ISOLATION OF SPONDWENI VIRUS FROM FOUR SPECIES OF CULICINE MOSQUITOES AND A REPORT OF TWO LABORATORY INFECTIONS WITH THE VIRUS

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Spondweni virus was first isolated in 1955 from a pool of *Mansonia* (*Mansoniodes*) *uniformis* (Theo.) mosquitoes collected in northern Zululand, South Africa.[1] It was shown then that Spondweni virus belonged to Casals' group B of the arthropod-borne viruses, and a survey of human sera collected from residents of northern Zululand showed the presence of neutralizing antibodies against this virus.

Shortly after the isolation of this virus, a field station was established by this Unit in northern Zululand where periodic field investigations have been made in a continuation of our study of the arthropod-borne viruses. This field station is at Ndumu which is about 20 miles from the Simbu area where the original isolation of Spondweni virus was made.

During the period March — June 1958, intensive field investigations were carried out at Ndumu, since previous experience had shown that overt viral activity is at its peak at this time of the year, namely, the late summer and autumn.

From mosquitoes collected at Ndumu during this period 9 strains of Spondweni virus were isolated. While these strains were being handled in the laboratory, 2 cases of illness occurred among laboratory personnel. Evidence is presented which indicates that these illnesses were caused by Spondweni virus.

MATERIALS AND METHODS

The main investigations at Ndumu consisted of the following activities:

1. Collection of mosquitoes for virus isolation.
2. Collection of tissues from small mammals and birds for virus isolation.
3. Collection of blood specimens for virus isolation from persons with symptoms suggestive of a virus infection who attended clinics in the area.
4. Collection of blood specimens from small mammals and birds for antibody studies.
5. Use was made of human beings and animals as sentinels. Antibody studies on sera obtained from bleedings at the beginning and end of the observational period were made to investigate possible antibody conversions.

The mosquitoes were caught off vegetation by hand in glass test-tubes, 90×15 mm., which were then plugged with cotton wool. At the field station the mosquitoes were grouped according to species and collecting sites. They were then stored on CO_2 ice until processed in the main laboratory in Johannesburg for inoculation into mice. For this the mosquito lots were processed in 0·75% bovine plasma albumin and the suspension from each lot was inoculated intracerebrally (IC) into 2 litters of infant mice, 2 - 3 days old, and into 6 adult mice. The dose of inoculum was 0·03 ml. for mice of both ages. Further details on the techniques used for the collection and handling of mosquitoes for virus isolation are given elsewhere.[2,3]

During the period March — June 1958, almost daily mosquito collections were made at 12 collecting sites. Since the establishment of the field station at Ndumu, many different collecting sites had been tried and the ones exploited during 1958 were those which had yielded the greatest number of mosquitoes. A collecting site is a small area usually about 50 yards in diameter. It is a clump of the somewhat denser scrub or trees and is relatively well shaded. Some of them are not in the immediate vicinity of water.

The serological procedures used in the neutralization (N), complement-fixation (CF), and haemagglutination-inhibition (HI) tests follow the methods described by Smithburn,[4] Casals *et al.*,[5] and Clarke and Casals,[6] respectively.

For use in the N test, the antiserum against the Spondweni prototype strain (AR 94) was obtained from a vervet monkey following 2 intraperitoneal inoculations of the virus. The AR 1038 antiserum was a pool from 2 rabbits following a single IC inoculation of virus. In CF and HI tests mouse hyperimmune antisera were used.

ISOLATION OF THE VIRUS STRAINS

Between 18 March and 5 June a total of 13,123 female mosquitoes was caught and inoculated into mice. Nine strains of virus, which were subsequently identified as Spondweni virus, were isolated from 4 species of mosquitoes as indicated in Table I. Included in this table are 3 mosquito species from which virus was not isolated, but which were obtained in relatively large numbers. The mosquitoes listed in this table comprise 98% of the total catch.

