

Characterization and Identification of Tick-Borne Viruses in Thailand

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OBJECTIVE: This study is designed to study the characteristics of and identify unknown viruses isolated from ticks.

BACKGROUND: Tick-borne viruses are well known causes of serious and often fatal human infections in many parts of the world. Throughout Asia tick-borne agents have been found which cause fever, hemorrhagic fever and encephalitis. Some, however, are not human pathogens. Two viruses previously found in Thailand, Nyamanini and Pathum Thani as well as Langat from Malaysia, are not known to cause human disease.

The causes of some cases of fever, hemorrhagic fever and encephalitis are not explained. It is possible that some are due to tick-borne viruses not previously identified. For this reason, a study was initiated in 1972 to attempt isolation of viruses from ticks and to identify them. Five strains of transmissible agents were recovered. This report describes their characteristics.

DESCRIPTION: Ticks of the same species, collected from the same area at the same time, were pooled in groups of 10 to 20. Virus isolations were made in suckling mice. Each tick pool was ground in a mortar and pestle and suspended in bovine albumin borate saline (2 ml/10 ticks) containing penicillin and streptomycin. The suspension was clarified by centrifugation at 1000 rpm for 30 minutes and the supernate inoculated into 1-2 day old suckling mice in a dosage of 0.02 ml intracerebrally. Two litters of mice were inoculated for each tick pool. The mice were observed daily for signs of illness or death for at least 3 weeks.

Mice that showed signs of neurologic disease were sacrificed and the brains harvested. A 20% suspension of mouse brain was prepared for inoculation of new litters of mice to demonstrate the presence of a transmissible agent and to establish a large supply of infectious seed virus.

Low passage infectious suckling mouse brain was used to attempt infection of a continuous line of LLC-MK2 cells in plaque flasks and glass tubes to determine and compare optimum media, plaque formation and cytopathic effect (CPE). A direct and delayed plaque technique was used which has been highly successful for the isolation of group A and B arboviruses. In an attempt to compare the unknown agents to other tick-borne arboviruses, the mouse brain isolates were tested for size by Millipore filtration, a lipid envelope by ether sensitivity, and acid lability at pH 3.0.⁽²⁾ Agents which grew in tissue cell culture were tested for sensitivity to 5-bromo-2-deoxyuridine (BUDR), a DNA inhibitor.

Identification of agents was based on neutralization by specific antisera. Each agent was used to prepare CF and HI antigens by a sucrose-acetone extraction technique.⁽³⁾ The antigens were used to make hyperimmune mouse ascitic fluid (HMAF) with Freund's adjuvant and Sarcoma 180 cells in adult mice.⁽⁴⁾ Plaque reduc-

tion neutralization tests were performed using homologous HMAF, a panel of 12 typing antisera prepared by the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, and other reference antisera. Isolates have been sent to reference laboratories to assist in the identification.

PROGRESS: A total of 14,268 ticks were collected from 12 provinces (i.e., Chantaburi, Chonburi, Chumpon, Loel, Mae Hong Sorn, Nakorn Nayok, Nakorn Ratchasima, Phatum Thani, Phangnga, Prachinburi, Ranong and Trang), identified and pooled for virus isolation attempts. Included in the 1267 pools tested were 17 species of ticks belonging to the genera *Amblyomma*, *Aponomma*, *Argas*, *Boophilus*, *Dermacenter*, *Ixodes*, *Haemaphysalis*, *Ornithodoros* and *Rhipicephalus* (Table 1). The largest number of ticks tested belonged to the genera *Argas* and *Haemaphysalis*, respectively. A total of 1885 ticks were collected from 815 mammals trapped and/or examined during this period. In addition, 442 ticks were collected from a total of 33 birds trapped or shot during the same period. The largest number of ticks, however, was collected from vegetation.

Eleven transmissible agents were recovered. Six came from pools of *Argas robertsi* collected in the vicinity of nests of night herons at Wat Pai Lom in Pathum Thani province. At the present time these agents have not been characterized further.

