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ECOLOGY OF VIRUSES ISOLATED FROM PANAMANIAN PHLEBOTOMINE SANDFLIES

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Abstract. A total of 269 virus strains was obtained from both male and female phlebotomine sandflies collected at two localities in Panama between 1969 and 1971. These isolates represented nine different virus types (Changuinola, VSV-Indiana, Punta Toro-CoAr 3319, Chagres, VP-175A, VP-437R, VP-161A, VP-488A and VP-118D) as well as several unidentified agents. Five of the virus types are new. Changuinola and Punta Toro-CoAr 3319 were the most frequently encountered agents and represented 52% and 19% of the isolates, respectively. Of man-biting sandflies processed by species, highest overall isolation rates were obtained from *Lutzomyia trapidoi*. Analysis of seasonal distribution of virus isolates indicated that activity of Changuinola, Chagres, VP-175 and VP-437R was continuous, while isolations of VSV-Indiana and Punta Toro-CoAr 3319 were intermittent. Seventeen isolates, representing six different virus types, were obtained from male sandflies. Isolation rates by sex for three of the virus types were similar in collections yielding comparable numbers of male and female insects. The frequency of virus isolations from male sandflies suggests that transovarial transmission of several of these agents occurs in nature. Neutralization tests on sera from inhabitants of seven rural Panamanian communities demonstrated a significant amount of human infection with VSV-Indiana, Punta Toro and VP-437R. Infections of caged sentinel animals, exposed at one of the collecting sites, were observed with VSV-Indiana, Chagres, VP-175A and VP-437R. The study demonstrated superiority of the Vero cell culture system over newborn mice for primary isolation of sandfly virus isolates. By using infected Vero cells as complement-fixing antigen for typing isolates, the necessity of blind passing many viruses in order to establish mouse pathogenicity was eliminated.

The role of phlebotomine sandflies as vectors of pappataci fever in the Mediterranean region has been well documented,[1-5] and preliminary work in India,[6] Pakistan,[7] Brazil,[8,9] and Panama[10,11] suggests that many different viruses are transmitted by these insects. During field studies on the ecology of vesicular stomatitis virus (VSV) in Panama between 1969 and 1971, a total of 252,512 sandflies was collected and processed for virus isolation, yielding 269 virus strains. Infection of caged sentinel animals exposed at one of the study sites and the presence of neutralizing antibodies among humans living in nearby communities were demonstrated for several of the viral agents. This paper describes results of these studies and their implications regarding the biology of certain sandfly-associated viruses.

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MATERIALS AND METHODS

Description of Study Areas and Collection Methods

Field studies were carried out at two localities in Panama between January 1969 and March 1971. The first site, El Aguacate, is a small, rural community in central Panama, located 300 m above sea level; the surrounding terrain is hilly and is covered with patchy areas of moist tropical forest and scrubby secondary growth. The region has an abundant wild animal population. About 200 human residents live in palm-thatched houses scattered throughout the forest; there are few domestic animals in the community. Most of the adult inhabitants of Aguacate farm small plots of land which produce rice, corn, yuca, coffee and citrus fruits. Cutaneous leishmaniasis is endemic

FIGURE 1. This photograph shows the abandoned coffee plantation which served as our collecting site in Aguacate. Coffee plants and dense undercover are seen in the foreground.

FIGURE 2. Partial view of the primary forest at the Limbo site. This photograph was taken from a clearing at the entrance to the forest.

in the human population, active cases being seen mainly in young children.

Insect collections at Aguacate were made in an abandoned coffee plantation (Fig. 1). In addition to coffee plants (leafy shrubs ranging from 2 to 3 meters in height), the study area contains a number of tall shade trees (mainly *Gustavia gigantica* and *Ficus* sp.) and a dense undercover. Collections at Aguacate were made on several consecutive nights each month during the study period.

The second study site, Limbo, is an uninhabited area of moist climax forest in the Canal Zone (Fig. 2). This site has been described in detail in a previous publication.[12] The Limbo forest is tall with two distinct canopies: one at about 40 m formed by several species of gigantic trees, the other at about 20 m made up of medium-sized trees and lianas. There are few plants in the understory and only scant ground cover as a result of heavy shading; epiphytic ferns, orchids and bromeliads are abundant. The forest is relatively undisturbed and supports an abundant and diverse wild animal population. Sandfly collections at Limbo were made irregularly during the 26-month study period.

