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STUDIES ON AN OUTBREAK OF WESSELSBRON VIRUS IN THE FREE STATE PROVINCE, SOUTH AFRICA

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ABSTRACT. In early March 1996, Wesselsbron (WSL) virus caused mortality among lambs on a farm near Bultfontein in the northern Free State Province, South Africa. Mosquito collections were therefore undertaken from 27 March to 1 April to collect floodwater *Aedes* mosquitoes for attempts at virus isolation. In all, 4,732 floodwater *Aedes* were tested; 5 WSL, 1 Middelburg (MID), and 5 unidentified viruses were isolated from 3,052 *Aedes* (*Neomelaniconion*) *mcintoshi/luridus* (minimum infection rate [MIR] for WSL = 1.63) and 5 WSL, 1 MID, and 3 unidentified viruses from 1,478 *Aedes* (*Ochlerotatus*) *juppi/caballus* (MIR for WSL = 3.38). One of the authors developed WSL fever on 3 April; WSL virus was isolated from his serum, and he developed a titer of 1:640 in the hemagglutination inhibition (HI) test and became IgM positive against WSL virus. Among a sample of 44 sheep bled on 4–5 September, 59% were antibody positive by the HI test against WSL and 48% against MID viruses. Mosquito collecting was restricted to 2 discrete, shallow, grassy depressions that were the main floodwater *Aedes* breeding sites on the farm so they will be investigated further as possible foci of transovarial transmission of WSL and MID viruses.

KEY WORDS Wesselsbron virus, Middelburg virus, floodwater *Aedes,* isolations, epidemiology

INTRODUCTION

The arbovirus Wesselsbron (WSL) belongs to the family of Flaviviridae and genus *Flavivirus.* Wesselsbron disease in southern Africa is characterized by sporadic outbreaks in sheep with neonatal deaths and abortions. Adult sheep and cattle also become infected but usually without morbidity and mortality (Swanepoel and Coetzer 1994). The occasional human infection has also been diagnosed, usually in persons investigating WSL virus in the field or laboratory (Swanepoel 1989). The virus is transmitted among livestock and to humans by floodwater *Aedes* mosquitoes, usually in wet summers. In South Africa, these mosquitoes most commonly oviposit in pans that are natural, seasonally flooded, shallow grassland depressions with no outlets and are not associated with river drainage systems. Some infections in humans and animals probably also occur by contagion (Swanepoel 1989).

Table 1 indicates the viral isolations and antigen detection made in vertebrates in South Africa over the past 21 years. In addition to this, routine hemagglutination-inhibition (HI) tests done in sheep and goats from 1980 to 1989 have shown the virus present in South Africa during this period (Gerdes, Veterinary Research Institute, Onderstepoort, South Africa, personal communication). Apparently, WSL virus is intermittently active in South Africa but lies dormant some years until suitable climatic conditions prevail that cause its reappearance on farms.

During the 2nd half of February until the beginning of March 1996, about 30 neonatal lambs died on a farm in the Bultfontein district of the northern Free State Province 82 km south of Wesselsbron, and 1 carcass was dispatched on ice to the Pretoria University Veterinary Faculty for laboratory diagnosis. The WSL viral antigen was detected by immunoperoxidase staining in this lamb, and symptomatology in all the lambs was consistent with WSL disease (Van der Lugt, personal communication). We made 2 subsequent visits to this farm to investigate this outbreak further. The 1st of these visits was in the autumn, from 27 March to 2 April, to collect floodwater *Aedes* mosquitoes for attempted virus isolation and to investigate the pans from which these mosquitoes probably originated. The 2nd visit was in spring, during September, to collect blood specimens from a sample of the sheep that had grazed in and near the pans and had been exposed to the mosquitoes the previous summer; the sera were tested for antibodies against WSL virus. One of us contracted WSL fever as a result of exposure to infected mosquitoes while collecting these insects.