Table 1. Ticks Collected in Thailand and Tested for Viral Agents, 1973-74

SPECIES	No. of Pools	No. of Ticks
<i>Amblyomma testudinarium</i>	11	28
<i>Aponomma lucasi</i>	1	1
<i>Argas robertsi</i>	715	7,437
<i>Boophilus microplus</i>	20	206
<i>Dermacenter</i> sp.	35	61
<i>D. atrosignatus</i>	11	21
<i>D. auratus</i>	28	88
<i>Ixodes granulatus</i>	2	2
<i>Haemaphysalis</i> sp.	22	2,944
<i>H. atherurus</i>	13	53
<i>H. bandicota</i>	6	72
<i>H. cornigera</i>	163	1,191
<i>H. lagrangei</i>	181	1,504
<i>H. obesa</i>	18	42
<i>H. papuana</i>	10	21
<i>Ornithodoros capensis</i>	30	596
<i>Rhipicephalus h. haemaphysaloides</i>	1	1
TOTAL	1,267	14,268

Five isolates were obtained from *Haemaphysalis* ticks (Table 2). Four of them came from ticks collected in Khao Yai National Park (KYNP) in Nakhon Ratchasima Province and the other from a single tick removed from a domestic dog in Loei Province. T-867, T-868 and T-870 were obtained from the same species of tick collected at the same time from the same habitat and are considered to be identical. Studies on the identification of isolates have used T-870, T-1642 and T-1674 as prototype strains.

Table 2. Sources of Transmissible Agents from *Haemaphysalis* Ticks.

Tick Isolate	Tick	Region	Habitat
T-867	<i>H. conigera</i>	Khao Yai	Leaf
T-868	<i>H. conigera</i>	Khao Yai	Leaf
T-870	<i>H. conigera</i>	Khao Yai	Leaf
T-1642	<i>H. papuana</i>	Loei	Dog
T-1674	<i>H. papuana</i>	Khao Yai	Leaf

The three isolates showed distinctly different growth characteristics. Although each grew to high titer in suckling mice, T-870 did not produce either CPE or plaques in LLC-MK2 cells regardless of the medium used. Investigators at WRAIR reported they were able to plaque T-870 by adjusting the agar overlay medium. Plaques were not seen when the same medium was used in this department. All subsequent work with this agent was done in suckling mice.

T-1642 did produce CPE and formed homogeneous plaques with clear centers similar in appearance to those of Chikungunya virus (Fig 1). T-1674 produced no CPE but did form plaques of various sizes from small to large without clear centers. Neither of the latter two agents formed plaques similar to our more familiar group B arboviruses, such as dengue or Japanese B Encephalitis virus (JEV). Tissue culture work suggested the three prototype strains were different.

All three isolates were inhibited by exposure to ether and showed acid lability at pH 3.0 (Table 3). Each passed through a 50 nm Millipore filter. T-1642 and T-1674 were not inhibited by BUDR suggesting they are not DNA viruses. T-870 was not tested for BUDR sensitivity since it was not grown in tissue culture. These results give suggestive evidence that all three are viruses and that T-1642 and T-1674 are members of the Togavirus group.

Sucrose-acetone extraction produced strong complement fixing antigens from each virus (Table 4). T-870 formed no hemagglutinin. T-1642 showed weak hemagglutination of goose erythrocytes at pH 6.0. T-1674 formed a strong hemagglutinin with optimum activity at pH 6.7 (range 6.4-7.0) at 22°C.

CF block titrations with sucrose-acetone antigens and hyperimmune mouse ascitic fluid showed that T-870, T-1642 and T-1674 were antigenically unrelated (Fig 2). On the other hand, T-1674 showed considerable cross-reactivity with Dengue 1-4 and Japanese B Encephalitis (Table 5). Plaque reduction neutralization tests showed T-1674 was partially neutralized by a pool of 35 group B arbovirus antisera. It appears, then, that T-1674 is a group B arbovirus closely related antigenically to Dengue 4 and JEV.