Climatic conditions at the two sites are similar; mean annual temperature is 26° C with high humidity and relatively little variation throughout the year.[12, 13] There are two distinct seasons; a 4-month dry season (January to April) and an 8-month wet season (May to December). Annual rainfall averages 266 cm at Aguacate and 204 cm at Limbo with about 95% of the rain occurring during the wet season.

Sandflies were captured from human bait[14] and in light traps[15] during the night, and by direct aspiration during the day from their resting places in tree buttresses (Fig. 3).[16] Collections were made both at ground level and in the forest canopy. A large number of anthropophilic sandflies were collected during the night from two

FIGURE 3. This illustration demonstrates one of the massive tree buttresses present at the Aguacate site. During the day, large numbers of sandflies were aspirated from their resting places in dark crevices at the base of the trunk.

platforms (Fig. 4) and surrounding branches located 15 m (Aguacate) and 30 m (Limbo) above the ground.

After collection, insects were immediately frozen in liquid nitrogen for transport to the laboratory and storage at −60° C. Later, specimens were sorted under a stereoscope on a chill table into pools according to method of collection (i.e., human bait, light trap, tree trunk aspiration), locality, date of capture, and sex. Blood-engorged sandflies were removed from the collection and saved for host identification studies.[17,18] Where possible, sandflies were also sorted by species.* Because of the variety of species encountered and different collection methods used, it was not feasible to standardize the number of insects in each pool; the size of female pools varied from 50 to 100 insects, whereas male pools consisted of 50 to 250 flies.

Virus Isolation and Identification

Individual sandfly pools were triturated in sterile, 7-ml Ten Broeck tissue grinders containing 3.0 ml of 0.05 M phosphate-buffered (pH 7.6) distilled water with 25% heat-inactivated (56° C for 30 minutes) rabbit serum, penicillin (1,000 units/ml), and streptomycin (1.6 mg/ml). The

FIGURE 4. This photograph shows the canopy platform, situated 30 m above ground, at the Limbo study area. At night, sandflies active in the canopy were aspirated from human bait and from tree branches adjacent to the platform.

resultant suspension was centrifuged at 9,750 × *g* for 30 minutes at 4° C. The supernatant was withdrawn and divided into two parts; one portion was refrozen at −60° C for reisolation attempts, the other was inoculated for primary virus isolation. Initially, suspensions of each insect pool were inoculated intracerebrally (0.02 ml) into a litter of newborn albino mice and into two tubes of Vero (African green monkey kidney) cell culture. However, after processing a few hundred pools it became apparent that the cell culture system was more sensitive; animal inoculation was then discontinued and isolation attempts were conducted in three Vero tubes. Methods for propogation of Vero cells have been described previously;[19] maintenance medium consisted of medium 199 containing 1.4 g of $NaHCO_3$, supplemented with 1% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin

* Human bait and canopy platform aspirator collections yielded mainly *Lutzomyia trapidoi* and *L. ylephilator*; many of these collections were sorted into species on the basis of distinct external morphological characteristics. In contrast, light trap and tree buttress collections yielded a wide variety of species and were separated only by sex.

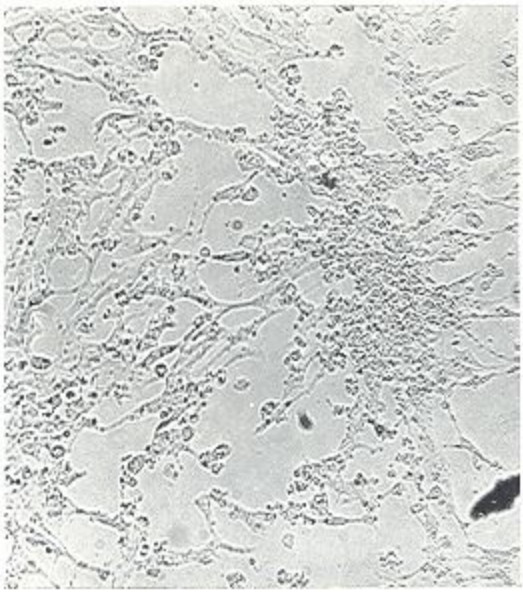

FIGURE 5. Photomicrograph showing late cytopathic effect (CPE) of virus VP-437R in Vero cells. The CPE of this virus began as focal areas of degeneration which gradually enlarged and coalesced to involve the entire monolayer; it is characteristic of the CPE produced by most members of the Phlebotomus fever group in Vero. × 100.

