VECTOR COMPETENCE EXPERIMENTS WITH ROCIO VIRUS AND THREE MOSQUITO SPECIES FROM THE EPIDEMIC ZONE IN BRAZIL

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ABSTRACT: First-generation progeny of field-collected *Psorophora ferox, Aedes scapularis,* and *Aedes serratus* from the Rocio encephalitis epidemic zone in S.Paulo State, Brazil, were tested for vector competency in the laboratory. *Psorophora ferox* and *Ae. scapularis* are susceptible to per os infection with Rocio virus and can transmit the virus by bite following a suitable incubation period. Oral ID_{50s} for the two species ($10^{4.1}$ and $10^{4.3}$ Vero cell plaque forming units, respectively) did not differ significantly. Infection rates in *Ae. serratus* never exceeded 36%, and, consequently, an ID_{50} could not be calculated for this species. It is unlikely that *Ae. serratus* is an epidemiologically important vector of Rocio virus. The utility of an *in vitro* feeding technique for demonstrating virus transmission by infected mosquitoes and difficulties encountered in working with uncolonized progeny of field-collected mosquitoes are discussed.

UNITERMS: Encephalitis viruses, physiology. *Psorophora ferox. Aedes scapularis. Aedes serratus.* Insect vectors, microbiology. Encephalitis, epidemic, transmission. Arbovirus infection.

Rocio virus was responsible for several epidemics of meningoencephalitis in coastal communities in Southern S.Paulo, Brazil, during 1975 and 1976. The natural transmission cycle has not been defined, but there is strong evidence to indicate that the virus is cycled between mosquitoes and birds (Iversson[8,9], 1977, 1980; Forattini et al.[4,5,6], 1978, 1981; Lopes et al.[10,11], 1978, 1981; Mitchell et al.[14], 1981; Mitchell & Forattini[13], 1984). Studies on mosquitoes captured during the epidemic yielded a single isolate of Rocio virus from a pool of *Psorophora ferox* (Von Humboldt) that contained engorged as well as unengorged specimens (Lopes et al.[10], 1981). Forattini et al.[3,4,5] (1961, 1978) reported that the predominant mosquito species in the epidemic area are *Aedes serratus* (Theobald), *Aedes scapularis* (Rondani), and *Culex (Melanoconion)* species. Mitchell & Forattini[13] (1984) demonstrated that *Ae. scapularis* from the epidemic zone in Brazil is an efficient vector of Rocio virus under experimental conditions. We report here on experiments with *Ps. ferox* and *Ae. serratus* and, for comparison, include additional data on *Ae. scapularis*.

MATERIALS AND METHODS

Virus. The Rocio virus strain used (SpH34675) was isolated at autopsy in 1975 from human brain tissue. Viral stocks were 10% suspensions of infected suckling mouse brain from the third passage.

Mosquitoes. Moesquitoes were collected aperiodically from December 1981 through March 1985 in "Pariquera-Açu" Township, S.Paulo State, Brazil, and brought to the laboratory at the University of S.Paulo. Females were given a blood meal and allowed to oviposit on filter paper. Eggs were conditioned for 10 days at 28°C in a humid atmosphere, then packaged in plastic bags and sent to the Division of Vector-Borne Viral Diseases, Centers for Disease Control, in Fort Collins, Colorado, via airmail. Usually, eggs were flooded in deionized water on the day received. Occasionally, a vacuum pump or nutrient broth was utilized in an attempt to improve hatching success. Tetramin*** fish food and commercial rabbit chow were given ad libitum until pupation occurred. Adult females were given 5% sugar water from the time of emergence until 24 h before a blood meal was offered. Only F_1-generation females were used experimen-

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tally, since an objective was to measure the susceptibility of field populations and not that of laboratory-adapted colonies.

Experimental procedure. The experimental design is essentially the same as that described in previous vector competence studies involving Rocio virus (Mitchell et al.[14], 1981; Mitchell & Forattini[13], 1984).