In mice these strains behaved similarly to each other and also to the original Spondweni virus strain, AR 94, isolated in 1955. By IC passage of infected mouse brain no difficulty

TABLE I. SPECIES OF MOSQUITOES COLLECTED AT NDUMU, MARCH-JUNE 1958, WHICH WERE PREVALENT AND/OR FROM WHICH STRAINS OF SPONDWENI VIRUS WERE ISOLATED

Species	*No. per lot*			*Total lots*	*Total mosq.*	*Spondweni virus isolations*	*Virally productive collection site*
	Min.	*Max.*	*Mean*				
Aedes (*Neomelaniconion*) *circumluteolus* (Theo.)	12	300	100	51	5,097	4	4, 16, 18, 21
Mansonia (*Mansonioides*) *africana* (Theo.)	1	166	30	31	926	3	4, 18, 21
Aedes (*Aedimorphus*) *cumminsi* ssp. *mediopunctatus* (Theo.)	3	25	10	3	31	1	18
Eretmapodites silvestris (I. and de M.)	1	8	2	7	16	1	16
Aedes (*Aedimorphus*) *albocephalus* (Theo.)	2	228	88	52	4,563	0	—
Culex (*Culex*) *univittatus* (Theo.)	6	138	38	37	1,396	0	—
Mansonia (*Mansonioides*) *uniformis* (Theo.)	3	173	33	25	835	0	—

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was encountered in establishing the 9 isolates in mice. In the case of the first strain isolated, AR 1038, the infant mice were noticed sick or dead on the 5th and 6th days following inoculation of the mosquito suspension. The adult mice inoculated with the same suspension sickened between the 8th and 10th days. On IC passage of brain from a sick infant mouse into further mice, infant mice sickened on the 4th day and died on the 5th and 6th days, while in adult mice deaths occurred a day or two later. From the 2nd passage, AR 1038 virus, as well as the other 8 strains, were fully lethal for mice of both ages. All the strains passed readily through Seitz asbestos filters.

In four instances where a mosquito suspension had yielded virus, the original suspension was inoculated into mice after storage at about −20°C. in an attempt at re-isolation. Of these suspensions, 3 proved infective for mice.

IMMUNOLOGICAL IDENTIFICATION OF THE ISOLATES

HI. From all 9 isolates a haemagglutinin was obtained from an alkaline aqueous suspension of infant mouse brain after centrifugation at 10,000 r.p.m. for one hour. This suggested their relationship to the group B viruses. HI tests with various specific antisera confirmed this relationship and their probable identity as Spondweni virus. In these tests their group B properties resulted in inhibition by several group B antisera, but by no other antisera. The highest titres occurred with a Spondweni hyperimmune mouse serum. Other group B viral antisera which gave inhibition were notably West Nile and, to a lesser extent, Uganda S and Wesselsbron.

CF. The identification of all 9 isolates as Spondweni virus was established in CF tests with the same antigens used in the HI tests and a range of mouse hyperimmune viral antisera. These antisera consisted of antisera against 4 group A viruses, 5 group B viruses and Rift Valley fever and Simbu viruses. Fixation occurred with Spondweni antiserum and with none of the other antisera.

N. The identity of the isolates was confirmed with the N test. All these tests were carried out in adult mice inoculated IC. As indicated in Table II a Spondweni virus antiserum significantly neutralized the 9 isolates. In addition there was reciprocal cross-neutralization between one of these isolates and Spondweni virus.

TABLE II. RESULTS OF N TESTS BETWEEN AR 94 STRAIN OF SPONDWENI VIRUS, 9 STRAINS OF VIRUS ISOLATED FROM MOSQUITOES, AND AR 94 AND AR 1038 VIRUS ANTISERA

Virus	*Serum*	
	AR 94	*AR 1038*
AR 94	3·3*	3·8
AR 1038	2·4	2·9
AR 1071	2·8	
AR 1077	3·6	
AR 1081	3·0	
AR 1084	2·1	
AR 1086	2·1	
AR 1163	3·4	
AR 1168	2·5	
AR 1266	3·3	

* Results expressed as the neutralization index.

LABORATORY INFECTIONS

As mentioned previously, while work with the Spondweni isolates was in progress in the laboratory, 2 cases of illness occurred among laboratory personnel. The details of these cases are as follows:

Case 1

J.L., a White female, 21 years old, became ill on the evening of 23 June 1958. Her symptoms were generalized aches and pains, rigors, and vertigo. Her temperature at 8 a.m. on 24 June was 100°F., but was normal when a blood specimen was obtained at 1.30 p.m. on the same day. It was observed that her blood clotted very slowly. Recovery was quick and complete.

