	51	5	28	(8,8/24,8)	(5,7/16,8)
	490,94	42	39	2	1		
	4909,4	42	42	0	0		
SABO ISOLATES							
1/70	0,31	84	41	1	42	0,346	0,327
#4smb	3,16	84	72	0	12	(0,18/0,5)	(0,16/0,5)
	31,69	25	25	0	0		
1262	0,28	84	14	0	70	7,2	6,0
#7smb	2,82	84	23	4	57	(0,3/?)	(3,5/12,5)
	28,25	32	24	0	8		
SHAMONDA ISOLATES							
LAMBRECHT							
#6smb	0,12	66	20	1	45	0,453	0,0432
	1,26	63	40	0	23	(0,2/0,7)	(0,24/0,74)
	12,6	15	15	0	0		
SHAMONDA							
#4smb	1,0	84	18	3	63	17,82	8,93
	10	80	20	5	59	(6,7/35)	(3,1/17,4)
	100	42	23	6	13		
	1000	42	37	1	4		

* The ED_{50} was determined by using the maximum likelihood
The figures in brackets are the 95% confidence limits of the ED_{50} for both deaths and deaths plus deformities

The percentage of mortalities and deformities are recorded on Table 12.

TABLE 12 - Deaths and deformities in chicken embryos recorded at different passage levels of Shuni virus strain 78/61

PASSAGE LEVEL (#smb)	TITER MLD_{50} /ml	% Deaths (log dilution) -5	-6	-7	C	% Deformities (log dilution) -5	-6	-7	C
3	*8,39	92	80	28	0	0	0	16,7	0
5	8,55	81	48	27	0	62,5	20	14	0
15	7,6	81	50	26,7	0	11,9	11,3	10,5	0
20	7,2	100	60	12	0	0	17	3	0
25	8,3	100	93	37	0	0	3,57	11,1	0
30	8,1	98	74	22,5	0	75	30	10	0

* in baby mice by intracerebral route.

It can be seen that the lethal effect as well as the teratogenic potential for the chicken embryo is maintained and in contradistinction to the peripheral infectivity for the suckling mouse showed a decrease at comparable passage levels.

The Probit analysis of these results are presented in Table 13.

TABLE 13 - Statistical analysis of the pathogenic potential of different passage levels of Shuni virus strain 78/61 in the chicken embryo

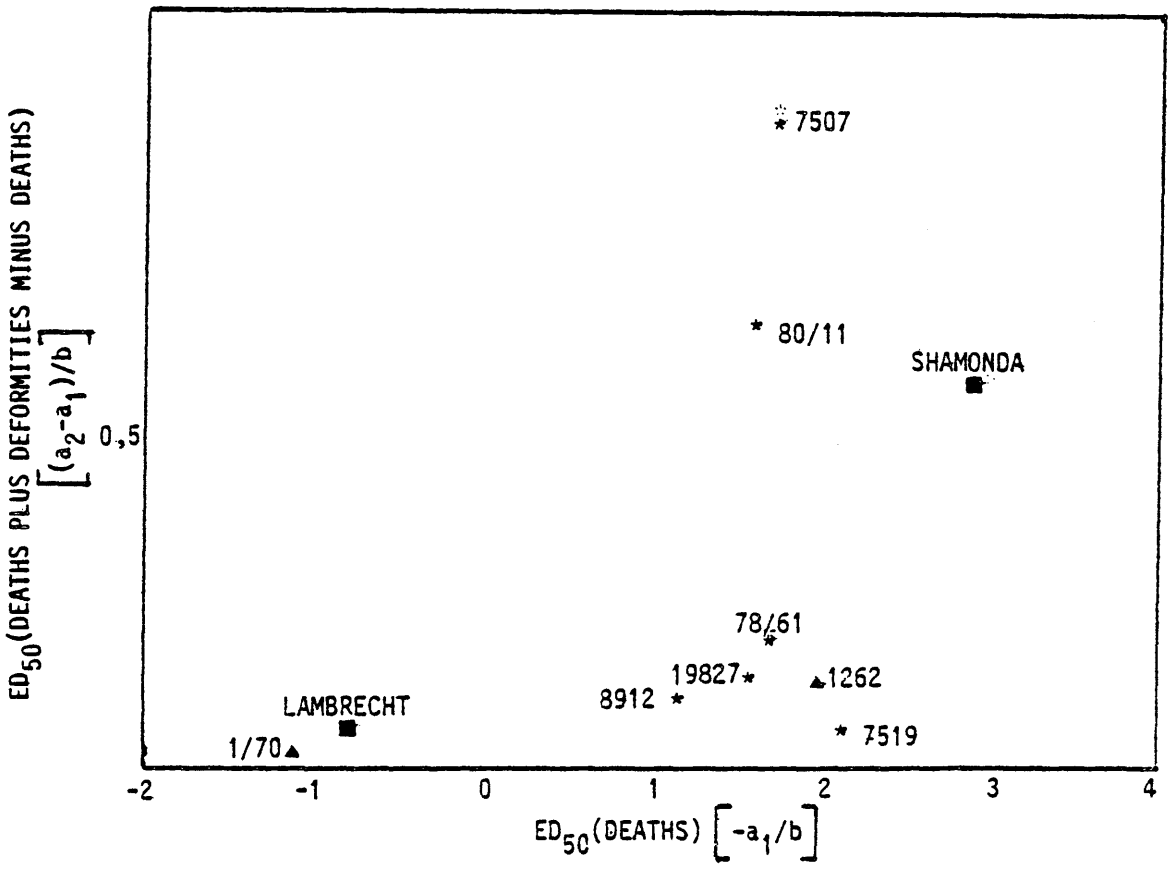
PASSAGE LEVEL	TITER MLD_{50} /EGG	NUMBER OF EGGS INOCULATED	DEAD	ALIVE ABNORMAL	ALIVE NORMAL	DEATHS:	ED_{50} DEATHS + ABNORMALS
#3smb	2,24	25	7	3	15	5,66* (2,8/10)	3,89* (1,4/7,4)
	22,44	25	20	0	5		
	224,40	25	25	0	0		
#5smb	0,63	49	6	3	40	9 (6,6/12)	6 (4,7/9)
	6,32	78	30	4	44		
	63,2	80	62	8	12		
	632,4	42	42	0	0		
#15smb	0,79	86	23	9	54	6,15 (3,7/10)	2,63 (1,4/4,2)
	7,96	84	40	9	35		
	79,62	42	34	5	3		
	796,2	42	42	0	0		
#20smb	2,00	78	9	2	67	12,19 (8,8/17)	9,7 (7/13,6)
	20,00	73	44	5	24		
	200,00	33	33	0	0		
#25smb	3,99	81	30	9	42	6,1 (4,3/8,2)	4,23 (2,8/5,7)
	39,90	84	78	3	3		
	399,0	84	84	0	0		
#30smb	1,32	80	18	6	56	4,83 (3,2/6,9)	3,07 (2,07/4)
	13,21	78	58	6	14		
	132,13	79	75	3	1		