Chicks less than 48 h old were infected with Rocio virus by subcutaneous inoculation of ca. 10,000 Vero cell plaque-forming units (PFU). Mosquitoes 3 to 9 days of age were allowed to feed overnight on viremic chicks 54 to 72 h postinoculation. Chicks were restrained on top of 1/2-pint cartons that were covered with fine-mesh nylon and that contained the mosquitoes. When feeding two species simultaneously on the same chick, the chick was sandwiched between two cartons that were taped together. Postexposure chick bloods were drawn by jugular venipuncture and frozen at -70°C until tested for virus. Engorged mosquitoes were segregated, provided oviposition dishes, and incubated at 26.7 ± 0.5°C, 75% to 80% RH, and a photoperiod of 16 h full light and 8 h of darkness. Some mosquitoes were given an opportunity to refeed individually on 1- to 2-day-old chiks following appropriate periods of incubation. The mosquitoes were frozen, and chicks that were bitten were banded and bled about 60 h later for virus assay.

In one series of experiments, the *in vitro* feeding technique described by Aitken[1](1977) was used to demonstrate virus transmission by mosquitoes. Briefly, glass capillary tubing drawn to a fine point in the flame of an alcohol lamp was marked with a rubber stamp at 1-mm increments. Each increment corresponded to an internal volume of 0.17 µl. Each capillary tube was loaded with 5 µl of fetal calf serum (FCS) at pH 7.2, and the proboscis of a test mosquito was inserted following removal of the mosquito's wings. The capillary tube with dangling mosquito was transferred to a styro foam rack, and the mosquito was allowed to feed for approximately 15 min. The amount of feeding suspension ingested by each mosquito was recorded. The remainder of the suspension was expressed into a microscope slide, loaded into another calibrated capillary tube, and infected parenterally into from one to four colony *Cx. pipiens* from Dayton, Ohio. The inoculation apparatus used was that described by Rosen & Gubler[15] (1974). These inoculated mosquitoes were given 5% sugar water and incubated at 26.7°C for 7 days. At that time, they were frozen at -70°C until processed and tested for virus as described below. Mosquitoes used in the *in vitro* feeding trials were frozen immediately after having their proboscis removed from the capillary. Virus content of each of these donor mosquitoes was determined by titration in Vero cell culture, and this information was collated with the amount of feeding suspension ingested.

General procedures used for processing mosquitoes for virus isolation tests have been described (Sudia & Chamberlain[17], 1967). Mosquitoes were disrupted by sonic energy in 1 ml of BA-1 diluent (0.2 M Tris, pH 8.0, 0.15 M NaCl, 1% BSA, 10 mg/litre phenol red, 50 g/ml Gentamicin, and 1 g/ml Fungizone). Suspensions were centrifuged at 2000 rpm for 20 min. The supernatant was frozen at -70°C until tested. Blood samples (0.1 ml) from chicks were taken by jugular venipuncture diluted in BA-1, centrifuged at 1500 rpm for 15 min, dispensed into screw-cap vials, and stored at -70°C.

Specimens were screened for virus or were titrated as appropriate. Viral assays were done by inoculating Vero cell cultures and counting plaques. Briefly, tenfold dilutions were made in BA-1, and samples (0.1 ml) were inoculated into Vero cell cultures in six-well plates, adsorbed for 1 h at 37°C, and overlaid with 1% Noble agar in M-199 supplemented with 2% FCS, 2.0 g/litre of $NaHCO_3$, 150 g/ml of DEAE-dextran, and 1:40,000 neutral red. Cell cultures were then examined for 10 days for characteristic plaques.

The virus infection rate in mosquitoes, expressed as a percentage, is the proportion of mosquitoes tested that contained virus. The ID_{50} value was estimated using probit regression. The virus transmission rate, also expressed as a percentage, is the proportion of infected mosquitoes that transmitted virus upon refeeding after a suitable extrinsic incubation period. Differences in infection rates between species fed simultaneously on the same viremic chick were tested for significance by Fisher's Exact Test (Snedecor & Cochran[16], 1967).

RESULTS

Hatching rates among batches of F_1-generation mosquito eggs received in Fort Collins were low (0 to 50%), and this was followed by further mortality during the rearing process. Variable feeding rates of adult females feeding on viremic chicks and additional mortality during incubation (Table 1) further reduced the size of samples available for virus infection and transmission assays.