METHODS

Study area: Bultfontein is located at the eastern edge of the Highveld region (28.17°S, 26.10°E) in the Free State Province. The term Highveld refers to the high inland plateau region with an altitude of 900–1600 m. It is grassland with a temperate climate and summer rains; mean annual temperature is 13–17°C. The Bultfontein area is generally flat country but has a number of shallow pans that flood during summers with good rainfall. In January 1996, 87.5 mm of rainfall caused the pans to become flooded, and by the end of this month, the farmers noticed substantial mosquito activity. In February, rainfall increased to 104 mm, and mosquito populations peaked at the beginning of March. Mosquitoes were collected from 2 of these pans on a farm south of Bultfontein. Most collecting was carried out at pan 1, in which the central sedge area, where the largest numbers of mosqui-

Table 1. Isolations and specific diagnoses of Wesselsbron virus in South Africa, 1975–1996.

Vertebrate	No. isolations	Town and province	Year	Reference
Calf	1	Irene, Gauteng	1975	Swanepoel 1989
Lamb	2	Harrismith, Free State	1975	Swanepoel 1989
Human	1	Johannesburg, Gauteng	1976	Swanepoel 1989
Ostrich[1]	1	Oudtshoorn, Western Cape	1992	Allwright et al. 1995
Lamb[2]	1	Frankfort-Cornelia, Free State	1994	Van der Lugt et al. 1995
Lamb[2]	1	Bultfontein, Free State	1996	Van der Lugt, personal communication 1996

[1] One isolation made from a pool of several spleens from young ostriches.
[2] Antigen detection by immunoperoxidase staining.

toes rested, was about 1 ha, whereas pan 2, with a central sedge area of about 0.3 ha, was exploited only on the last day of the fieldwork.

Mosquito collecting: Three methods were used: net traps baited with solid carbon dioxide (CO_2) (Jupp and McIntosh 1967), battery operated NIV light-suction traps also baited with CO_2 (Jupp 1986), and landing-biting catches on human bait using battery operated aspirators (Mechanical aspirator made by Hauscherr's Machine Works, Toms River, NJ). The latter catches were done by 2 operators, each collecting those mosquitoes alighting on himself or on his companion. The different methods were employed for various lengths of time as explained in the Results section. Culicine mosquitoes were identified according to the keys recently published in the book by Jupp (1997).

Collection of sheep sera: On 4–5 September, sheep were bled from the humeral vein, and blood specimens were transported the same day in a polystyrene box back to the laboratory in Johannesburg. After overnight storage at 4°C, specimens were centrifuged to separate the sera, which in turn were stored at −20°C.

Virus isolation and serology: Because of the large number of mosquitoes collected and because a large sample of floodwater *Aedes* was sought for virus testing, identification of more than small samples of the mosquitoes in the field was impractical. Instead, mosquitoes were sorted roughly into genera, stored in liquid nitrogen, and brought back to Johannesburg. In the laboratory, a batch of mosquitoes was identified and the *Neomelaniconion* and *Ochlerotatus* subgenera segregated for testing the same day. Mean pool size was 49.4 (n = 106, SD = 8.78, SE = 0.86). Subsequently, suspensions of the pooled insects were inoculated intracerebrally into 2-day-old suckling mice in attempts to isolate virus. Brain from sick mice was used for serial passage of virus in mice and as a source of virus for identification. Virus isolates were identified by complement fixation (CF) tests in which hyperimmune mouse ascitic fluids were used as a source of known antibody. These tests were done according to the method of Bradstreet and Taylor (1962) adapted to a microtechnique using 0.025-ml volumes (Blackburn and Swanepoel 1980). Sera obtained from both mosquito collectors were tested for presence of virus, and virus was identified using the same techniques.

Sheep and human sera were tested for antibodies in micro-HI tests against antigens of Sindbis, West Nile (WN), WSL, Rift Valley fever, and Middelburg (MID) viruses using an adaptation of the technique described by Clarke and Casals (1958). Sera from the 2 operators were also tested for WSL IgM antibodies by the ELISA test following the procedure used by Besselaar et al. (1989). The statistical mosquito infection rate (IR) was calculated by the method of Chiang and Reeves (1962).