Table 3. Physical and Chemical Characteristics of Tick-borne Agents

Agent	Nucleic Acid (BUDR)	Envelope (Ether)	Size (Millipore)	Acid Lability (pH 3.0)	Presumptive Classification
Tick Isolate					
T-870	NT*	+**	50 nm	+**	Togavirus
T-1642	-	+	50 nm	+	
T-1674	-	+	50 nm	+	
Control Viruses					
Polio I				-	
Chikungunya	-			+	
Herpes	+				

* NT = not tested

** + indicates agent is sensitive to treatment

Table 4. Antigenic Characteristics of Tick-borne Viruses

Tick Isolate	Sucrose-Acetone Antigens		
	SMB Passage	CF	HA
T-870	5	+	0
T-1642	3	+	±
T-1674	4	+	+

Table 5. CF Titers of Suckling Mouse Brain Antigens and Hyperimmune Mouse Ascitic Fluid

Sucrose Acetone Antigen	HMAF					
	T-1674	D1	D2	D3	D4	JEV
T-1674	128*	32	32	16	16	32
	64	16	32	64	128	128
D1	8	128				
	4	512				
D2	8		64			
	4		1024			
D3	16			128		
	16			512		
D4	16				128	
	32				512	
JEV	32					512
	8					512

* Reciprocal antigen titer over antibody titer

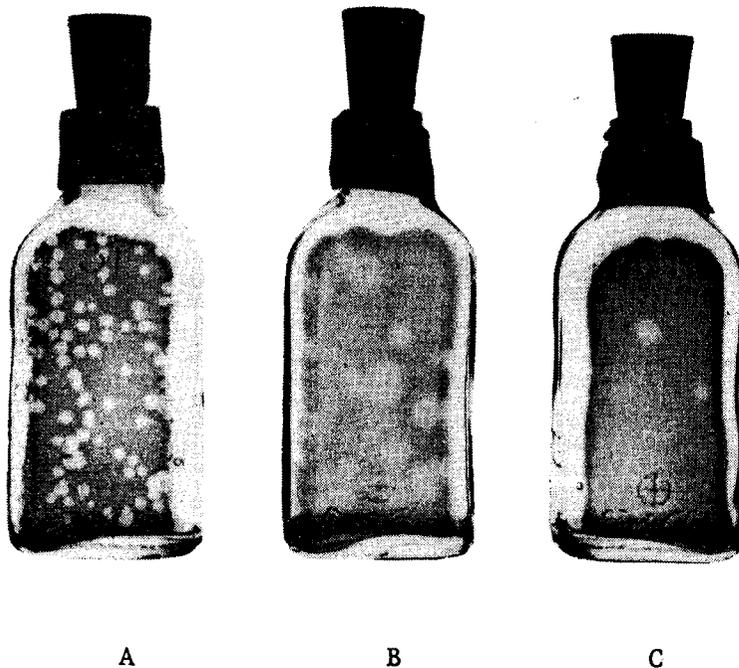


Figure 1. Comparison of plaque formation in LLC-MK2 cells of two strains of tick-borne viruses using a direct staining method. A. T-1642, passage SMB-4, TC-1 stained on day 3, formed uniform plaques 1.8-2.4 mm in diameter with clear centers. Photo was taken on day 7. B. & C. T-1674, passage SMB-4, TC-4 stained on day 8, formed plaques without clear centers that ranged from 3-5 mm in diameter. This pass was made by picking a single large plaque. Photo was taken on day 11.