Case 2

C.B., a White male, 19 years old, became ill on 27 April 1958. His symptoms were severe headache, weakness, nausea, and slight epistaxis. On the morning of 28 April his temperature was 99°F. and in the afternoon 102·3°F., when a blood specimen was collected. On the 29th his temperature was 99·5°F. and a slight headache was present. The patient remained in bed until 1 May when he returned to work. However, he went off duty that afternoon owing to a severe headache and weakness and stayed away from work for a further 3 days.

Serum obtained from the acute-phase blood specimens of both individuals was inoculated into infant mice in an attempt at virus isolation. A virus was isolated from J.L.'s serum, but not from that of C.B. An attempt at re-isolation by inoculation into mice of J.L.'s serum after 14 days' storage at −20°C. was unsuccessful. However, this can possibly be explained by a loss of infectivity during storage, since its original infectivity was such that only 6 out of the 12 infant mice inoculated sickened. As is evident from the serological studies on this serum mentioned below, the group B virus antibody present in this serum may also have been a factor in the low infectivity.

The virus obtained from J.L. was identified as Spondweni virus in an N test. In this test a Spondweni virus (AR 94) antiserum neutralized 2·5 logs of the virus.

The antibody response following the illnesses of the 2 patients was studied by means of the N, CF and HI tests.

Owing to the antigenic overlap between the group B viruses, 5 other group B viruses, apart from Spondweni virus, were included in the CF and HI tests with sera from both individuals. Also included in these 2 tests was a pre-illness serum from both patients as well as a second convalescent serum from J.L.

The results obtained with sera from C.B. and J.L. in the CF and HI tests are shown in Tables III and IV.

TABLE III. CF RESULTS WITH SEVERAL GROUP B VIRUS ANTIGENS AND VARIOUS SERA FROM INDIVIDUALS J.L. AND C.B.

Antigen	*Sera from J.L.*				*Sera from C.B.*		
	7 Feb. 1958 pre-illness	*24 June 1958 acute*	*10 July 1958 1st conv.*	*10 Mar. 1959 2nd conv.*	*28 Mar. 1958 pre-illness*	*28 April 1958 acute*	*28 May. 1958 conv.*
Spondweni (AR 94)	0	0	0	4*	0	0	8
Wesselsbron	0	4	32	8	0	0	0
H 336†	0	32	128	64	0	0	0
West Nile	0	8	32	16	0	0	0
Zika	0	8	8	8	0	0	0
Yellow fever	0	16	128	16	0	0	0

* Reciprocal of highest serum dilution giving +3 fixation.

† A group B virus isolated from man in South Africa and related to, but not identical with, Uganda S virus.[11]

TABLE IV. HI RESULTS WITH SEVERAL GROUP B VIRUS ANTIGENS AND VARIOUS SERA FROM INDIVIDUALS J.L. AND C.B.

Antigen	*Sera from J.L.*				*Sera from C.B.*		
	7 Feb. 1958 pre-illness	*24 June 1958 acute*	*10 July 1958 1st conv.*	*10 Mar. 1959 2nd conv.*	*28 Mar. 1958 pre-illness*	*28 April 1958 acute*	*28 May 1958 conv.*
Spondweni (AR 94)	0	40*	640	320	0	640	1,280
H 336	0	1,280	2,560	1,280	0	0	40
Wesselsbron	0	320	640	320	0	0	40
Zika	0	10	40	10	0	0	0
West Nile	0	1,280	2,560	2,560	0	0	160
Yellow fever	10	160	320	160	0	0	0

* Titre expressed as reciprocal of highest serum dilution which gave inhibition.

From Table III it can be seen that Spondweni CF antibodies were present in the convalescent serum and absent in the acute and pre-illness sera. Also CF antibodies against the other 5 group B viruses were absent in the convalescent serum. In Table IV it can be seen that Spondweni virus HI antibodies were absent in the pre-illness serum, while there was a two-fold rise in antibodies following the illness. Of the group B viruses tested, the HI titre was highest against Spondweni virus.