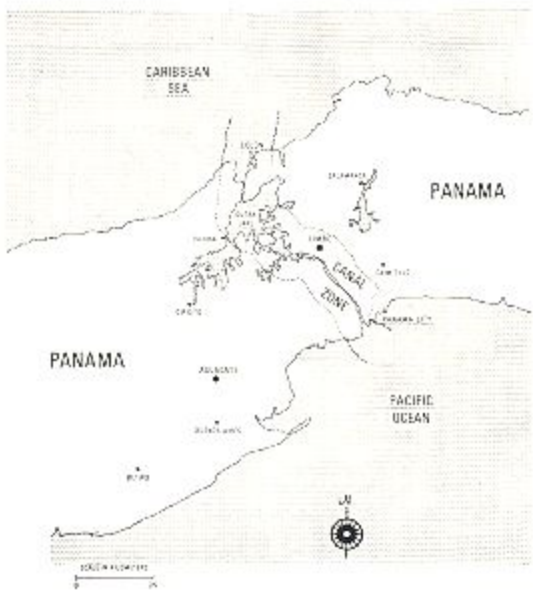

FIGURE 6. Map showing the Isthmus of Panama and locations of the Aguacate and Limbo collecting sites. The six other rural communities included in our serologic survey are also noted.

(100 μg/ml). Cell culture tubes from which fluid medium had been removed were inoculated with 0.1 ml of the sandfly supernatant and incubated for 1 hour at 33° C. After incubation, maintenance medium (1.5 ml) was replaced. Vero cultures were then incubated on a roller drum at 37° C, examined periodically for virus cytopathic effect, and held for 14 days before being discarded. Maintenance medium was changed twice a week.

Virus isolates were identified by complement fixation (CF) or neutralization (N) test using hyperimmune hamster serum or mouse ascitic fluid. Vesicular stomatitis virus isolates were identified by N test in a micro plaque method[19] with Vero cells, using varying dilutions of virus versus fixed dilutions of mouse hyperimmune ascitic fluid prepared against three VSV serotypes (Indiana, New Jersey and Cocal). VP-118D was also typed by N test. The remaining viral agents were identified by micro CF test, using sera of adult hamsters hyperimmunized with 10% crude brain suspensions of newborn hamsters infected with Changuinola, Punta Toro, CoAr 3319 and Chagres viruses as well as selected representatives of our sandfly virus isolates. VP-437R antiserum was prepared by hyperimmunizing adult hamsters with a virus pool prepared in MA-111 (rabbit kidney) cell culture.

In attempting to prepare brain antigens for CF test, it soon became apparent that many of the sandfly virus isolates required multiple blind passages to establish pathogenicity for infant mice or hamsters. To eliminate the necessity of animal-adapting each of the isolates, infected Vero cells were used as the source of antigen for most CF tests. Concentrated tissue culture antigens were prepared following the method of Schell et al.[20] Vero cells grown in 2-oz plastic flasks containing 5.0 ml of maintenance medium were inoculated with virus and were harvested when they showed 3 or 4+ viral cytopathic effect (Fig. 5). After centrifugation at 1,000 × *g* for 10 minutes and removal of 4.5 ml of the supernatant, flasks containing the concentrated cell suspensions were then frozen and thawed three times. Following a final centrifugation at low speed, the resultant supernatant was used as CF antigen.

Neutralization Studies of Sentinel Animal and Human Sera

During 1968 and 1969, most of the human residents of Aguacate were bled for virus studies.

TABLE 1

Comparison of isolation results from sandflies, using Vero and suckling mouse culture systems, by virus type

Culture system		Number of isolates			
Vero	Suckling mice	Phlebotomus fever group	Changuinola	VSV-Indiana	Total
Pos.	Pos.	0	9	5	14
Pos.	Neg.	5*	1	0	6
Neg.	Pos.	0	1	0	1
Total		5	11	5	21

* Includes 3 isolates of VP-175, 1 of Chagres and 1 of VP-437R.

In addition, adult human sera from six other rural communities located within a 40-km radius of Aguacate or Limbo (Fig. 6) were examined for neutralizing antibodies to selected sandfly virus isolates. These latter sera were obtained between 1964 and 1972 in conjunction with other Middle America Research Unit projects.

