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FLAVIVIRUSES IN SOUTH AFRICA: DIAGNOSTIC PROCEDURES

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ABSTRACT

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Employing rabbit immune serum, 10 flaviviruses known to be present in South Africa could be divided into 5 serological subgroups. The subgroups conform to the general pattern described for the group. Sera from experimentally infected calves and lambs were monospecific in neutralization tests, but cross-reacted in haemagglutination inhibition tests. These results suggest that sheep and cattle sera from the field can best be tested by microneutralization tests. The greater sensitivity of embryonated hen's eggs for some viruses and of one-day-old mice for other viruses necessitates the employment of both systems for the isolation of flaviviruses from field specimens.

INTRODUCTION

Of the more than 63 distinct flaviviruses now recognized, at least 10 are known to be present in South Africa, but only Wesselsbron (WSL) has hitherto been regarded as an important cause of livestock disease (Weiss, Haig & Alexander, 1956).

Numerous outbreaks of abortion have been investigated over a period of several years. Some abortions were associated with hydranencephaly, arthrogryposis and weak dumb calves, or lambs that died soon after birth (Coetzer & Barnard, 1977). In a small percentage of cases, WSL virus or other agents could be incriminated, but in most cases no viral aetiological diagnosis could be established. However, on 3 occasions, Bagaza (BAG) virus was isolated from aborted calves, and occasionally haemagglutination-inhibition (HI) antibodies, presumable against other flaviviruses, were detected in foetal serum (Barnard, 1978, unpublished results).

Flaviviruses are grouped together on the basis of their cross-reactivity in serological tests (Casals, 1957). To investigate the role played by flaviviruses in South Africa it was considered necessary to observe the cross-reactions which occur in the commonly employed serological tests and to evaluate and select suitable diagnostic procedures.

MATERIALS AND METHODS

Viruses

The 10 flaviviruses that are known to be present in South Africa and have been examined are shown in Table 1. The viruses were passaged intracerebrally (i.c.) in one-day-old mice and stored as freeze-dried suspensions of 10 % infective mouse brain in phosphate buffer containing 10 % peptone and 5 % lactose (BLP).

Cell culture

The CER cell, developed by Tsunemasa Motanashi at the Nippon Institute for Biological Science, Tokyo, was cultivated in Eagle's medium containing 5 % bovine serum free of antibodies against flaviviruses, benzyl penicillin (200 i.u./mℓ), streptomycin sulphate (200 μg/mℓ) and Fungizone (Amphotericin B) (2,5 μg/mℓ). Monolayers in roller tubes were inoculated and incubated at 35 °C–37 °C. When approximately 75 % of cells showed cytopathic (CP) changes, the cultures were harvested, mixed with 25 % BLP, freeze-dried and stored at 4 °C.

Virus titration

Virus titrations were carried out in plastic trays of 96 wells each 15 mm in diameter and in microtitre cell culture plates.* Trays with a confluent monolayer were inoculated with tenfold dilutions of virus. Adsoprtion was allowed for 30 min at room temperature before the medium was replaced by on 0,58 % agarose overlay. The cultures were incubated at 37 °C in an atmosphere of 3 % CO_2. A second overlay containing trypan blue was applied 3–6 days later. The tests were read when plaques were clearly visible in control wells.

Microtitre plates were inoculated with tenfold dilutions of virus, after which the plates were seeded with sufficient cells to form a confluent monolayer within 8–12 h. The plates were incubated at 37 °C in an atmosphere of 3 % CO_2 and inspected microscopically every day. When optimum CP changes were seen, the plates were rinsed in tap water. The remaining cells were fixed with alcohol for 5 min and stained with 1 % aqueous basic fuchsin. The plates were examined visually for CP changes.

The viruses were also titrated intracerebrally (i.c.) and intraperitoneally (i.p.) in one-day-old and in three-week-old mice. In addition, four-day-old embryonated eggs were inoculated via the yolk-sac route, and twelve-day-old embryos were inoculated intravenously (i.v.). The eggs were incubated at 34 °C. Embryos that died within 48 h were discarded. The remaining eggs were candled daily until they were due to hatch. The surviving eggs were refrigerated and the embryos were removed and examined for abnormalities. All virus titres were calculated according the the method of Reed & Muench (1958).