In the N test C.B.'s acute serum gave no neutralization of Spondweni virus, whereas the convalescent serum was fully protective against 158 LD_{50} of virus.

In the case of J.L., preliminary tests indicated that the antibody response following her illness was by no means clear-cut. This individual had been immunized some 5 years previously against yellow fever and it appeared likely, from the HI and CF tests on her acute-phase serum, that she had had a group B virus infection between the time of commencement of her employment in this laboratory in February 1958, and her illness in June 1958. During this period she had been intensively engaged in the preparation of several group B virus HA antigens so that opportunity for such infection to have occurred had existed.

Four sera were available from J.L. for antibody tests. These included a pre-illness serum collected on 7 February 1958, when she started work in this laboratory; the acute serum, from which Spondweni virus was isolated, obtained on 24 June 1958, the day following the onset of the illness; and 2 convalescent sera. The one was obtained on 10 July 1958, 16 days after the onset of the illness, and the other on 10 March 1959. During January 1959 she had received a second yellow fever immunization. The results with the CF, HI and N tests on these sera are shown in Tables III, IV and V.

TABLE V. RESULTS OF N TESTS WITH SPONDWENI VIRUS, A NORMAL MONKEY SERUM, AND THE ACUTE AND CONVALESCENT SERA FROM PATIENT J.L.

Serum	*Logs virus titre*	*Logs virus neutralized*
Normal monkey	5·5	—
Acute, 24 June 1958	4·1	—
Convalescent, 10 July 1958 ..	2·8	1·3

With the CF test (Table III) it can be seen that, while her pre-illness serum was devoid of antibody against all the group B agents tested, her acute serum contained antibody against 5 of these agents, but not against Spondweni. It is noteworthy that after 16 days following the commencement of her illness Spondweni antibody was still absent, but there was an increase in antibody titre against 4 of the other agents. Also, whereas her second convalescent serum showed the appearance of Spondweni antibody, the antibody titres against the remaining group B agents either remained constant or decreased.

From Table IV it is evident that at the time of her illness the patient possessed quite a degree of group B virus HI antibody which reached a considerably higher level at the time her first convalescent serum was obtained. In this increase it should be noted that there was a sixteen-fold rise in antibody titre against Spondweni, while only a two-fold or four-fold rise occurred against the other antigens. The 1 : 10 HI titre against yellow fever antigen in the pre-illness serum is possibly the result of her first yellow fever immunization.

The results of the N test on J.L.'s acute and first convalescent sera shown in Table V, indicate a slight rise in Spondweni virus antibody during convalescence. While a neutralization index of only 1·3 was obtained, this figure was reproducible and this low value could possibly be accounted for by the presence of group B virus antibody in the acute-phase serum. The difference in titre of the virus in this serum and a normal monkey serum supports this contention.

DISCUSSION

The isolation of Spondweni virus from 9 different pools of mosquitoes caught at Ndumu was conclusive evidence of the presence of this agent in the immediate vicinity. These isolations occurred after more than 2 years of work at Ndumu during which Spondweni virus had not been isolated. The apparent sporadic appearance of viruses in Tongaland has been observed with other arthropod-borne viruses during the course of our studies in this area.

Of the 12 collecting sites at Ndumu which were exploited, 4 yielded virus. The productive sites are located within an area about 6 miles in diameter which was virtually the area of our field activities at Ndumu at this time. Within such narrow limits, these isolations must reflect an intense type of viral activity.

The non-arthropod hosts involved in this outbreak can only be speculated upon. Our observations suggest that birds and small mammals should be excluded. Attempts at virus isolation were made from 120 wild birds and 85 small mammals caught during the period March — June 1958, with no isolation of Spondweni virus. Although the birds were obtained just outside the perimeter of the mosquito-collecting area, the mammals were trapped at the actual collecting sites. Furthermore, antibody studies on sera obtained from birds and rodents have shown that Spondweni-neutralizing antibody is not prevalent in these animals from this area.

The susceptibility of cattle, goats and sheep, of which there are large numbers in the area, is not known. Of 234 sera collected in Tongaland in 1955 from cattle, sheep, and goats, tests for neutralizing antibody against Spondweni virus were positive in only 6 instances.[7] However, only 2 of these positive sera were monotypic for Spondweni virus alone, since the other sera were also positive against other group B viruses. In the area of the 1958 Spondweni outbreak, use was made of 31 cattle and 8 sheep as sentinels. Antibody studies with the N test on paired sera from these animals showed that 3 cattle and 2 sheep had possibly been infected with Spondweni virus during the outbreak.