Caged spider monkeys (*Ateles geoffroyi*) and laboratory-reared rodents (*Tylomys nudicaudatus*) were exposed at the Aguacate collecting site from October 1968 to February 1971. Serum specimens were obtained from the animals at intervals ranging from 4 to 12 weeks. Due to death or escape, the number of animals exposed varied from month to month; thus, from 3 to 6 monkeys and from 6 to 14 rodents were present at any given time in the study site.

Following heat inactivation at 56° C for 30 minutes, human sera were screened by a modified plaque-neutralization method,[19, 21] using Vero cell monolayer cultures, against the following viral agents: VSV-Indiana, Punta Toro, Chagres, VP-175A, VP-437R and VP-161A. Sentinel animal sera were tested by the same method against VSV-Indiana, Punta Toro, Chagres, VP-175A and VP-437R. Sera tested against VSV-Indiana were screened at a 1 in 16 dilution, using phosphate-buffered saline solution, pH 7.1, containing 0.5% gelatin as diluent; plaque reduction of 95% or more was recorded as a positive test, indicating the presence of specific neutralizing antibodies. For the other viruses, all sera were screened at a 1 in 8 dilution and plaque reduction of 90% or more was the criterion for a positive test.[21]

TABLE 2

Summary of sandfly virus isolates, Panama, 1969–1971

Species identification	Sex	No. pools	No. sandflies	No. virus isolates	Virus identification									
					Changuinola	VSV-Indiana	Punta Toro-CoAr 3319	Chagres	VP-175A	VP-437R	VP-161A	VP-488A	VP-118D	NI*
AGUACATE														
Lutzomyia sp.	M	362	64,183	8	1	0	2	1	3	1	0	0	0	0
(mixed)	F	1,306	81,383	103	55	6	15	1	10	7	0	0	0	10
L. trapidoi	M	8	1,223	0	0	0	0	0	0	0	0	0	0	0
	F	944	52,415	98	52	3	24	2	10	7	0	0	0	0
L. ylephilator	F	418	24,376	21	12	0	1	3	3	0	0	1	0	1
Subtotal		3,038	223,580	230	120	9	42	7	26	15	0	1	0	11
LIMBO														
Lutzomyia sp.	M	52	5,860	1	0	0	0	0	0	0	0	0	1	0
(mixed)	F	120	4,819	3	1	0	0	0	0	0	1	0	1	0
L. trapidoi	M	58	8,683	8	0	0	2	0	4	1	0	0	0	0
	F	187	9,570	27	18	0	5	0	1	3	0	0	0	0
Subtotal		417	28,932	39	19	0	8	0	5	4	1	0	2	0
Total		3,455	252,512	269	139	9	50	7	31	19	1	1	2	11

* NI, unidentified.

RESULTS

Virus Isolations

Suspensions of the first 366 sandfly pools were inoculated intracerebrally into 1 litter of newborn mice and into 2 tubes of Vero cell cultures. Table 1 compares isolation results by virus type in the two culture systems. Fourteen virus strains were isolated in both systems; these were all Changuinola group agents or VSV-Indiana. One Changuinola isolate was obtained from suckling mice but not in Vero; however, during reisolation attempts on the original insect suspension, this agent was reisolated in Vero cell culture. Six virus strains were isolated in Vero but not in suckling mice; 5 of these were Phlebotomus fever group agents (Chagres, VP-175A and VP-437R), the sixth was a strain of Changuinola. On the basis of these results, indicating greater sensitivity of the Vero culture system, animal inoculation was discontinued and all subsequent isolation attempts on sandfly pools were done in Vero.

Table 2 summarizes our insect collection and virus isolation results. Sixty-eight percent of all sandflies captured were females; the paucity of males reflects our collecting methods, since human bait and light traps yielded predominantly females. A total of 252,512 sandflies was collected and processed as 3,455 pools for virus isolation. From these, a total of 269 virus strains were obtained, 17 from pools of male sandflies and 252 from females. Most of the isolates have been identified and represent at least 9 different virus types. Four of these, Changuinola, VSV-Indiana, Punta Toro-CoAr 3319 and Chagres, are previously known viruses; the other five (VP-175A, VP-437R, VP-161A, VP-488A and VP-118D) appear to be new viral agents and are listed by their prototype numbers. Characterization of the new viruses will be described in another publication.[23] Eleven isolates failed to react in CF or N test with antisera prepared against any of the nine virus types listed above and are still unidentified. On the basis of growth characteristics and cytopathic effect in Vero tissue culture, they appear to be different from the other isolates. One sandfly pool yielded two viral agents, Chagres and VP-437R. This was a pool of 250 male sandflies (mixed species) from Aguacate.