RESULTS

Mosquitoes: Table 2 shows the numbers of different mosquito species or species groups sampled by each collection method. Of the 13,048 mosquitoes collected, 7,306 were *Culex theileri* and 5,399 floodwater *Aedes.* Only a sample of the latter could be identified to species due mainly to their advanced age with the concomitant rubbed ornamentation of most of the specimens. The identifications done (Table 2) showed that *Aedes* (*Ochlerotatus*) *juppi* and *Aedes* (*Ochlerotatus*) *caballus* occurred in the proportion 3:1, whereas *Aedes* (*Neomelaniconion*) *mcintoshi* and *Aedes* (*Neomelaniconion*) *luridus* were present in the proportion 15:1. Progeny were reared from some of the gravid *Neomelaniconion* mosquitoes to confirm their identifications. A very few *Aedes* (*Neomelaniconion*) *unidentatus* may have remained undetected in the collections, but this is considered unlikely, particularly because this species is usually larger in size than the other 2 *Neomelaniconion* species.

As mosquito collecting progressed, it became clear that, of the 3 collecting methods for obtaining floodwater *Aedes,* the landing-biting catch was the most productive in relation to effort. Hence, on the last 2 of the 5 collecting days, only this method was used, and on the last day, collecting moved to pan 2, where 8 of the total 25 man-hours of catches were carried out. Four light traps were set overnight the 1st day (27 March) and yielded about 6,000 mosquitoes per trap. This catch had to be discarded because the preponderance of *Cx. theileri* made

Table 2. Number of mosquitoes collected by 3 different trapping methods at Bultfontein.

Mosquito species	Net trap + CO_2 (64 trap-hours)[1]	Light suction + CO_2 (8 trap-hours)	Landing-biting (25.0 man-hours)	Total
Aedes (Aedimorphus) dentatus	3	0	10	13
Aedes (Aedimorphus) mixtus/ microstictus	1	0	0	1
Aedes (Neomelaniconion) mcintoshi/luridus	340	236	2,590	3,166
Aedes (Neomelaniconion) luridus	0	0	7	7
Aedes (Neomelaniconion) mcintoshi	5	0	107	112
Aedes (Ochlerotatus) juppi/caballus	366	121	1,484	1,971
Aedes (Ochlerotatus) caballus	6	0	24	30
Aedes (Ochlerotatus) juppi	28	0	71	99
Anopheles (Cellia) squamosus	260	24	5	289
Culex (Culex) theileri	7,056	162	88	7,306
Culex (Culex) univittatus	46	2	0	48
Culex (Culex) pipiens	6	0	0	6
Total				13,048

[1] This included 4 traps that were set overnight on one occasion.

separation of *Aedes* from the catch impractical, and the excessively large catch caused further damage to the already old floodwater *Aedes* specimens, making identification unfeasible. On 28 March, 4 light traps were set only from 1700 to 1900 h; this change reduced the number of *Cx. theileri* and was a feasible way of collecting the *Aedes*. However, as can be seen in Table 2, the landing-biting catches were superior for *Aedes*: 171.3 mosquitoes per man-hour compared with 44.6 per trap-hour. Similarly, the net traps were overall also much less efficient, 11.6 mosquitoes per trap hour, although when they were set in the late afternoon until sunset, the *Aedes* yield was greater and that of the *Cx. theileri* greatly reduced.

The most rewarding methods for the landing-biting catch were as follows: 1) an individual operator walked a few paces through the sedge to disturb the resting mosquitoes, halted, and then collected the mosquitoes that alighted on his body, and 2) 2 operators stood close together so that 1 could collect off himself as well as off his companion. Most mosquitoes were collected before they could engorge. The late afternoon until sunset was found to be the best time of day; 182 mosquitoes per man-hour were taken during this period compared with only 101 mosquitoes per man-hour in the early morning. It was also notable that *Cx. theileri* appeared and started biting only at dusk about 15 min before sundown. Conversely, the number of floodwater *Aedes* decreased dramatically after sunset, showing they were predominantly daytime biters.