What rôle the several thousand human beings resident in the Ndumu area may have played in the outbreak was not determined. Our attempts at virus isolation from 53 febrile patients who attended the clinics and from whom blood samples were taken, resulted in the isolation of a strain of West Nile virus,[8] but no Spondweni virus. N tests on paired sera from 7 African boys employed as mosquito catchers showed that one of these boys had an antibody conversion to Spondweni virus. On the other hand, the incidence of Spondweni antibody was found to be extremely low in 111 sera collected in December 1958 from Africans resident in the Ndumu area. These sera were initially tested in an HI test when 23 were positive at a dilution of 1 : 10 to Spondweni antigen. When these 23 sera were tested in an N test, only 2 were fully protective against Spondweni virus.

Aedes circumluteolus and *Mansonia africana* both frequent the shady bush habitat, although the latter species is also known to enter dwellings irregularly at night.[2] Visual observation[2] and the results of precipitin tests on recently engorged mosquitoes of these 2 species caught in the Ndumu area have shown that they possess somewhat similar host preferences, namely, they feed mainly on man and the larger domestic and wild animals, but infrequently on birds and rodents.[9]

Despite the host preferences of 2 species of mosquito implicated in this outbreak, our failure to isolate virus from the human beings who attended the clinics and the lack of more definite serological evidence incriminating man, cattle, and sheep means that the vertebrate host must remain conjectural.

Theiler and Casals[10] reported on the nature of the antibody response following infection with yellow fever in human beings, some of whom had apparently been infected previously with another group B virus. A distinction was made between the antibody response following a primary group B virus infection and that following a secondary infection. In a primary infection the response was more specific to the infecting agent, especially with the N test, and, if heterologous antibodies developed, they did so to a lower titre than the homologous antibodies. In a secondary infection the antibody response was to a variety of agents, and the only conclusion that could be derived from serological evidence was that a group B agent was involved. In these cases a specific diagnosis can be made only if the causal group B virus is isolated during the illness. Often the highest antibody levels following secondary infections were heterologous.

In accordance with these findings and the results of our serological tests on their sera, C.B.'s illness is considered a primary group B infection and J.L.'s illness a secondary or even multiple group B infection if the first yellow fever immunization is taken into account. Hence, the diagnosis of Spondweni infection in these cases would depend on the following evidence:

C.B.

1. The absence of Spondweni and group B, CF antibodies in the acute serum.

2. The presence of neutralizing Spondweni and CF antibody in the convalescent serum and the absence from this serum of CF antibody against 5 other group B agents.

3. The fact that HI antibody titre in the convalescent serum was highest against Spondweni virus.

L.J.

1. The isolation of Spondweni virus from the acute serum.
2. The rise in group B virus antibody during convalescence.

SUMMARY

1. Nine strains of Spondweni virus were isolated in mice from 13,123 female mosquitoes collected in Tongaland, South Africa, in 1958.

2. Four strains were isolated from *Aedes* (*Neomelaniconion*) *circumluteolus* (Theo.), 3 strains from *Mansonia* (*Mansoniodes*) *africana* (Theo.), and one strain each from *A. cumminsi* ssp. *mediopunctatus* (Theo.) and *Eretmapodites silvestris*, Ingram and De Meillon.

3. With the haemagglutination-inhibition, complement-fixation and neutralization tests, these strains were found to be immunologically similar to the strain of Spondweni virus isolated in Tongaland in 1955.

4. The outbreak of Spondweni virus activity in mosquitoes in 1958 followed a 2-year period when the presence of this virus had not been detected in Tongaland.

5. The non-arthropod host involved in the outbreak was not apparent.

6. At the time that strains of Spondweni virus were being handled in the laboratory, 2 cases of illness occurred among laboratory personnel. Spondweni virus was isolated from one individual. The nature of the antibody response in the convalescent sera of both was studied with the neutralization, complement-fixation, and haemagglutination-inhibition tests.

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