Most frequently encountered were Changuinola-complex viruses which accounted for 52% (139/269) of all isolates. These were identified by CF test which is not type specific. Nine strains of VSV-Indiana were obtained; three of the isolates were made from pools of female *Lutzomyia trapidoi*.

TABLE 3

Complement fixation test results with Punta Toro, CoAr 3319 and selected Panamanian sandfly virus isolates

Antigen	Antiserum	
	Punta Toro	CoAr 3319
Punta Toro*	32/128†	4/4
CoAr 3319*	16/16	64/64
VP-422G	16/64	16/64
VP-266K	16/64	32/64
VP-210A	16/128	32/256
VP-419T	8/16	32/64
VP-334K	4/8	64/64
V-269Q	4/16	64/256

* Antigen prepared from plaque-purified virus pool.
† Reciprocal of highest antiserum dilution/antigen dilution.

Viruses identified as related to Punta Toro-CoAr 3319 represented 19% of our isolates; these were identified by CF test. Both Punta Toro and CoAr 3319 are members of the Phlebotomus fever group of arboviruses and are closely related serologically.[21] Table 3 shows CF results with Vero cell antigens for Punta Toro, CoAr 3319 and six selected sandfly isolates from our study. Because of varying patterns of cross reactivity among many of the isolates with immune sera prepared against the two prototype viruses, we did not attempt to separate them. Therefore, the 50 virus isolates reacting in CF test with Punta Toro and/or CoAr 3319 antisera are listed together in Table 2.

Seven isolates of Chagres virus were obtained as well as 31 of VP-175A, 19 of VP-437R, 2 of VP-118D, and 1 each of VP-161A and VP-488A (Table 2).

Recovery of Virus from Male Sandflies

A total of 17 isolates, representing 6 different virus types (Changuinola, Punta Toro-CoAr 3319, Chagres, VP-175A, VP-437R and VP-118D), was made from male sandflies (Table 2). Because our collection methods were designed principally to capture female sandflies, relatively few collections yielded comparable numbers of males. However, Table 4 compares male-female isolation rates for four agents from those collections at Aguacate

TABLE 4

Sandfly virus isolations by sex from two comparable collections at Aguacate and Limbo

Sex	No. sandflies	No. pools	Changuinola	Punta Toro-CoAr 3319	VP-175A	VP-437R
Aguacate—mixed species from tree buttresses (ground)						
Male	39,463	193	1	2	3	0
Female	26,217	381	12	3	1	3
Limbo—*Lutzomyia trapidoi* from canopy platform (30 m)						
Male	8,683	58	0	3	4	1
Female	9,754	191	18	5	1	3

and Limbo which did yield comparable numbers. Isolations of Punta Toro-CoAr 3319 were similar for both sexes, while more isolates of VP-175A were actually made from male sandflies than from females in these two collections. In contrast, only one male isolate each of Changuinola and VP-437R was obtained. In addition, two isolations of VP-118D were made from sandflies (mixed species) captured at Limbo, one each from 5,860 male and 4,819 female insects collected from tree buttresses (Table 2).

Seasonal Distribution of Isolates

Table 5 shows the seasonal distribution of virus isolates from female sandflies at Aguacate. In this table the year is divided into three seasons, corresponding to periods when sandfly population densities reflect effects of dry, early and late rainy seasons.[12] The paucity of specimens collected between January and April reflects the marked decline in the sandfly population that occurs during the dry months in Panama. Isolations of Changuinola group viruses were made in almost every month, and activity was demonstrated during all but one of the 4-month periods. Likewise, isolation rates for VP-175A, VP-437R and Chagres virus showed little variation throughout the study period. In contrast, isolation rates of Punta Toro-CoAr 3319 showed significant differences. During the first 16 months of the study, only 1 isolate of Punta Toro-CoAr 3319 was obtained, and then 49 isolates were made in the next 11 months. Five isolations of VSV-Indiana were obtained during August 1969 and 4 more were made between June and September of 1970. These months (June to September) correspond to the period of highest sandfly population density.[12] Interestingly, the 11 unidentified virus isolates from Aguacate were all made between August and October of 1970.

