Growth of a Candidate Arbovirus (Tsuruse) in *Aedes aegypti* Mosquitoes Following Intrathoracic Inoculation[1] (36351)

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A virus, tentatively named Tsuruse virus, was isolated in Japan in 1954 from mice inoculated with blood of a Blue Magpie (*Cyanopica cyaneus*).[2] The virus has physical, chemical and biologic properties described below which are compatible with arboviruses, and therefore its ability to grow in mosquitoes was determined to help classify it further.

Materials and Methods. Viruses. Tsuruse virus, strain Mag 271580, was used as infected suckling, 1–4 day-old, mouse brains representing passages 7, 8, 10 or 14–16. Supernatant fluids (10,000*g*, 20 min) from 10 or 20% brain suspensions were made in 1% bovine albumin in Hanks' solution (H) containing penicillin and streptomycin, 100 units and 100 μg/ml, respectively (BA). Swiss albino mice were from Taconic Farms, Germantown, NY. Two other viruses employed in control experiments were vaccinia and Sindbis, strain AR 339 (1); they were used as fluids from infected chicken embryonic cell cultures at an unknown, but high passage level of vaccinia virus and at passage 10 of Sindbis virus. Viruses were stored in multiple small aliquots in an electric freezer at −60°. Each aliquot was used only once.

Virus assays. Tsuruse virus was usually assayed by counting plaques in primary chicken embryonic cell (CEC) cultures prepared essentially as described elsewhere (2). Cultures were incubated at 37° for 1 or 2 days and then 0.2 ml volumes of serial decimal dilutions of virus in H were adsorbed to 18 sq cm cell sheets at 37° for 1 hr. Cells were covered with 5 ml of agar medium containing 1.5% Difco Noble agar in 85 ml H without phenol red, 2% heat-inactivated calf serum, 0.002% neutral red, 0.15% lactalbumin hydrolysate, 300 units of penicillin and 300 μg of streptomycin per ml and 0.02% diethylaminoethyl dextran (Sandoz). Plaques were counted after 2 and 3 days at 37°. Tsuruse virus was also sometimes assayed in groups of eight 1–4 day-old suckling or five 3–4 week-old weanling mice (SM or WM) inoculated intracranially (ic) with 0.01 or 0.03 ml, respectively, of each decimal dilution of virus per mouse. After observation for 14–21 days, 50% lethal end points ($SMicLD_{50}$ or $WMicLD_{50}$) were calculated (3). Vaccinia virus was assayed like Tsuruse virus, by counting plaques in CEC except that cells were covered with 5 ml of liquid maintenance solution (MS) (2) containing 300 units of penicillin, 300 μg of streptomycin, and 100 μg of Nystatin per ml, incubated undisturbed for 60 hr at 37°, the fluid removed, cells stained with Gram's crystal violet solution and plaques counted. Sindbis virus was assayed in CEC in 16 × 125 mm screw-cap test tubes. Growth medium was decanted, 0.1 ml of each decimal virus dilution in H added to each culture (3 tubes per dilution) and 1 ml of MS added per tube. The virus dilution at which 2 or 3 cultures showed cytopathic effects after 60 hr at 37° was taken as the end point.

Size estimation of Tsuruse virus by Mil-

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[2] The original isolation was made by Dr. C. Southam and associates at the U.S. Army 406th Medical General Laboratory, Tokyo, Japan.

TABLE I. Size of Tsuruse Virus Estimated by Filtration.

Expt.	Virus titer of filtrate[a] (% previous filtrate passing filter)				
	Unfiltered	450 mμ	300 mμ	100 mμ	50 mμ
A	4.5	4.2 (50.0%)		1.8 (0.4%)	<0.7 (<12.0%)
B			5.8	4.1 (2.0%)	

[a] $-\log_{10}$ CEC PFU/ml for experiment A and $-\log_{10}$ WMicLD$_{50}$/0.03 ml for B.

lipore filtration. Virus was 10% suckling mouse brain (passage 10) centrifuged 10, 000*g*, 20 min 5°. Experiment A employed serial filtration, and experiment B used direct filtration through each filter. Experiment B was done by T. Izumi at the University of Minnesota. In experiment A virus titers were $-\log_{10}$ CEC PFU/ml and in experiment B they were $-\log_{10}$ WMicLD$_{50}$/0.03 ml.

