Table 1. Antigenic pattern of dengue viruses, including new types, by neutralization and by complement fixation tests.

Dengue virus type	Neutralization index, monkey sera, by type				Complement fixation titer, mouse sera, by type			
	1	2	3	4	1	2	3	4
1	5400	170	160	140	16	8	4	<4
2	10	1000	72	27	<4	128	<4	<4
3	4	2	1000	3	4	32	32	4
4	4	2	1	>4350	<4	8	8	32

and immunity was frequently demonstrated.

From the Manila area, 14 viruses were adapted to suckling mice; in each case adaptation required 10 to 20 passages. Incubation periods shortened from 20 or more days to 3 or 6 days, and titers rose from $<10^0$ to 10^4 or 10^6 LD_{50}. These all proved to be antigenically related to dengue viruses types 1 and 2 (*5*) but, except for a single type 2 strain, represent new types. Designation as dengue types 3 and 4 is recommended. Antigenic relations of the group as determined by complement fixation and by neutralization are shown in Table 1 (*6*). Human sera yielded nine type 3 and two type 4 viruses, and one type 2 virus, while *Aedes aegypti* and *Culex tritaeniorhynchus* each yielded one type 3 virus; these were the first dengue viruses to be reported from wild-caught mosquitoes.

From Bangkok, agents were isolated with less difficulty both from human sera and from *Aedes aegypti.* Of nine isolations made and identified in our laboratory, six were found, by neutralization and complement fixation tests, to be similar antigenically to dengue type 2, and three, to chikungunya, a group-A virus causing a dengue-like febrile disease in Africa and apparently transmitted there by *A. aegypti* (*7*). Three isolations typed as dengue 2 were from *A. aegypti,* while all other dengue and chikungunya viruses were from serum. Among viruses isolated from serum by other workers in Bangkok and sent to us for identification were two of the chikungunya type (*8*) and one tentatively identified as dengue type 1 (*9*). One of our isolations from human serum behaves like still another agent. Thus, three or possibly four virus types have been isolated. Other sera are still under test.

Serological studies of patients are as yet incomplete, but by demonstrating a rise of antibody titer in suitably spaced paired specimens in a combination of neutralization, complement fixation, and hemagglutination-inhibition tests, we have obtained ample confirmation of concomitant infection with one or more of these viruses and the disease syndrome. A number of patients from Bangkok showed a simultaneous rise in titer in response to both chikungunya and dengue viruses. A few patients having brief, principally mild, illnesses, without shock, showed no rise in antibody titer in response to the group A or B agents employed.

It is too early to evaluate completely the possible etiological significance of all these agents isolated from blood sera of patients with the broad clinical syndrome of "H" fever and from mosquitoes of the two epidemic urban areas. However, it is difficult to draw any conclusion other than that of probable etiologic relationship when the following points are considered: (i) viruses of the same type were isolated from several patients with the same syndrome; (ii) homologous rises in antibody titer were demonstrated in these and in numerous other patients; (iii) the epidemic pattern of the disease, with seasonal occurrence and *A. aegypti* association, was similar to that expected for related agents; (iv) several of the agents were isolated from *A. aegypti* mosquitoes; (v) no classical dengue-like disease was observed simultaneously in the areas; and (vi) older persons in these communities were probably immune to these or closely related agents; this would explain the unusual age distribution for the observed disease. Such immunity had been partially demonstrated in previous surveys, dengue antibody having been found in Manila (*10*) and Semliki Forest virus (related antigenically to chikungunya) antibody in Bangkok (*11*).

The next question of major interest which these observations raise is the adequacy of antigenic analysis, now generally accepted as the final method of identification for the ar-bo virus group. Several of the incriminated agents are identical with, or very closely related antigenically, to agents causing either classical dengue (dengue types 1 and 2) or chikungunya disease (bent bones). The "H" fevers do not resemble the classical dengue syndrome. It is true, however, that in association with a few epidemics called dengue on a clinical basis, an occasional hemorrhagic case, sometimes fatal, has been reported. Such cases could either have represented other infections or have been an indication that hemorrhagic variants of the common viruses occasionally arise. Sabin (*12*) noted occasional petechiae in cases of dengue among volunteers. Further study is obviously required.