At the Aguacate study site, *L. trapidoi* and *L. ylephilator* were the most abundant species; they accounted for over 90% of sandflies captured from human bait and were also the predominate species obtained in aspirator and in light trap

TABLE 5

Seasonal isolation rates—female sandflies, Aguacate

Year	Season	No. pools	No. sandflies	Changuinola	PT-CoAr	VP-175A	VP-437R	VSV-Indiana	Chagres
1969	Jan–Apr	16	1,937	–	–	5.0	–	–	–
	May–Aug	139	13,412	7.5*	–	–	1.0	4.7	–
	Sep–Dec	328	26,239	5.7	–	1.9	0.4	–	1.1
1970	Jan–Apr	63	4,681	4.3	2.1	–	–	–	–
	May–Aug	803	44,835	6.0	2.4	1.5	0.7	0.4	0.2
	Sep–Dec	966	48,936	8.8	4.7	1.6	1.6	0.4	0.4
1971	Jan–Apr	353	18,197	11.5	2.2	1.1	0.6	–	–
	Total	2,668	158,237	7.5	2.4	1.4	0.9	0.6	0.3

* Isolates/10,000 female sandflies.

TABLE 6

Species isolation rates from female sandflies—Aguacate

Virus	Species	
	L. trapidoi	*L. ylephilator*
Changuinola	10.0*	5.0
Punta Toro-CoAr 3319	4.6	0.4
VP-175A	1.9	1.2
VP-437R	1.3	0.0
Chagres	0.4	1.2
VSV-Indiana	0.6	0.0
Total no. sandflies	52,415	24,376
Total no. pools	944	418

* Isolates/10,000 sandflies.

collections. Table 6 compares isolation rates of 6 agents from female *L. trapidoi* and *L. ylephilator* from Aguacate. Both species yielded Changuinola, Punta Toro-CoAr 3319, VP-175A and Chagres, but isolation rates for all but Chagres were higher in *L. trapidoi*.

Serologic Studies

Table 7 shows the prevalence of VSV-Indiana, Punta Toro and VP-437R neutralizing antibodies in adult residents (≥20 years of age) of Aguacate and six other rural communities located within a 40-km radius of the Aguacate or Limbo collecting sites (Fig. 6). In Aguacate, 95% (54/57) of adults had neutralizing antibodies to VSV-Indiana, 27% (17/64) to Punta Toro, and 13% (8/61) to VP-437R. Neutralizing antibody rates among Aguacate inhabitants less than 20 years of age (predominantly school children) were VSV-Indiana 37% (42/116), Punta Toro 5% (6/123) and VP-437R 6% (7/114). In general, infection rates in the other six communities were lower but followed the same pattern observed in Aguacate. None of the human sera from any of the communities demonstrated neutralizing activity against Chagres, VP-175A or VP-161A.

TABLE 8

Comparison of sandfly isolations, sentinel animal conversions, and prevalence of neutralizing antibodies in adult human residents from Aguacate for five virus types

Virus	No. sandfly isolations	No. sentinel conversions	Human antibody rate (%)
VSV-Indiana	9	12	95
Punta Toro	42	0	27
VP-175A	26	3	0
VP-437R	15	7	13
Chagres	7	3	0

Table 8 shows serologic conversions among sentinel animals exposed at the Aguacate collecting site by virus type. Twelve sentinels developed neutralizing antibodies to VSV-Indiana, 0 to Punta Toro, 7 to VP-437R and 3 each to VP-175A and Chagres. Except for VSV-Indiana, conversions of sentinel animals were sporadic and showed no obvious seasonal pattern or clustering. Paradoxically, only 1 sentinel animal was infected with VSV-Indiana during months in which the 9 isolates of this virus were made (5 isolates obtained in August of 1969 and 1 each month during June,

TABLE 7

Prevalence of VSV-Indiana, Punta Toro, and VP-437R neutralizing antibodies among adult residents of selected rural Panamanian communities

Community	VSV-Indiana		Punta Toro		VP-437R	
	No. pos./ tested	Percent pos.	No. pos./ tested	Percent pos.	No. pos./ tested	Percent pos.
El Aguacate	54/57	95	17/64	27	8/61	13
Ciricito	11/15	73	6/15	40	2/15	13
Escobal	1/12	8	2/15	13	1/15	7
El Retiro	6/16	37	1/17	6	0/17	0
Buenos Aires	4/14	27	1/16	6	0/16	0
Salamanca	12/15	48	6/26	23	0/26	0
Nuevo Caimitillo	8/27	30	7/27	26	2/27	7
Total	96/166	58	40/180	22	13/177	7

July, August and September of 1970). The remaining 11 VSV-Indiana serologic conversions occurred between December and March of 1969, 1970 and 1971.