Effect of 5-iododeoxyuridine (IdU) on Tsuruse virus growth. Tsuruse, vaccinia, and Sindbis viruses were diluted decimally in H and placed in 0.1 ml volumes on 1 day-old CEC monolayers in 16 × 125 mm screw-cap test tubes. Three tubes from each dilution were immediately covered with 1 ml MS, three with 1 ml MS containing 10 μg/ml IdU and three with 1 ml MS containing 10 μg/ml IdU and 50 μg/ml of thymidine. Fluids were harvested after 60 hr at 37° and titrated for virus content.

Hemagglutinin preparation and tests for hemagglutination-inhibition (HI), complement-fixation (CF) and neutralization (N) antibodies. Previously described methods were used for these tests with Tsuruse virus (4, 5). Tsuruse virus antisera were made by multiple intraperitoneal (ip) inoculations of adult mice.

Cell cultures tested with Tsuruse virus. Cultures were prepared as described elsewhere (6, 7). MS was used (a) without serum for CEC and primary human amnion cells, (b) with 2% calf serum and 0.5% lactalbumin hydrolysate for primary monkey kidney cells and mouse embryonic cells, (c) with 2% swine serum and 10% yeast extract for swine embryonic kidney cells and (d) with 10% horse serum for continuous porcine kidney cells. The agar medium is described above under virus assays.

Maintenance and inoculation of female Aedes aegypti mosquitoes and preparation for virus assay. Colonial maintenance and intrathoracic inoculation are described elsewhere (8). Tsuruse virus was also fed to female mosquitoes as virus-blood mixtures on cotton. At selected times mosquitoes were immobilized with carbon dioxide, placed individually in 15 × 48 mm screw-capped vials and frozen at −60° within 5 min of recovery from anesthesia. Within 2 months, individual mosquitoes were ground in chilled mortars in 1 ml of BA containing 300 units of penicillin and 300 μg of streptomycin per ml. After centrifugation (10,000*g*, 20 min, 5°), supernatant fluids were decanted and stored at −60°. These fluids were considered to be 10^0 dilutions in virus assays. However, since only a portion of each mosquito's suspension was actually assayed (*e.g.*, about 0.08 ml of 1 ml), the level above which virus was detectable was about 3 LD$_{50}$ per mosquito.

TABLE II. Growth of Tsuruse Virus in CEC in the Presence of 5-Iododeoxyuridine (IdU).

Test and control viruses	Titer in CEC of fluid harvested after 60 hr at 37°[a]		
	Untreated	IdU, 10 μg/ml	IdU, 10 μg/ml and thymidine, 50 μg/ml
Tsuruse	3.0	3.0	3.0
Vaccinia	4.4	<1.0	4.4
Sindbis	6.5	6.5	7.0

[a] −Log 10 TCID$_{50}$/0.1 ml of fluid harvest based on qualitative production of plaques (vaccinia and Tsuruse) or CPE (Sindbis).

TABLE III. Arboviruses Compared Immunologically with Tsuruse Virus.

GROUP A — 8 viruses
- eastern encephalitis[c]
- Getah (Sagiyama)[c,d]
- Mayaro[d]
- Ndumu[a]
- Pixuna[a]
- Semliki Forest[d]
- Sindbis[a,c,d]
- western encephalitis[a,c]

Group B — 14 viruses
- dengue types 1 and 2[c,d]
- Ilheus[c,d]
- Japanese encephalitis[c,d]
- *Langat*[c]
- Murray Valley encephalitis[c,d]
- Ntaya[d]
- Russian encephalitis[d]
- St. Louis encephalitis[c,d]
- Tembusu[d]
- Uganda[d]
- West Nile[c,d]
- Yellow fever[c,d]
- Zika[d]

BUNYAMWERA GROUP — 9 viruses
- *Batai*[c]
- Bunyamwera[a,c,d,e]
- Cache valley[a,c]
- *Germiston*[c]
- Guaroa[a,c]
- Ilesha[b,c]
- *Kairi*[c]
- *Sororoca*[c]
- Wyeomyia[c,d]

GROUP C — 9 viruses
- *Apeu*[c]
- *Caraparu*[c]
- *Itaqui*[c]
- *Madrid*[c]
- *Marituba*[c]
- Murutucu[a,c]
- *Nepuyo*[c]
- Oriboca[a,c,e]
- *Ossa*[c]