* The ED_{50} was determined using the maximum likelihood

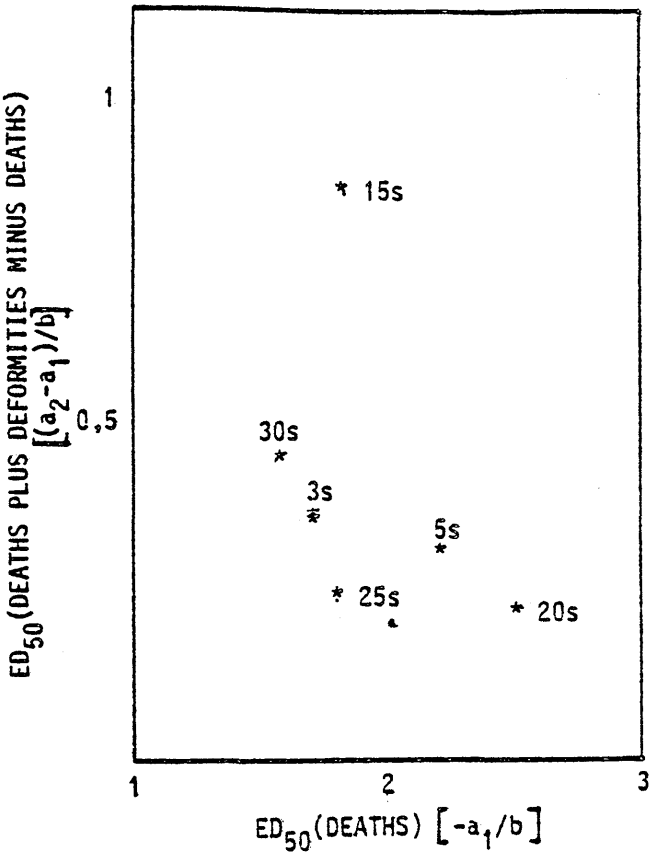
The figures in brackets are the 95% confidence limits of the ED_{50} for both deaths and deaths plus deformities.

The 50% Effective Dose (ED_{50}) values for both deaths and deaths plus deformities are given by $-a_1/b$ and $-a_2/b$ respectively.

From these ED_{50} values a graphic representation was prepared. The horizontal axis is the natural logarithm of virus dose to cause 50% deaths. The vertical axis $[(a_2-a_1)/b]$ is the difference between natural logarithm of the dose that causes 50% deaths and the natural logarithm of the dose that causes 50% deaths plus deformities.