Per os infection rates were determined for three mosquito species from the Rocio encephalitis epidemic zone (Table 2). Using the infection rates and titers of infective meals shown in the table, and an estimated blood meal volume of 3 µl, ID_{50} values of $10^{4.1}$ (95% confidence limits from $10^{3.4}$ to $10^{4.9}$) for *Ps. ferox* and $10^{4.3}$ (95% confidence limits from $10^{3.8}$ to $40^{5.4}$) for *Ae. scapularis* were derived

TABLE 1

Initial feeding rates on viremic chicks by three species of mosquito and subsequent mortality during the 14 - to 21 - day incubation period.

Species	No. of Feeding Trials	Feeding Rates		Mortality Rates	
		No. Fed/ No. Tested	%	No. Died/ No. Fed	%
Ae. scapularis	17	635/1088	58	154/635	24
Ae. serratus	9	145/360	40	21/145	14
Ps. ferox	14	293/593	49	89/293	30

TABLE 2

Rocio virus infection rates in three mosquito species from Pariquera-Acu, S.Paulo State, Brazil.*

Titer of Infective Meal**	No. of Days Incub.	*Ps. ferox*		*Ae. scapularis*		*Ae. serratus*	
		No. Pos./ Tested	% Pos.	No. Pos./ Tested	% Pos.	No. Pos./ Tested	% Pos.
3.2	18	0/14	0				
4.0	13			1/33	3		
4.1	13			0/26	0		
4.6	21	0/1	0	1/16	6		
5.0	17	3/9	33	3/16	19		
5.1	20	6/21	29	4/29	14		
5.2	14			5/28	18	0/10	0
5.7	21	0/4	0	2/6	33		
5.8	19	1/9	11				
5.8	20			5/23	22		
5.9	20			5/20	25		
6.0	20			17/47	36		
6.1	20	1/10	10	22/40	55+		
6.2	14					2/26	8
6.2	20			3/29	10	0/10	0
6.6	20	12/23	52+	10/47	21		
6.6	20	19/29	66	9/17	53		
6.7	17	4/28	14			1/11	9
6.7	20	7/12	58++	4/28	14		
6.8	20			22/35	63++	1/20	5
7.2	14	2/3	67			8/22	36
7.6	20			38/41	93++	3/13	23
7.9	14	14/17	82+			0/3	0
8.3	14	23/24	96++			1/9	11

* Data on the same line represent simultaneous feedings by two species on the same viremic chick.

** Log_{10} Vero cell PFU/ml.

\+ Significantly higher ($P < 0.05$) than corresponding result for other species fed on same chick; comparisons made by Fisher's Exact Test.

++ Same as above, but $P < 0.01$.

using probit regression. The difference is not statistically significant ($P > 0.05$). Infection rates in *Ae. serratus* never exceeded 36% in nine feeding trials; therefore, an ID_{50} could not be calculated for this species.

Statiscally significant differences in infection rates were observed in three of eight feeding trials in which *Ps. ferox* and *Ae. scapularis* fed on the same chick simultaneously (Table 2). In one trial, the infection rate was significantly higher in *Ae. scapularis* than in *Ps. ferox*, whereas in the other two instances the reverse was true. Two of four paired feeding trials involving *Ps. ferox* and *Ae. serratus* and two of four involving the latter species and *Ae. scapularis* yielded statistically significant results (Table 2). In all four cases, infection rates in *Ae. serratus* were significantly lower than in the other species (Table 2).

In experiments designed to measure Rocio virus transmission by *Ps. ferox*, sample sizes were small because not all mosquitoes refed, and not all of those that did refeed were infected (Table 3). We attempted to circumvent the problem of refeeding by using an *in vitro* technique to measure transmission. Samples of 14 and 22 infected *Ps. ferox* were tested, and 43% and 36%, respectively, were shown to transmit virus in this fashion (Table 3). It was of interest to determine whether there was a correlation between the amount of feeding suspension ingested by *Ps. ferox* and Rocio virus transmission rates using the *in vitro* system. Arbitrarily, transmission rates among 17 mosquitoes that ingested from 0.17 µl to 1.02 µl were compared to those of 19 mosquitoes that ingested more than 1.02 µl of feeding suspension (Table 4). The observed difference was not significant in Fisher's Exact test ($P<0.5$).