Japanese quail (Coturnix coturnix japonica)

Japanese quail were injected intramuscularly (i.m.) with 100 Mouse LD_{50} and observed for 3 weeks. Brain, spleen and liver of quail that died were titrated in mice and in embryonated eggs.

TABLE 1 Flaviviruses known to be present in South Africa

Virus	Abbreviation	Origin
Wesselsbron	WSL	Calf[1]
Bagaza	BAG	Calf foetus[1]
Turkey meningo encephalitis	TME	Turkey[1]
Ntaya	NTA	B. McIntosh[2]
Banzi (H 336)	BAN	B. McIntosh
Uganda-S (AR 19786)	UG-S	B. McIntosh
Unidentified (AR 5189)	AR 5189	B. McIntosh
West Nile	WN	Dog[1]
Usutu (AR 1776)	USU	B. McIntosh
Spondweni (AR 94)	SPO	B. McIntosh

[1] Isolated during routine diagnostic procedures

[2] Kindly supplied by Dr B. M. McIntosh, National Institute for Virology, Sandringham 2131, Republic of South Africa

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* Nunc Laboratory Products

TABLE 2 Prozone formation by flaviviruses in microtitration on CER cells

Virus dilution	Cytopathic changes in microtitre plates inoculated with:							
	WSL	BAG	TME	NTA	BAN	UG-S	AR 5189	WN
10^{-1}	—[1]	—	—	—	100[2]	100	—	100
$10^{-1,5}$	—	25	—	—	100	100	—	100
10^{-2}	25	100	—	—	100	100	—	100
$10^{-2,5}$	50	100	25	—	100	100	50	100
10^{-3}	100	100	100	—	100	100	100	100
$10^{-3,5}$	50	SP[3]	100	25	100	100	100	100
10^{-4}	SP	.	50	50	100	50	SP	100
$10^{-4,5}$	.	.	SP	100	50	SP	.	100
10^{-5}	.	.	.	SP	SP	.	.	50

[1] Prozone = no cytopathic change
[2] Percentage of cell destruction
[3] Single plaques

TABLE 3 The influence of different sera on virus titre and prozone effect with Wesselsbron, Turkey meningo encephalitis and AR 5189 titrated on CER cells in microtitre plates

Virus prozone	Virus titre[1] and prozone titre with 5 different sera				
	Serum 1[2]	Serum 2	Serum 3	Serum 4	Serum 5
WSL	3,0	4,0	3,0	3,5	3,0
Prozone	1,0	2,0	2,0	2,0	NP[3]
TME	4,0	4,0	3,0	4,0	3,0
Prozone	2,0	2,0	2,0	2,0	NP
AR 5189	3,0	4,0	4,0	3,0	4,0
Prozone	2,0	2,0	2,0	2,0	NP

[1] Expressed as Log_{10} of infective units/0,05 mℓ
[2] Serum 1. Serum from an ox kept under insect-free conditions for 6 years
Serum 2 + 3. Two different batches of pooled serum obtained from an abattoir
Serum 4. Same as serum 3 treated with polyethylene glycol
Serum 5. Commercially available serum (Flow Laboratories)
[3] No prozone

Antisera

Guinea-pigs and rabbits were injected intraperitoneally (i.p.) with 1 mℓ of a 10 % suspension of infected mouse brain, followed 14 days later by an intracardial injection. After another 10 days they were anaesthetized, bled and the serum separated for use in serological tests.

Calves and lambs (9–12 months old), reared under insect-free conditions, were injected i.v. with mouse brain containing approximately $10^{3,5}$ mouse infective doses of virus. They were bled before and 30 days after inoculation. All sera were first inactivated for 30 min at 56 °C before being used in serological tests.