Graphical representation of ED_{50} values of the South African Simu serogroup viruses : * Shuni virus strains ; ■ Shamonda virus ; ▲ Sabo virus



Graphical representation of ED_{50} values of Shuni virus strain 78/61 after several passages in suckling mouse brain

As can be seen, the local strains of Shuni, Sabo and Shamonda virus are predominantly lethal, and the deformities were only observed in those dilutions where minimal amounts of infective virus could be expected to be present.

DISCUSSION

The local strains of Shuni and Sabo viruses show a variable peripheral infectivity for suckling mice Unfortunately, only

one local strain of Shamonda virus was available and thus no comparasions were possible. These results are similar to those obtained for different strains of Akabane virus by Kurogi *et al.* (1978) in Japan. By studying the effect of an increased intracerebral passage level on one of our local Shuni virus strains, it was observed that, as the passage level increased so the peripheral infectivity decreased, comparable to the results obtained by the Japanese with Akabane virus.

Using the chicken embryo model, the local strains of Shuni, Sabo and Shamonda virus were studied with respect to their capability of inducing congenital malformations. Although mortality rather than deformities was the principal feature, the local strains were also able to induce malformations in the chicken embryo. In natural infections, these viruses have to infect and circulate in the pregnant mother and only after crossing the placenta can they infect the foetus and produce congenital deformities. Unfortunately when using the chicken embryo as a model to assess the teratogenic potential of a strain of a virus isolate, the so called "placental barrier" is bypassed and the virus is in direct contact with the developing foetus and so able to produce its pathogenic more readily.

In this group of viruses, the main effects observed in natural infections are hydranencephaly and arthrogryposis in domestic ruminants. Akabane virus, in the chicken embryo, also produces hydranencephaly and arthrogryposis, and the same effects were also observed in association with our local Shuni and Sabo virus strains as well as with the only Shamonda strain tested. It can

be concluded, therefore, that the chicken embryo model is a very pratical method to assess the teratogenic potential of local strains of Simbu serogroup viruses.

One of the interesting results that emerged from these experiments utilizing the chicken embryo model, is that although the "peripheral infectivity" of one of the local Shuni strain decreased with intracerebral passages, the "teratogenic" capacity of the virus, at least for the chicken embryo, was maintained. It seems that the "peripheral infectivity" exhibited by these viruses and which characterizes their pathogenicity for mice, is distinct from their teratogenic potential, as far as the chicken embryo model is concerned.

GENERAL SUMMARY AND DISCUSSION

In order to evaluate the pathogenicity of the different strains of Akabane virus, Japanese workers used suckling mice and three-week-old mice as experimental models. They demonstrated that Akabane virus had variable *peripheral infectivity* under the conditions of their tests. Using similar methods, it has been established that the South African Simbu group viruses, Shuni, Sabo and Shamonda, also exhibit a variable peripheral infectivity for mice (Kurogi *et al.*, 1978).

The Japanese were also the first to use the chicken embryo model to study the teratogenic effect of Akabane virus, demonstrating that it induces gross pathological changes similar to those of the Arthrogryposis-Hydrananecephaly Syndrome observed in domestic ruminants. More recently, the Australians extended the use of this laboratory model for the evaluation of the teratogenic potential of their regional Bunyaviruses (Ikeda *et al.*, 1978; Miah *et al.*, 1978; McPhee *et al.*, 1982; McPhee *et al.*, 1984).

The advantages of this economic laboratory model are obvious- it is easy to handle large numbers of inoculated eggs and the viruses are placed in direct contact with the developing embryo,

since there is no placental barrier. In 2 weeks a polychotomous quantal response is obtained - which includes deaths, surviving and normal as well as surviving but deformed embryos. Such a response can then be submitted to statistical analysis and the pathogenic potential of the viruses under study can be determined. By the application of similar methods, the South African strains of Shamonda, Sabo and Shuni viruses were also shown to induce dramatic teratogenic effects in the chicken embryo. The southern African strains seem to have a similar pathogenic index without the wide diversity of pathogenic potential exhibited by the the Australian viruses.

Kurogi, Inaba, Takahashi, Sato, Akashi, Satoda & Omori, 1979, when choosing an attenuated strain of Akabane virus as a candidate for a live virus vaccine, selected a strain of virus that had undergone 20 passages in HmLu-1 cell cultures because at this passage level it still possessed immunogenic capacity. The *peripheral infectivity* for mice was one of the criteria of safety used by the Japanese workers to assess the genetic stability of this vaccine strain. This property was shown to be a more realiable marker *in vivo* because it remained unaltered after further passages of the strain in cell culture or in suckling mice.