GUAMA GROUP — 4 viruses
- *Bimiti*[c]
- *Catu*[c]
- Guama[a,c,e]
- *Moju*[c]

CAPIM GROUP — 5 viruses
- Acara[b]
- *Bushbush*[c]
- *Capim*[c]
- *Guajara*[c]
- *Morichc*[c]

SIMBU GROUP — 6 viruses
- Akabane[a,b,e]
- Ingwavuma[b]
- Manzanilla[a]; Oropouche[c]
- Sathuperi[b]
- Simbu[b]

BWAMBA GROUP — 2 viruses
- Bwamba[c,e]
- Pongola[b]

CALIFORNIA GROUP — 6 viruses
- California[c,e]
- *La Crosse*[c]
- *Melao*[c]
- *Snowshoe hare*[c]
- Tahyna (*Lumbo*)[a,c]
- *Trivittatus*[c]

ANOPHELES A GROUP — 3 viruses
- Anopheles A[b,c,e]
- Lukuni[b]
- Tacaiuma[a,b]

PHLEBOTOMUS FEVER GROUP — 10 viruses
- Anhanga[b,c]
- *Bujaru*[c]
- Candiru[b,c]
- Chagres[a,c]
- Icoaraci[a,b,c]
- *Iran 58*[c]
- *Iran 81*[c]
- *Itaporanga*[c]
- Naples[c]
- Sicilian[a,b,c]

TIMBO GROUP — 2 viruses
- Chaco[b]
- Timbo[b]

CHANGUINOLA GROUP — 2 viruses
- Irituia[b]
- BeAr 41067[b]

VESICULAR STOMATITIS GROUP — 3 viruses
- Indiana[b,e]
- Piry[b]
- New Jersey[b,c]

TACARIBE GROUP — 3 viruses
- *Amapari*[c]
- *Junin*[c]
- Tacaribe[b,c]

12 OTHER GROUPS — 12 viruses
- Anopheles B[b,e]; Bluetongue[c]
- Corriparta[e]; Ketapang[a]
- Mossuril[b]; Nyando[b]
- Palyam[b]
- Quaranfil[b]
- Tete[b]
- Turlock[a,c]
- Wad Medani[b]
- Wongal[b]

UNGROUPED — 14 viruses
- Colorado tick fever[b,c]
- Hart Park[b]
- Jurona[b]
- Kern Canyon[b]
- Lebombo[b]
- Marco[b]
- Minnal[b]
- Navarro[b]
- Nodamura[c,d]
- Nyamanini[b]
- P 422[c]
- Pacui[b]
- Wanowrie[b]
- Witwatersrand[a,b]

Italics signifies that the arbovirus antibody was only in polyvalent group specific antiserum kindly supplied by Dr. R. Shope at Yale University and Dr. T. Work while at CDC.

[a,b] Arboviruses tested by HI and CF, respectively, against Tsuruse virus antiserum; [c,d] Arbovirus antisera tested against Tsuruse virus by CF and N methods, respectively; [e] Arboviruses tested by N against Tsuruse virus antiserum.

Virus stability in test tubes at insectary temperatures. When inoculated into mosquitoes, virus suspensions were also placed in multiple 0.3 ml quantities in 15 × 48 mm screw-cap test tubes and incubated at 27°. Then, when mosquitoes were frozen for virus

TABLE IV. Animal Host Range of Tsuruse Virus.

Animal and age	Route of inoc.[a]	Dose as $SMicLD_{50}$	Fraction dying	Incubation period (days)	Fraction of survivors developing antibody[b]
Mice 1–3 days	ic	>1	8/8	3–5	
	sc	20,000	1/8	21	5/5
3–4 weeks	ic	1	5/5	3–7	
	sc,ip	25,000	0/4[e]		[d]
	in	2500	0/4		
8 weeks	sc	20,000	0/4		0/4
Hamsters 4–6 weeks	ic	20,000	4/5	4–7	1/5
	sc	20,000	0/5		1/3
Chicken embryos					
7–8 day	ys	50,000	5/5[c]	3–7	
9 day	cam	50,000	0/11		
Chicks 1–3 days	ic,sc,ip,in, iv,im	20,000	0/5[e]		0/5[e]
6 weeks	sc	10,000	0/4		0/4

[a] ic = intracranial, sc = subcutaneous, ip = intraperitoneal, in = intranasal, ys = yolk sac, cam = chorioallantoic membrane, iv = intravenous, im = intramuscular.