Haemagglutination-inhibition

The haemagglutination-inhibition (HI) technique (Clark & Casals, 1958) was used to determine antibody titres. A sucrose-acetone extract of infected mouse brain was used in the tests, which were performed at predetermined pH values.

Plaque inhibition

For plaque inhibition (PI) tests, equal volumes of twofold dilutions of serum and virus suspensions, containing sufficient virus to produce approximately 10–30 plaques per well, were mixed and kept at room temperature for 30 min. A constant volume of 0,2 mℓ was then transferred onto preformed monolayers in 15 mm wells in plastic trays. The cultures were treated and stained as described above. The tests were read when plaques were clearly visible in wells containing negative control serum. The reciprocal of the highest dilution of serum showing 75 % inhibition of plaques was taken as the antibody titre.

Microneutralization

Twofold serum dilutions, starting at 1:4, were made in microtitre plates. An equal volume of a virus suspension, containing a calculated 10–30 plaque-forming units of virus, was added to each well. The serum-virus mixtures were left at room temperature for 30 min before seeding with sufficient cells to form a confluent monolayer within 8–12 h at an incubation temperature of 35 °C–37 °C. The plates were treated, stained and read by the procedure already described. The reciprocal of the highest dilution of serum showing 75 % of inhibition of CP was taken as the antibody titre.

Neutralization tests in mice

Serial twofold dilutions of serum were prepared in BLP, and a volume unit of each dilution was mixed with an equal volume of virus, containing approximately 30–100 Mouse LD_{50}. This was then incubated at 37 °C for 30 min. Each serum-virus mixture was injected i.c. into 2 families of 7 one-day-old mice, each receiving 0,02 mℓ. Deaths were recorded from the 2nd to the 12th day. The reciprocal of the highest dilution of serum showing 50 % protection was taken as the end-point.

Susceptibility of mice and embryonated hen's eggs

Infected mouse brain and blood collected from calves reacting to experimental infection with WSL and WN virus was titrated simultaneously in mice and in embryonated hen's eggs.

RESULTS

All the viruses readily adapted to growth on CER cells in roller tubes. Multiplication was characterized by rounding of cells, followed by detachment after 5–15 days. In many instances it was not possible to distinguish between detachment caused by viral activity or by normal degeneration after prolonged incubation. Consequently, it was not possible to determine end-points accurately. Yellowing of infected cultures was a constant feature, and this was usually discernible before CP

TABLE 4 Haemagglutination inhibition cross-reactions in sera from rabbits injected with flaviviruses known to be present in South Africa

Antigen	HI antibody titres[1] in sera from rabbits injected with:									
	WSL	BAG	TME	NTA	BAN	UG-S	AR 5189	WN	USU	SPO
WSL	**1 280**	40	640	20	640	1 280	80	640	80	20
BAG	1 280	**1 280**	1 280	80	160	1 280	80	1 280	80	40
TME	1 280	640	**1 280**	40	320	320	80	320	40	20
NTA	640	20	80	**80**	80	320	20	320	10	20
BAN	640	20	640	20	**1 280**	640	20	160	40	40
UG-S	80	10	640	10	160	**1 280**	10	40	10	10
AR 5189	80	10	320	10	160	80	**320**	40	10	10
WN	320	10	80	10	40	80	10	**160**	10	20
USU	640	20	80	10	160	640	10	80	**40**	10
SPO	20	10	20	10	80	80	10	80	80	**80**

[1] Titres expressed as reciprocals of serum dilution

TABLE 5 Haemagglutination inhibition cross-reactions in sera from calves and lambs experimentally infected with Wesselsbron virus

Antigen	Antibody titres[1] in sera from calves and lambs infected with WSL			
	Calf 1	Calf 2	Lamb 1	Lamb 2
WSL	1 280	320	40	320
BAN	640	80	10	10
BAG	80	20	10	10
TME	160	20	10	10
USU	320	20	10	10

[1] Titres expressed as reciprocals of serum dilution

changes became evident. In PI tests, all the viruses, except SPO and USU, regularly formed plaques on CER cells, although the time of appearance, size and clarity varied with different viruses. Plaque formation by SPO and USU was very inconsistent and could not be utilized for routine PI tests. It was often difficult, or even impossible, to count plaques formed by BAG and TME because of their small size. Some batches of bovine serum also inhibited the development of normal sized plaques. This effect was observed with serum used in the overlay as well as with serum used in PI tests. It appeared to be a non-specific effect, as several virsues were inhibited by the same serum.