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1. F. N. Quintos *et al., Philippine J. Pediat.* **3**, 1 (1954).
2. We now prefer this abbreviation for the term *arthropod-borne zoonoses viruses* to the commonly used "arbor," thought erroneously by many to be of Latin derivation and to mean tree.
3. This investigation was carried out in cooperation with the American Armed Forces; the Philippine Health Department; the School of Hygiene, University of the Philippines; and the Philippine National Museum. The work was done in part under the sponsorship of the Commission on Viral Infections, Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General, Department of the Air Force, by the Office of the Surgeon General, Department of the Army, and by a research grant from the National Institutes of Health, U.S. Public Health Service.
4. W. McD. Hammon *et al., Abstr. 9th Pacific Sci. Congr. Bangkok* (1957), p. 187.
5. ———, *Abstr. 6th Intern. Congr. Trop. Med. and Malaria,* Lisbon (1958), p. 163.
6. These relationships were subsequently confirmed by hemagglutination inhibition and by complement fixation by Jordi Casals of the Rockefeller Foundation.
7. M. C. Robinson, *Trans. Roy. Soc. Trop. Med. Hyg.* **49**, 28 (1955); W. H. R. Lumsden, *ibid.* **49**, 33 (1955).
8. The viruses were isolated by Sriprapai Phong-Aksara of the Queen Saovabhas Memorial, Pasteur Institute, Bangkok, Thailand.
9. The virus was isolated by Sman Vardhanabhuti, Faculty of Medicine in Chiengmai, Siriraj Hospital, Dhonburi, Thailand.
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Species Differentiation of Insects by Hemolymph Electrophoresis

Abstract. By means of microelectrophoresis in agar gel, we studied the hemolymph proteins of different species of the family Triatomidae (class Insecta) and different species of the families Ixodidae and Argasidae (class Arachnoidea). The results show a specific electrophoretic pattern for each species. Insects infected with pathogenic organisms have the same pattern as normal insects.

The hemolymph of blood-sucking insects has a high protein content (7 to 8 gm-percent by refractometric determination); 2 to 3 μl hemolymph are easily taken by cutting a leg of the in-

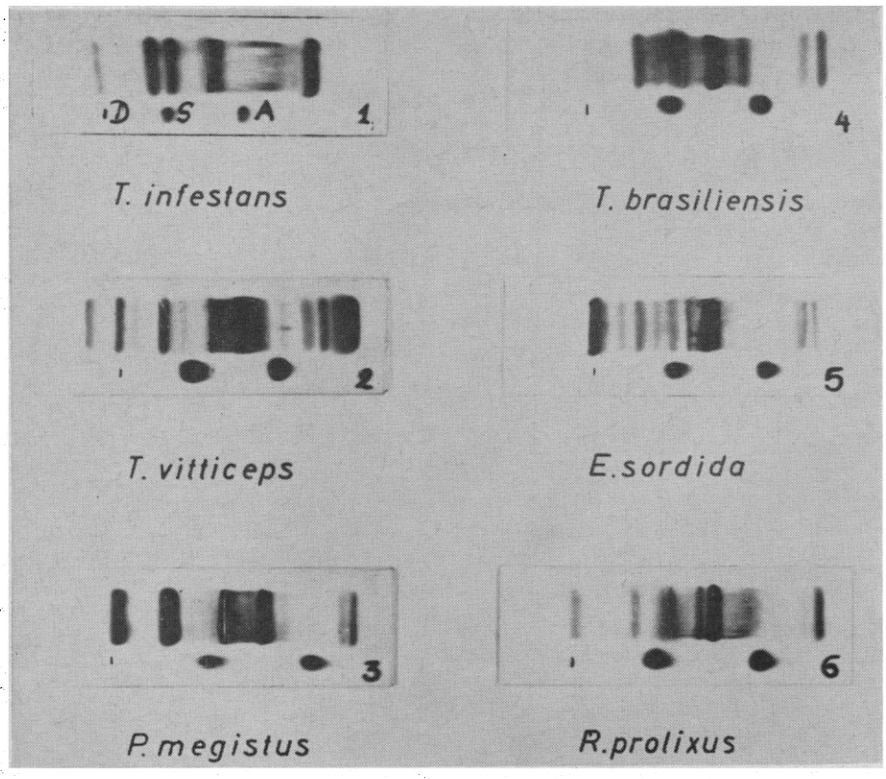

Fig. 1. Electrophoretic patterns for six species of the family Triatomidae.