HYPERIMMUNE MOUSE ASCITIC FLUIDS

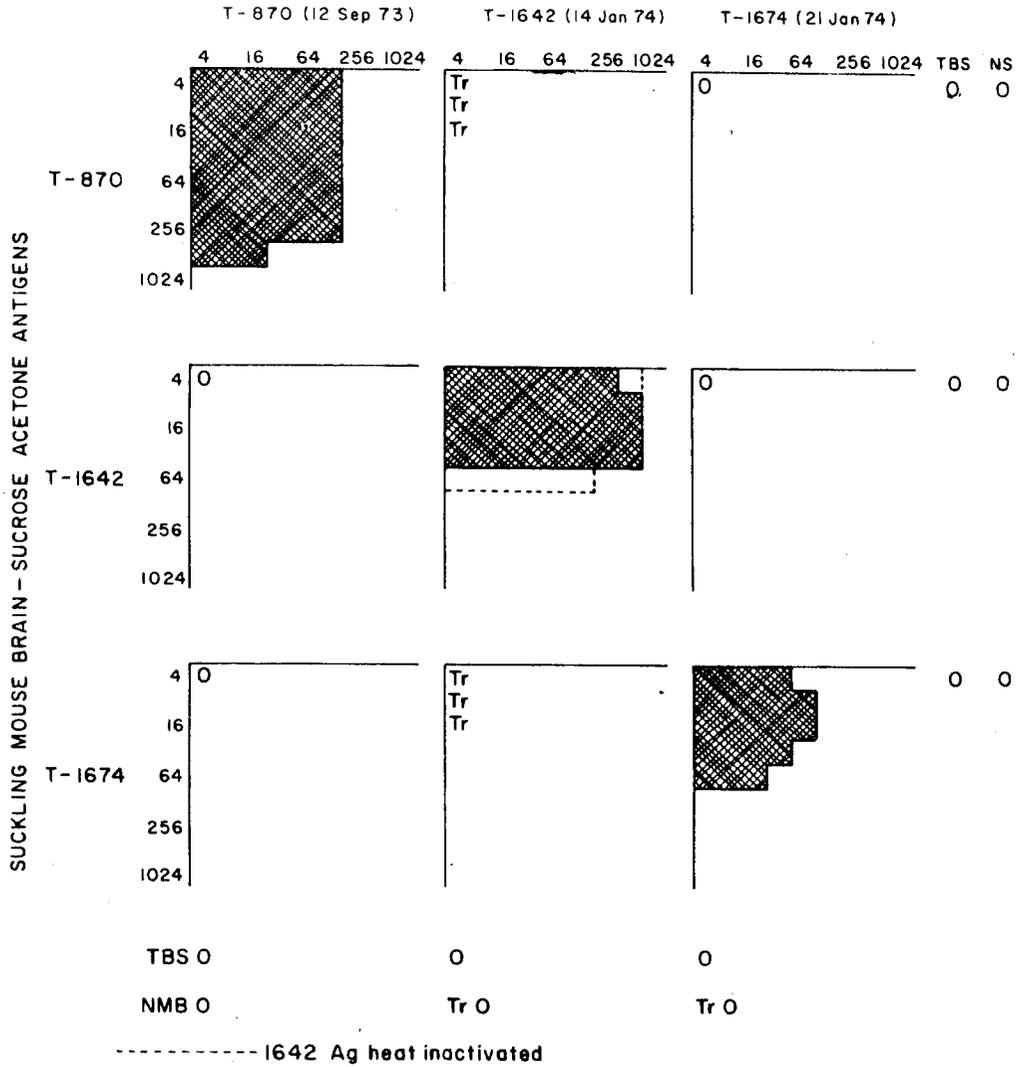


FIGURE 2. COMPLEMENT FIXATION BLOCK TITRATIONS COMPARING THREE VIRUSES ISOLATED FROM TICKS. ANTIGENS WERE PREPARED BY SUCROSE - ACETONE EXTRACTION OF SUCKLING MOUSE BRAINS. ANTIBODY WAS PREPARED IN MOUSE ASCITIC FLUID. NO CROSS-REACTIVITY BETWEEN THE VIRUSES WAS FOUND.

T-1642 did not react with any of the 12 different arbovirus grouping antisera. The identity of this virus is totally unknown.

T-870 has been studied by Dr. Robert Shope of the Yale Arbovirus Research Unit (YARU). A preliminary report said he found that in CF tests T-870 was not closely related to any of over 200 reference arboviruses he tested. It did show a weak cross-reaction with Wad Medanii virus, a tick-borne virus that has been found in Malaysia and Singapore in the past.

Strains of all three viruses and their homologous hyperimmune ascitic fluids have been provided to YARU for further attempts at identification.

DISCUSSION: At least four different viruses have been isolated from ticks during this study. The isolate from the Argas tick in Pathum Thani has not been characterized but is probably related or identical to Pathum Thani virus of birds.

The other three agents have characteristics of Togaviruses and may be pathogenic for mammals. Two viruses, T-870 and T-1642, may be entirely new, since neither show a definite antigenic relationship to any virus included in the pools of grouping antisera.

Incomplete characterization of these agents does not reveal if any of these viruses are pathogenic. Nevertheless, since T-1674 is a group B arbovirus, it must be considered potentially infectious for man.

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