Comparable results were obtained with plaque titration and titration on microtitre plates, but a prozone effect, observed with most viruses (Table 2), was constant feature in microtitrations, where only the higher dilutions of virus produced visible CP changes. This effect was more pronounced with certain sera (Table 3) and could not be eliminated by polyethylene glycol treatment or heat inactivation of the sera. No prozone effects was observed with a commercially available serum.

Antibody production

Rabbits and guinea-pigs reacted to all the viruses by the formation of antibodies. However, the antibody titres in serum from rabbits were 10–20 times higher than those of guinea-pigs (results not shown). The titres of antibodies produced by calves and lambs were lower and were limited to animals injected with certain viruses. High levels of antibody were produced by calves and lams injected with WSL, BAN and WN. The antibody titres of animals injected with UG-S, AR 5189 and BAG were much lower. No antibody was detected in serum of calves and lambs injected with USU, TME, NTA and SPO.

Cross-reactivity in haemagglutination inhibition tests

The results of the HI tests performed with rabbit serum showed that rabbit serum (Table 4) and calf serum (Table 5) are broadly cross-reactive and that sheep serum is virtually monospecific (Table 5). Only the results of lambs and calves infected with WSL are shown, but a similar pattern was observed with the sera of animals reacting to other viruses.

Cross-reactivity in neutralization tests

Table 6 shows the results when rabbit immune serum was tested against the homologous and heterologous viruses. Antibody titres obtained with PI are significantly higher than titres obtained with microneutralization or with neutralization tests in mice. Slightly more cross-reactivity was observed with the mouse neutralization test. It was possible to divide the 10 viruses into the following 5 subgroups: Group 1-WSL, Group 2-BAG, TME and NTA, Group 3-BAN, UG-S, and AR 5189, Group 4-WN and USU and Group 5-SPO.

Susceptibility of embryonated hen's eggs and mice

All the viruses multiplied in eggs and in mice (Table 7). Virus titres obtained in one-day-old and three-week-old mice injected, i.c., were reasonably similar. Lower titres were obtained in mice, injected i.p. This was especially noticeable in three-week-old mice which reacted poorly to WSL and BAG and not at all to USU, SPO and NTA.

Virus titres obtained in eggs injected with WSL, BAN, USU, SPO, UG-S and AR 5189 were similar to, or lower than, the titres obtained in mice. On the other

TABLE 6 Virus neutralizing cross-reactions amongst flaviviruses known to be present in South Africa