DISCUSSION

Perhaps the most striking data obtained in this study were the number and diversity of viral agents isolated from sandflies. From a total of 172,563 females processed, 253 virus strains were recovered; this represents 1 isolate per 685 female sandflies (Table 2). Similar rates have been reported previously for sandflies from Panama,[10,11] Brazil,[8] and Pakistan.[7] The diversity of virus types active simultaneously and obtained from collections comprised mainly of two anthropophilic sandfly species (*L. trapidoi* and *L. ylephilator*) indicate the biological complexity of these agents and suggest that the vector potential as well as public health importance of phlebotomine sandflies has not been fully appreciated.

A second important finding was virus infection of male sandflies. A total of 17 isolates, representing 6 different virus types, were made from male sandflies (Table 2). While the single Changuinola male isolate may represent an error in our sorting or processing; this possibility seems unlikely to account for the other 16 virus isolates from male sandflies. The number of male isolates is even more impressive when one considers that males comprised only 32% of our total sandfly collection and that the average size of male pools (167) exceeded that of female pools (58). From collections yielding comparable numbers of male and female insects (Tables 2 and 4), isolation rates of Punta Toro-CoAr 3319 and of VP-118D were similar, while isolation rates of VP-175A were actually higher in males. Other workers[5,7,9] have also reported isolations of Phlebotomus fever group agents and of Pacui, an ungrouped arbovirus, from male sandflies and have suggested that transovarial transmission of some of these viruses may occur in nature as we have demonstrated experimentally with VSV-Indiana.[22] The fact that male sandflies do not suck blood would appear to be additional evidence for transovarial transmission. Presumptive evidence that *Phlebotomus papatasi* transmit "sandfly fever virus" to their offspring actually was first reported in 1924 and again in 1937; however, little attention has been given to this work because virus isolation and identification were not done.[2,23] Collectively these observations imply that transovarial transmission of certain sandfly-associated viruses occurs in nature and that the ecology of some of these agents is quite different from that of mosquito-borne viruses.

One of the characteristics of viruses in the Phlebotomus fever arbovirus group is low virulence for animals, and multiple blind passages in newborn mice have been required to establish pathogenicity for a number of these agents.[3,7,24] Our experience supports these observations; Phlebotomus fever group viruses were recovered only in cell culture when both Vero and mice were used for primary isolation (Table 1), and initial sandfly isolates of Punta Toro-CoAr 3319, Chagres, VP-175A and VP-488A all required serial blind passages in newborn mice or hamsters to establish pathogenicity. Isolate VP-488A, for example, did not produce illness or death in infant mice until the fourth serial passage (intracerebrally). Furthermore, the two new prototype viruses, VP-437R and VP-118D, could not be mouse-adapted despite repeated blind passages. We were also unable to mouse-adapt the 11 unidentified isolates. In contrast, most of the Changuinola complex sandfly isolates and all of the VSV-Indiana strains were lethal to newborn mice on initial passage. These results indicate that the Vero cell culture system is more efficient for primary isolation of certain sandfly viruses than are newborn mice; the number and diversity of viruses isolated in this study can be attributed in part to use of the Vero cell culture system, since several new viruses were found—some plainly not mouse pathogenic. The suitability of concentrated cell culture antigen for CF tests also eliminated the necessity of mouse-adapting each of the isolates in order to produce potent brain antigen.