Thirty sucessive passages of a strain of Shuni virus (78/61) in mouse brain did not seem to influence the teratogenicity for the chicken embryo, although the peripheral infectivity for suckling mice decreased substantially. This observation suggests that we are evaluating two unrelated biological properties of

this virus: a) peripheral pathogenicity for mice and b) teratogenic potential for the chicken embryo.

All these studies provided a positive indication of the relative potential pathogenicity of these viruses. However, it remains to be shown how these observations will correlate with the pathogenic potential of these viruses for the foetus of the domestic ruminant and it is inevitable that the final criteria will have to be determined in ruminants. It will be a prerequisite to establish how these viruses are able to circulate in the dam and after overcoming the placental barrier, infect the developing foetus and induce the congenital defects previously described.

In Japan and in Australia, Aino virus was shown to be one of the viruses implicated in congenital malformations in cattle and sheep. Antigenically, Shuni virus is considered a subtype of Aino virus and therefore they are very closely related. A preliminary serologic survey based on a random collection of cattle sera has shown that antibodies to Shuni and Sabo virus are widely distributed throughout South Africa. It is therefore anticipated that Shuni virus on the African continent may play the same role as its conterpart in Japan and Australia.

The picture in South Africa is furthermore complicated by the fact that the Simbu group viruses are prevalent, together with many strains of Bluetongue and Wesselsbron virus under similar ecological conditions. Breeding and management systems also ensure that a large proportion of the sheep population are

pregnant during the period of highest vector activity.

In order to evaluate the importance of all these viruses in producing congenitally defective calves and lambs it is necessary to collect more information from the field. This would include a concerted effort to obtain samples from the congenitally affected animals as well as from their dams. The detection of antibody would appear to be the most pratical method of study and must of necessity involve selected and strategically placed sentinel herds of cattle and flocks of sheep.

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THE ISOLATION AND IMPORTANCE OF SIMBU GROUP VIRUSES IN SOUTH AFRICA

by

VASCO MANUEL DA COSTA MENDES

PROMOTER: Prof. P. G. Howell

For the degree M. Med. Vet. (Vir.)

Department of Infectious Diseases, Faculty of Veterinary Science

University of Pretoria

PRETORIA 0002.

SUMMARY

The five viruses used in this study were isolated from cattle. An additional virus was recovered from a pool of biting midges, *(Culicoides spp.)* . The six viruses shared common group specific antigens in the cross-complement fixation test but in the cross-serum-virus neutralization test, they were shown to be three distinct viruses. In the cross-serum-virus microneutralization test utilizing reference sera and viruses of the Simbu serogroup it was demonstrated that they represented local strains of Shamonda, Shuni and Sabo virus.

Using suckling mice and the chicken embryo as a model it was shown that the South African strains of Shuni and Sabo virus

also exhibited variable *peripheral infectivity* for suckling mice, a characteristic which was distinct from their teratogenic properties as determined in chicken embryos. Although mortality was higher the local strains of Shuni, Sabo and Shamonda virus also induced dramatic deformities in the chicken embryo. Additional mouse-brain passage of a local strain of Shuni virus, markedly reduced the *peripheral infectivity* of the virus, without apparently affecting its teratogenic potential at least for the chicken embryo.

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LEIER: Prof. P. G. Howell

Vir Die Graad M. Med. Vet. (Vir.)

Departement Infeksiesiekte, Fakulteit Veeartsenykunde

Universiteit van Pretoria

PRETORIA 0002

OPSOMMING

Die 5 virusse wat in hierdie studie gebruik is, oorspronklik uit beeste geisoleer. 'n Bykomstige virus is uit 'n poel van bytende muggies (*Culicoides spp.*) herwin. Die 6 virusse deel gemeenskaplike groepspesifieke antigene in die kruis-komplementbindingstoets, maar in die kruis-serum-virus neutralisasietoets is hulle as drie aparte virusse opgewys. In die kruis-serum-virus mikroneutralisasietoets met verwysings sera en virusse van die Simbu serogroep is gedemonstreer dat hulle lokale stamme van die Shamonda, Shuni en Sabo virusse verteenwoordig.

Deur die gebruik van die baba-muis en kuiken embrio model, is

daar getoon dat die Suid-Afrikaanse stamme van Shuni en Sabo virus, ook 'n wissellende *perifere besmetlikheid* vir baba-muise toon, 'n kenmerk wat skynbaar apart is van hul teratogene kenmerke soos bepaal in kuiken embrios.

Alhoewel mortaliteit hoer was, het die lokale stamme van Shuni, Sabo en Shamonda virusse ook dramatiese misvormings in die kuiken-embrio geinduseer. Verdere muisbrein passasie van 'n lokale stam van Shuni virus, het die *perifere besmetlikheid* van die virus verminder, skynbaar sonder om sy teratogene potensiaal vir die kuiken-embrio in elk geval, te affekteer.