Virus	Neutralization test	Virus neutralizing antibody titres[1] in serum from immune rabbits injected with:									
		WSL	BAG	TME	NTA	BAN	UG-S	A5	WN	USU	SPO
WSL	Mouse	80	—[2]	—	—	—	—	—	—	—	—
	Plaque inhibition	320	—	—	—	—	—	—	—	—	—
	Microtitre	64	—	—	—	—	—	—	—	—	—
BAG	Mouse	—	640	40	—	—	—	—	—	—	—
	Plaque inhibition	—	1 280	640	—	—	—	—	—	—	—
	Microtitre	—	512	128	—	—	—	—	—	—	—
TME	Mouse	—	80	640	—	—	—	—	—	—	—
	Plaque inhibition	—	640	1 280	—	—	—	—	—	—	—
	Microtitre	—	256	256	—	—	—	—	—	—	—
NTA	Mouse	—	T[3]	20	80	—	—	—	—	—	—
	Plaque inhibition	—	—	—	40	—	—	—	—	—	—
	Microtitre	—	—	—	32	—	—	—	—	—	—
BAN	Mouse	—	—	—	—	640	20	80	—	—	—
	Plaque inhibition	—	—	—	—	1 280	320	—	—	—	—
	Microtitre	—	—	—	—	512	32	—	—	—	—
UG-S	Mouse	—	—	—	—	40	640	80	—	—	—
	Plaque inhibition	—	—	—	—	320	1 280	—	—	—	—
	Microtitre	—	—	—	—	32	256	—	—	—	—
AR 5189	Mouse	—	—	—	—	20	40	640	—	—	—
	Plaque inhibition	—	—	—	—	—	—	640	—	—	—
	Microtitre	—	—	—	—	64	—	128	—	—	—
WN	Mouse	—	—	—	—	—	—	—	160	T	—
	Plaque inhibition	—	—	—	—	—	—	—	1 280	—	—
	Microtitre	—	—	—	—	—	—	—	128	—	—
USU	Mouse	—	—	—	—	—	—	—	T	40	—
	Plaque inhibition	—	—	—	—	—	—	—	ND[4]	ND	—
	Microtitre	—	—	—	—	—	—	—	—	32	—
SPO	Mouse	—	—	—	—	—	—	—	—	—	40
	Plaque inhibition	—	—	—	—	—	—	—	—	—	ND
	Microtitre	—	—	—	—	—	—	—	—	—	ND

[1] Titres expressed as reciprocals of serum dilution; [2] — = less than 10; [3] T = Trace; [4] Not done

TABLE 7 Susceptibility of mice and embryonated hen's eggs to 10 flaviviruses known to be present in South Africa

Source of virus	Virus titres of flaviviruses titrated:					
	Intracerebrally in mice		Intraperitoneally in mice		Embryonated eggs	
Infected mouse brain	1 day old	3 weeks old	1 day old	3 weeks old	4 days old	12 days old
WSL	7,0[1]	6,5	6,6	1,2	5,5[2]	6,0
BAN	10,8	10,6	9,5	8,3	7,5	7,5
USU	5,0	4,5	Neg.[3]	Neg.	5,5	5,0
SPO	5,3	5,5	2,6	Neg.	2,5	3,5
UG-S	7,6	6,8	6,9	6,6	5,0	6,5
AR 5189	6,5	5,6	4,3	4,0	6,0	6,3
BAG	6,9	6,8	5,0	3,1	8,3	7,8
TME	5,7	5,7	5,7	4,0	7,1	7,6
NTA	4,7	4,7	Neg.	Neg.	6,8	6,3
WN	7,1	6,9	6,8	6,5	9,7	9,4
Calf blood						
WSL	1,5	1,0	1,0	Neg.	1,0	1,0
WN	1,6	1,0	1,0	1,0	1,2	1,5

[1] Virus titres expressed as Log_{10} mouse LD_{50}; [2] Including deformed embryos
[3] Neg. = negative

hand, embryonated eggs yielded significantly higher titres with BAG, TME, NTA and WN.

In four-day-old embryos, varying percentages of abnormalities were encountered. Stunted growth and poor feather development were regularly observed with all the viruses. Other abnormalities encountered were oedema, beak deformities, hydranencephaly and imperfectly closed crania. Arthrogryposes was occasionally observed in embryos injected i.v. at 12 days. When only embryos that died were taken into account, virus titres in four-day-old embryos were lower with BAN, USU, SPO, UG-S and AR 5189. If deformed embryos are included as well, virus titres were very similar in embryos injected i.v. or via the yolk-sac route. Deformed embryos seldom yielded virus when they were titrated in embryonated eggs or in mice.

Susceptibility of Japanese quail

Quail injected i.m. were killed by BAG, TME, NTA and WN. On isolation, all dead quail yielded virus. Quail injected with the other viruses did not show any symptoms of disease. Virus isolation from these birds was not attempted.