TABLE 3

Experimental transmission of Rocio virus by *Ps. ferox* and *Ae. scapularis* to baby chicks or to an *in vitro* assay system.*

Titer of Infective Meal**	No. of Days Incub.	*Ps. ferox*			*Ae. scapularis*		
		Proportion Refed	Transmission Proportion+	%	Proportion Refed	Transmission Proportion+	%
		(Transmission to baby chicks)					
5.1	20	9/21	1/2	50	17/29	3/3	100
5.8	19	6/9	1/1	100			
6.1	20	4/10	0/1	0	12/40	3/7	43
6.6	20	10/23	1/7	14	15/21	4/5	80
6.7	20	3/12	1/1	100	21/28	1/4	25
6.8	20				16/35	6/10	60
7.6	20				17/41	13/15	87
		(Transmission to *in vitro* assay)					
7.9	14	17/17	6/14	43	Not Done		
8.3	14	23/24	8/22	36	Not Done		

* Data on the same line are from experiments in which both mosquito species were infected by feeding on the same viremic chick.

** Log_{10} Vero cell PFU/ml.

\+ No. Mosquitoes that transmitted virus/no. infected mosquitoes that refed.

Among the 36 infected *Ps. ferox* tested for their ability to transmit Rocio virus by *in vitro* feeding, 14 transmitted virus and 22 did not. To determine whether mosquitoes that transmitted contained significantly more virus than nontransmitters, we ranked the mosquitoes into four groups according to the amount of feeding suspension ingested and according to whether they transmitted virus (Table 5). Although the observed differences in median and average titers between transmitters and non-transmitters are not great (median 5.7 vs 5.5, mean 5.7 vs 5.4, respectively), these differences are significant in the Mann-Whitney and t-tests ($P<0.5$).

Meaningful data concerning Rocio virus transmission rates by *Ae. serratus* were not obtained because infection rates were low, and few infected mosquitoes were available for testing (Table 2). This species was very reluctant to refeed on chicks at the end of

the incubation period despite being given the opportunity to oviposit following the infective meal. An attempt was made, however, to measure transmission by *in vitro* feeding. Twelve mosquitoes were tested; only one was infected, and it failed to transmit the virus.

TABLE 4

Relationship between amount of feeding suspension ingested by *Ps. ferox* and Rocio virus transmission rates on day 14 post-infection.

Amt. of Feeding Suspension Ingested	No. Tested	No. Trans. Virus To Feeding Susp.	%
<0.17 µl	2	1	
0.17 to 0.51 µl	7	3	35.5*
0.52 to 1.02 µl	8	2	
>1.02 µl	19	8	42.1*

* Fischer's Exact Test, $P > 0.5$.

TABLE 5

Rocio virus titers* in *Ps. ferox* that transmitted virus *in vitro* compared with mosquitoes that ingested feeding suspension but did not transmit virus.

Amt. of Feeding Suspension Ingested	Mosquitoes That Transmitted Virus			Mosquitoes That Did Not Transmit Virus		
	Sample Size	Virus Titers* Range	Avg.	Sample Size	Virus Titers* Range	Avg.
< 0.17 µl	1	–	5.9	1	–	5.8
0.17-0.51 µl	3	5.3-5.7	5.5	4	4.7-5.9	5.6
0.52-1.02 µl	2	5.7-5.8	5.8	6	5.0-5.8	5.5
> 1.02 µl	8	5.6-6.0	5.7	11	4.3-6.3	5.3
TOTALS	14	5.3-6.0	5.7**	22	4.3-6.3	5.4

* Log_{10} Vero cell PFU/ml.

** Significantly higher ($P<0.05$) than in counterpart group that did not transmit virus.

DISCUSSION

Our results indicate that both *Ps. ferox* and *Ae. scapularis* from the Rocio encephalitis zone are susceptible to per os infection with Rocio virus and that infected mosquitoes can transmit the virus by bite following a suitable incubation period (Tables 2 and 3). The great amount of variability in infection rates following ingestion of viremic blood meals precludes a rigorous comparison of the susceptibility of the two species to infection per os. In any event, since both species become infected and transmit Rocio virus by bite under experimental conditions, accumulation of field evidence for involvement of either or both species in natural transmission cycles will be required to determine their relative importance as vectors in nature. On the other hand, our results suggest that *Ae. serratus* can be discounted as an epidemiologically important vector of Rocio virus because of its relative lack of susceptibility to per os infection, even following the ingestion of high-titered blood meals (Table 2).