Changuinola complex viruses accounted for 52% of our isolates (Table 2). Viruses of this group have been recovered previously from sandflies in Panama, Colombia and Brazil,[8,10,11] (R. E. Shope, 1972. Yale University School of Medicine, New Haven, Connecticut, personal communication). Because of their distinctive physical and serological properties (a double-stranded RNA genome, relative resistance to lipid solvents, lability at acid pH, and antigenic independence from other known virus groups), it has been proposed that they be included in a newly designated

arbovirus group, Orbiviruses, which includes bluetongue, African horse sickness, Colorado tick fever and a number of other viral agents.[25-29] Like bluetongue[27] and African horse sickness[28-29] viruses, members of the Changuinola complex are indistinguishable by CF test, although by neutralization test a number of different serotypes have been shown.[30]

A few human sera from Aguacate were examined by N test for antibodies to Changuinola complex viruses; all were negative. However, studies in our laboratory and elsewhere,[30] (R. E. Shope, 1972, personal communication), indicate that hamsters and mice develop Changuinola complex neutralizing antibodies only after repeated inoculations of virus and adjuvant. For this reason and because viruses of this complex do not produce hemagglutination,[8] we were unable to determine whether Changuinola complex viruses infect humans in Aguacate.

Fifty isolations of Punta Toro-CoAr 3319 were made. Punta Toro was originally isolated from a sick human exposed in Panama;[31] CoAr 3319 was obtained from sandflies in Colombia.[32] Because of serologic cross-reaction of many of our isolates with one or both of these prototype viruses we did not attempt to separate them; however, preliminary data suggest that the Punta Toro-CoAr 3319 complex may contain several distinct serotypes. Serologic studies of human sera from adult residents of Aguacate and 6 nearby rural communities demonstrated Punta Toro neutralizing antibody rates ranging from 6% to 40% (Table 7). In addition, we have recently made a second isolate of Punta Toro virus from the blood of a sick person. These data suggest a significant amount of human infection with this agent in Panama.

Seven isolates of Chagres virus, a member of the Phlebotomus fever group,[8,21] were obtained. Since previous isolations of this virus have been made only from sick humans,[33,34] the present results represent the first isolates of this agent from sandflies, the presumed vector. No Chagres neutralizing antibodies were found in the human populations sampled, although three serologic conversions occurred among sentinel animals exposed at the Aguacate study site.

Nine strains of VSV-Indiana were obtained, bringing to 14 the total number of isolations of this virus made from Panamanian sandflies.[10,11] Three of our VSV-Indiana isolates were made from pools of female *L. trapidoi*. Recent experimental studies indicate that this anthropophilic species can transmit the virus by bite to susceptible animals and to its offspring via the egg (transovarially).[22,35] These facts suggest that sandflies play a role in the epidemiology of vesicular stomatitis in this area of Panama.

The other five virus types isolated from sandflies (VP-175A, VP-437R, VP-161A, VP-118D and VP-488A) seem to be new agents and will be described in detail in another publication.[21] By CF or N test, all appear to be new members of the Phlebotomus fever group.

Table 8 compares the number of sandfly virus isolations, sentinel animal conversions, and prevalence of neutralizing antibodies in adult human residents from Aguacate for VSV-Indiana, Punta Toro, VP-175A, VP-437R and Chagres. There are several apparent paradoxes in these data. While 95% of the adult population of the community had antibodies to VSV-Indiana and 12 sentinel animal conversions were demonstrated, only 9 isolates of this virus were obtained from sandflies. Furthermore, 11 of the 12 sentinel conversions occurred between December and March, while all VSV-Indiana insect isolations were obtained between June and September, indicating virus activity in the study area when it was not detected in sandflies. If sandflies are indeed the sole vector of VSV-Indiana, one would expect a much higher insect isolation rate than was actually observed. Collectively, these observations imply that there may be another yet undetected source of the virus in nature.

The data for VP-437R (15 sandfly isolates, 7 sentinel animal conversions, and 13% human infection rate) are more compatible. In contrast, 42 isolations of Punta Toro-CoAr 3319 were made and 27% of Aguacate adult residents had neutralizing antibodies to Punta Toro, yet no sentinel conversions were recorded to this virus. The data for VP-175A and Chagres are equally puzzling, since 26 and 7 isolations of these viruses were made, respectively. Three sentinel conversions occurred to each, yet no human infection was detected with either agent. In view of the number of sandfly isolates of both agents, the sentinel animal conversions, and the known infectivity of Chagres virus for humans,[33,34] a higher human infection rate might be expected. One possible

explanation for this discrepancy may be that VP-175A and Chagres infections do not produce detectable or persistent neutralizing antibodies in man.

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