DISCUSSION

Antibodies against flaviviruses are most specific for individual viruses following primary infection and become more cross-reactive after reinfection, even with the same virus (Casals, 1957). When rabbit immune serum was employed to determine serological cross-reactions between the 10 flaviviruses known to be present in South Africa, sera were broadly cross-reactive in HI tests (Table 4) and more specific in VN tests (Table 5). The results further indicated that the relationship between these 10 viruses, which could be divided into 5 subgroups, conforms to the general pattern described for the group (Casals, 1957; De Madrid & Porterfield, 1973) (Table 6).

In serum from calves and lambs reacting to a single injection of virus, virtually no VN cross-reactions were detected, even among viruses within a particular subgroup. With the HI test (Table 5), very few cross-reactions were encountered with lamb serum. However, cross-reactions were commonly encountered in calf serum, even between members of different subgroups. Because of these cross-reactions and because of the rapid decline of HI antibodies, VN tests are preferred for routine diagnostic tests.

Blackburn & Swanepoel (1980) remarked on the differences in cross-reactions observed in serum from cattle, sheep and guinea-pigs. They maintained that some of the differences are related to variations in immunizing schedules, test procedures and the maturity of the host in which the sera are prepared. They found a weak cross-reaction with VN tests between WSL and BAN, and were in agreement with Smithburn, Kokernot, Heymann, Weinbren & Zentkowsky, (1959). De Madrid & Porterfield (1973), however, failed to demonstrate any relationship between WSL and BAN but showed that UG-S is closely realted to BAN. The results obtained in the present study are in agreement with those of De Madrid of Porterfield (1973). In addition, we also found that the unidentified virus AR 5189 is closely related to BAN and UG-S.

The immunological response of calves and lambs was very similar. High levels of homologous antibody were present in serum of both species injected with WSL, BAN, UG-S and WN. Low levels of antibody were present against BAG and AR 5189. No measurable antibodies could be detected in serum of calves and lambs injected with TME, NTA, USU and SPO.

Initially, the PI test was performed, but non-specific inhibition was encountered. Several viruses were inhibited by certain sera incorporated in the overlay. This resulted in the formation of minute plaques only. Consequently the test was abandoned in favour of the microneutralization test. Virus titrations in micro culture plates led to the detection of a prozone effect (Table 2). Cytopathic changes could be seen only with higher dilutions of virus. The inhibition was related to the incorporation of certain sera in the growth medium (Table 3). Limited attempts to determine the cause of inhibition indicated that it was not related to the method of antigen production as it was observed with antigen prepared in mouse brain as well as on CER cells. Heat treatment of serum for 30 min at 56 °C or treatment with polyethylene glycol did not diminish the effect. It became clear, however, that in order to obtain reliable results it is important to employ only optimum dilutions of antigen and to carefully select serum for use in the growth medium.

Virus titres obtained in one-day-old and three-week-old mice injected i.c. were reasonably similar. The lower titres obtained in mice injected i.p. with WSL and BAG, and the lack of infectivity of USU, SPO and NTA may be helpful in the initial identification of newly-isolated

virus. Results also showed that, for isolation purposes, the i.c. route must be used.

BAG, TME, NTA and WN yielded significantly higher titres of virus in embryonated eggs than in mice. These 4 viruses also killed Japanese quail injected i.m. These results show the greater sensitivity of birds for these viruses and indicate that they may be primarily bird viruses.

Four-day-old and twelve-day-old embryos seemed to be equally susceptible to infection with the viruses used (Table 7). However, in the results shown, abnormal embryos were regarded as infected, but they seldom yielded virus when subcultured either in eggs or in mice. Consequently, twelve-day-old embryos injected i.v. are preferred for virus isolation.

The greater sensitivity of embryonated hen's eggs for some viruses and of mice for others necessitates the employment of both systems for the isolation of flaviviruses in South Africa.

In four-day-old and in twelve-day-old embryos, varying percentages of abnormal embryos were encountered. The abnormalities are similar to those seen in outbreaks of abortions in cattle and in sheep. It must, however, be realized that in order to infect a bovine or sheep foetus, the virus must be able to multiply in the mother and to cross the placental barrier. The susceptibility of cattle and sheep will be the subject of another report of flaviviruses in South Africa.

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