The susceptibility of the *Ps. ferox* tested in this study cannot be compared directly with the published information on colonized *Ps. ferox* from Louisiana (Mitchell et al.[14], 1981) because the latter were fed only relatively low-titered blood meals. The oral ID_{50} for *Ae. scapularis* tested in this study ($10^{4.3}$) is significantly different ($P<0.05$)

from that reported earlier ($10^{3.5}$) for the same species from the same area (Mitchell & Forattini[12], 1984). Arnell[2] (1976) noted variation in the presence or absence of a small retrorse process on the outer angle of the claspette filament of the male genitalia of *Ae. scapularis*. He considered these variations to be characteristic of individuals rather than populations. Such variation was noted in males from the last batch of *Ae. scapularis* that we tested*; whether this is indicative of variation that could also affect vector competence of females from the same collections in unknown, but it is an important area for future investigation. The high degree of variation in per os infection rates in all the species tested may be due, in part, to seasonal variation, but our sample sizes are too small to test this hypothesis. Also, mosquitoes ranging in age from 3 to 9 days were used in the present study in an attempt to increase sample sizes in the feeding trials, and this may have contributed to the variation. A probable major source of variation was fluctuating virus titers during the overnight feeding period and the fact that mosquitoes of different species used in paired feeding trials did not necessarily feed at the same time.

Finally, we should recognize that widespread variation in vector competence among field populations may be a real phenomenon with far-reaching epidemiological implications (Hardy et al.[7], 1983; Mitchell[12], 1983). The desirability of using field-collected mosquitoes or their progeny for vector competence studies is obvious when one wishes to relate the results to field situations. However, the logistical problems involved, especially when distances between the field sites and the laboratory are great, are significant factors affecting the outcome of such experiments. These problems are compounded by difficulties associated with rearing progeny from field-collected mosquitoes, especially when synchronous broods are required. One may opt for more rigorously controlled experiments in which colonized mosquitoes are used or accept the limitations of more variable, but perhaps more realistic, assessments derived from using field specimens or their progeny.

The *in vitro* feeding technique proved very useful in demonstrating Rocio virus transmission. It is noteworthy that the amount of feeding suspension ingested by the test mosquitoes had no significant effect on whether virus was transmitted to the feeding suspension. The fact that *Ps. ferox* that transmitted Rocio virus *in vitro* had significantly higher titers than those that did not transmit virus is in agreement with results from *in vivo* transmission experiments conducted with *Culex pipiens* L. (Mitchell et. al.[14], 1981).

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MITCHELL, C.J. et al. [Ensaios sobre a capacidade vetora para o vírus Rocio, de três espécies de culicídeos da zona epidêmica no Brasil.] **Rev.Saúde públ.**, S.Paulo, **20** : 171-7, 1986.

RESUMO: Em condições de laboratório procedeu-se a ensaios visando testar a capacidade vetora para o vírus Rocio, da primeira geração de *Psorophora ferox, Aedes scapularis* e *Aedes serratus* obtida a partir de especimens coletados na região epidêmica do Estado de São Paulo, Brasil. *Psorophora ferox* e *Aedes scapularis* revelaram-se suscetíveis à infecção por via oral e capazes de transmitir o vírus mediante a picada após período adequado de incubação. Para as duas espécies, as ID_{50} orais não diferiram significativamente. Em *Ae. serratus* as taxas de infecção nunca ultrapassaram os 36,0% o que impossibilitou o cálculo da ID_{50} para essa espécie. É improvável que *Ae. serratus* seja vetor epidemiologicamente importante do vírus Rocio. Discute-se a utilidade da técnica de alimentação "in vitro" para demonstrar a transmissão por mosquitos infectados, e também as dificuldades encontradas ao trabalhar com gerações não colonizadas originárias de especimens coletados no campo.

UNITERMOS: Vírus da encefalite, fisiologia. *Psorophora ferox. Aedes scapularis. Aedes serratus.* Insetos vetores, microbiologia. Encefalite epidêmica, transmissão. Arboviroses, transmissão.

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