Of the 5,399 floodwater *Aedes* collected, 4,732 (87.6%) were tested for virus (Table 3). None of the *Culex* and *Anopheles* species was tested because previous studies had indicated that they did not play a role as vectors of WSL or MID virus (Jupp et al. 1987; Swanepoel 1989). Five WSL viruses and 1 MID virus were isolated from each *Aedes* subgenus. Eight further isolates were made that are still to be identified. Table 4 reports the infection rates with WSL virus determined for samples of each mosquito duo collected as well as rates for pan 1 and pan 2. The overall rate for *Ae. juppi/ caballus* (2.78) was considerably higher than that for *Ae. mcintoshi/luridus* (1.67). Furthermore, IRs for *Neomelaniconion* and *Ochlerotatus* mosquitoes, respectively, were higher for mosquitoes collected at pan 2 than pan 1: 2.33 and 3.72 as compared with 0.61 and 2.33.

Sheep and human infections: Out of a sample of 44 sheep (ewes), 26 (59%) had antibodies against WSL virus by the HI test, none against WN virus, and 21 (48%) against MID virus. Titer ranges in this HI test for WSL and MID viruses were 1:20–1:640.

We returned from the mosquito collecting trip on 2 April, and one of us (P.J.) became ill on the evening of 3 April with headache, myalgia, and fever (temperature 38.6°C). On 4 April, these symptoms

Table 3. Number of mosquitoes tested for virus and number of isolations made at Bultfontein.

Mosquito species	No. mosquitoes tested	No. pools	Virus isolations		
			WSL	MID	Unidentified
Aedes (Neomelaniconion) mcintoshi/luridus	3,052	60	5	1	5
Aedes (Neomelaniconion) luridus	7	1			
Aedes (Neomelaniconion) mcintoshi	100	2			
Aedes (Ochlerotatus) juppi/caballus	1,478	40	5	1	3
Aedes (Ochlerotatus) caballus	24	1			
Aedes (Ochlerotatus) juppi	71	2			
Total	4,732				

persisted, although temperature dropped to 37.7°C, and an acute serum tested negative for WSL and WN antibodies by both HI and IgM ELISA tests. On 5 April, temperature was about normal (36.6°C) although myalgia persisted, and there was slight arthralgia in the lumbar region. On 6 April, an erythematous rash appeared on the trunk together with small ulcers on the gums. By 7 April, the rash had cleared, but mouth ulceration persisted until about 9 April and lassitude until 10 April. Convalescent sera were obtained on 17 April, 23 April, 2 May, and 24 May showing WSL HI antibody titers of 1:1,280, 1:320, 1:320, and 1:640, respectively. Tests for IgM antibody on serum of 17 April was positive for WSL but negative for WN virus, proving illness had been due to WSL virus. The WSL virus was isolated in the acute serum on 2nd passage in infant mice and identity was confirmed by the CF test. Sera tested from the 2nd author (A.K.) remained negative for antibody against both WSL and WN viruses.

DISCUSSION

The use of different trapping methods showed that, at the end of summer at the close of the mosquito season, the landing-biting catch was the most efficient way to collect floodwater *Aedes* mosquitoes. Unfortunately, the likelihood of the collectors becoming infected with WSL virus is high using this method, and this risk should be taken into account if landing-biting catches are utilized. However, light traps and net traps baited with CO_2 may prove useful for collecting floodwater *Aedes* at the beginning of the season, when a low *Cx. theileri* density is expected.

Most of the previous isolations made from mosquitoes in southern Africa came from the *Neomelaniconion* and *Ochlerotatus* aedine subgenera (McIntosh 1980, Swanepoel 1989). Nine isolations were made from 108,297 *Aedes (Neomelaniconion) circumluteolus* (minimum IR [MIR] 0.08) collected at Ndumu in Kwazulu-Natal (Worth et al. 1961)

Table 4. Mosquito infection rates with Wesselsbron virus at Bultfontein.