sect, or by puncturing a lymphatic gland. Electrophoresis was carried out on a microscopic slide covered with a 1-mm layer of buffered agar gel (1 percent agar in a barbital buffer at *p*H 8.3. μ 0.05) (*1*). The electrophoretic run took place at 140 volts and required 25 minutes. After fixation and drying the plates were stained with amidoblack 10 B (Bayer). The relative mobilities of the different fractions were obtained by comparing their migration rates with those of a standard mixture containing human albumin, siderophilin, and depolymerized dextran. The distance between albumin and dextran (a molecule with zero charge) was taken as a unit.

For the class Arachnoidea, we studied two species of the genus *Hyalomma* belonging to the Ixodidea: *H. excavatum* (Koch, 1844) and *H. impeltatum* (Schulze and Schlottke, 1930). Electrophoretic patterns with 11 fractions were obtained. There was a marked difference between the patterns for the two species studied.

The two most important species in each of two genera belonging to the Argasidae were studied: from the genus *Argas, A. reflexus* (Fabricius, 1794) and *A. persicus* (Oken, 1818); from the genus *Ornithodoros, O. moubata* (Murray, 1877) and *O. savignyi* (Audouin, 1827). Here, the differences between the species were very striking, showing, however, a great analogy between the two genera. The hemolymph proteins of *A. reflexus* give three fractions; the relative mobility (m_r) of the most important fraction is 0.880. *Argas persicus* shows five clearly distinguishable fractions. Here, the most important fraction quantitatively (m_r, 1.002) migrates at the same rate as human albumin. For the genus *Ornithodoros*, we studied the two species which, when infected with *Borrelia hispanicum*, are very important agents in the transmission and dissemination of human disease. *Ornithodoros moubata*, the eyeless tampan, was investigated in both normal and infected condition. Twenty-three adult female ticks give a pherogram characterized by a fraction with m_r of 0.813, which is quantitatively the most important fraction among 11 others. No changes could be observed in the pherograms of ticks infected with *Borrelia hispanicum*. The fluid released by the tick when biting has a low protein content. Electrophoresis of this secretion gave only one fraction; the m_r is 0.905, and thus, this fraction migrates faster than the main fraction of the tick's hemolymph.

Ornithodoros savignyi, a tick which closely resembles *O. moubata*, has a completely different pattern; only three fractions were observed, with m_r of 0.785, 0.888, and 1.019, respectively; the second is quantitatively quite distinctive.

Seven species belonging to four genera of Triatomidae (*2*) were studied: *Triatoma infestans* (Klug, 1834), *T. vitticeps* (Stål, 1859), *T. brasiliensis* (Neiva, 1911); *Panstrongylus megistus* (Burmeister, 1835); *Eutriatoma sordida* (Stål, 1859); *Rhodnius prolixus* (Stål, 1859) and *R. pallescens* (Barber, 1932). The electrophoretic patterns for six of these species are shown in Fig. 1. Even though there is a striking analogy among these different genera, the pattern is specific for each species. We wish to emphasize the fact that no differences are found that reflect sex, age, or infection with *Trypanosoma cruzi*. So we are led to assume that the parasite takes no part in the protein metabolism of the insect.

In a previous communication (*3*) it was shown that electrophoresis of the hemolymph proteins of these blood-sucking insects is not influenced by the species of the individual whose blood is utilized. This seems to show that these insects possess their own specific protein metabolism. On pherograms obtained after feeding, quantitative variations may be observed in the area of the slowest-moving fractions, but this increase does not alter the specificity of the findings for the species under study.

It is worth pointing out that, for the same species, we found repeatedly the same basic electrophoretic pattern. The constancy of our results for each insect individually emphasizes the specificity of this method. For instance, for 37 determinations carried out for *Triatoma infestans*, the m_r of the main fraction is 0.785, with a standard deviation (σ) of 0.018.

It appears that electrophoresis of the insect's hemolymph in agar gel could help confirm the findings of taxonomy (*4*).

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4. This investigation was supported in part by a grant (180-2) from the National Multiple Sclerosis Society, U.S.A.

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