	No. mosquito pools tested	No. pools positive	IR[1]	MIR[2]
Aedes (Neomelaniconion) mcintoshi/luridus overall	60	5	1.67	1.63
Aedes juppi/ caballus overall	40	5	2.78	3.38
Aedes (Neomelaniconion) mcintoshi/luridus				
Pan 1	37	1	0.61	0.53
Pan 2	23	4	2.33	3.47
Aedes (Ochlerotatus) juppi/caballus				
Pan 1	28	3	2.33	3.37
Pan 2	12	2	3.72	3.38

[1] Statistical infection rate/1,000 calculated by method of Chiang and Reeves (1962).
[2] Minimum infection rate = no. infected mosquitoes/1,000.

and 15 isolations from *Aedes (Neomelaniconion) lineatopennis* (= *Ae. (Neo.) mcintoshi*) (MIR 3.9) collected in Zimbabwe (McIntosh 1972, 1980). Three isolations were also made from an unrecorded number of *Aedes lineatopennis/albothorax* (probably = *Ae. mcintoshi/luridus*) and 13 from an unknown number of *Ae. caballus s.l.* collected in Middelburg in the Eastern Cape Province (Kokernot et al. 1960). In earlier laboratory tests, both *Ae. circumluteolus* (Muspratt et al. 1957) and *Ae. caballus s.l.* (Kokernot et al. 1958) transmitted WSL virus. However, such experiments need to be repeated quantitatively on these and the other common floodwater *Neomelaniconion* and *Ochlerotatus*. The present Bultfontein isolations are the first WSL isolates made from mosquitoes collected in the Free State Province. Although there were an equal number of isolations made from *Neomelaniconion* and *Ochlerotatus* mosquitoes, the MIR was higher for *Ochlerotatus* (3.38) compared with *Neomelaniconion* (1.63). Both these MIRs are much higher than that for *Ae. (Neo.) circumluteolus* collected at Ndumu in 1955, but the rate for *Ochlerotatus* was about the same as that determined for *Ae. lineatopennis* in Zimbabwe in 1969. Although mosquitoes were collected only on the last day of the study at pan 2, there were proportionately more isolates from this sample than from mosquitoes collected at pan 1. Comparison of the IRs at both pans (Table 4) indicated a significantly higher IR at the 2nd pan.

The high antibody positivity rate in the sample of sheep tested confirmed the high level of WSL virus activity at the 2 pans and also indicated a high level of MID virus activity. The reason there were not more human clinical cases of WSL fever is thought to be directly related to the behavior of the *Neomelaniconion* and *Ochlerotatus* mosquitoes: observations by the senior author over many years indicate that these mosquitoes tend to remain at or close to the pan habitat. Because the farmer and his laborers seldom enter and spend time in the pan habitat but usually work in the cultivated areas, it is unlikely that these individuals would receive multiple mosquito bites from floodwater *Aedes* in the same way as one of us who contracted WSL fever. Adult floodwater *Aedes* are usually encountered in or close to the breeding pans, but McIntosh (1971) made an observation on *Ae. (Neo.) luridus* that indicated this species may travel some distance to feed on sheep if there are none close to the pan.

The clinical symptoms recorded in the human case study were similar to those previously noted (Swanepoel 1989). However, this is only the 3rd time a rash has been reported, and mouth ulcers have not been noted in earlier studies.

In South Africa, the available evidence indicates that the occurrence of WSL virus in floodwater *Aedes* populations is an intermittent phenomenon throughout the inland plateau as well as in the eastern subtropical and tropical lowlands. The isolation of virus only from mosquitoes belonging to the genus *Aedes* points to the possibility that transovarial transmission is the mechanism of virus survival through the dry winters and drought periods. It is unlikely that birds would introduce virus into South Africa from tropical countries to the north because several species of passerines as well as ducks have been shown refractory to infection in the laboratory (McIntosh 1980).

It seems likely that there were some infected *Aedes* mosquitoes that emerged from pans 1 and 2 earlier in the season when they were first flooded, i.e., some of the aedine eggs may have carried virus through the dry winter in the course of transovarial transmission (TOT) of virus to the next generation of insects. The main value of the study is that a farm has been located where the breeding of floodwater *Aedes* is more or less restricted to 2 small pans, which enhances their value as sites for a future study to determine whether TOT of WSL virus occurs. The isolation of 2 MID viruses means that there is the possibility that TOT of this virus might also be demonstrated in the same field study. However, it is difficult to make field observations on the immature stages and newly emerged adults of floodwater *Aedes* because their development from egg to adult is extremely rapid, taking only a few days after inundation. This rapid development occurs because the surface temperature of water in a pan rises to in excess of 30°C during the summer. Often, one learns of local rainfall too late to be able to arrange the collection of the immature stages.

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