[b] Mice and hamsters were tested for CF antibody and chickens for N antibody in undiluted, unheated serum in CEC plaque reduction neutralization tests. Positive CF antibody titers were 1:2–64 at 3–4 weeks after inoculation.

[c] Embryos were hemorrhagic and yielded SM infectious virus which passed successfully to other eggs by yolk sac route.

[d] Three ip inoculations of 3–4 week-old mice during a three week period produced antibody.

[e] Results identical for each route of inoculation.

assay, a tube of virus was placed at —60° and subsequently assayed like mosquitoes at the time of mosquito tests. Titers of virus in test tubes were calculated per 0.0001 ml because this was the approximate volume of intrathoracic inoculum per mosquito. The minimal detectable level of virus for these suspensions was the same as for mosquito assays, 3 LD_{50} per ml or $10^{-3.5}$ LD_{50} per 0.0001 ml.

Results. Properties of Tsuruse virus. Virus passed 450, 300 and 100 but not 50 mμ Millipore filters (Table I). These results were similar to those with Colorado tick fever virus (9), which is 75–80 mμ by electron microscopy (10). Sodium deoxycholate (final concentration 0.5% in H, 37°, 1 hr reduced the titer of virus in passage 8 from $10^{-3.5}$ to less than $10^{-1.9}$ $WMicLD_{50}$/0.03 ml; diethyl ether (25%, 4°, 1 hr) reduced titers from $10^{-3.7}$ to $10^{-1.8}$. Growth of Tsuruse virus in CEC was undiminished by IdU as was the RNA control virus, Sindbis (Table II). The control DNA virus, Vaccinia, was completely inhibited, and the inhibition was reversed by thymidine. Goose or chick erythrocyte hemagglutinins have not been found in sucrose–acetone extracts of suckling mouse brain or liver. HI tests (using Tsuruse virus antiserum against known arboviruses), CF tests and neutralization (N) tests in suckling mice inoculated ic, demonstrated no antigenic relationship between Tsuruse virus and 112 arboviruses representing 27 groups and 14 ungrouped viruses (Table III).[3] All but three arboviruses known to occur in Japan were tested; however of these three, Negishi and Apoi are in group B and Aino is in the Simbu group, and Tsuruse virus was not related to 14 other group B or 6 other Simbu

[3] Many of these HI and CF tests were generously done by Dr. Robert Shope, N tests by Dr. E. L. Buescher and the Corriparta virus test by Dr. Ralph Doherty.

TABLE V. Comparison of Properties of Tsuruse Virus and Common Murine and Avian Viruses.

Virus[a]	Properties of Tsuruse virus incompatible (—), questionable (?) or compatible (+) with murine and avian viruses					
	Size 50–100 mμ	Contains RNA	Inactivated by lipid solvents	Antigenicity	Kills chick embryos	No pocks on CAM
Laboratory mouse						
GD VII	—	+	—	?		+
reo 3	+	+	—	?	±	+
adeno	+	—	—	?		
pox	—	—	—		+	—
hepatitis	—	+	+	—	—	+
Sendai	—	+	+	—	—	+
K	—	—	—	—	—	+
polyoma	—	—	—	—	—	+
LCM		+	+	—		+
PVM	—	+	+	—	—	+
EMC	—	+	—	—	+	
Avian						
pox	—	—	—		+	—
NDV	—	+	—		+	

[a] Information from Andrewes, Sir C. H., (1967), "Viruses of Vertebrates," 2nd Ed., Williams and Wilkins, Baltimore, 401 pages.

viruses. Tsuruse virus antigen or antiserum was also tested by CF, HI and N procedures against GD VII, reovirus type 3, mouse adenovirus, mouse hepatitis, Sendai, K, polyoma, LCM, pneumonia virus of mice, EMC, and psittacosis agent, with negative results. Mouse antiserum containing Tsuruse virus antibody reacted at low titers (80, less than 80 and 80) with the first three viruses, probably due to the presence of these viruses in the source mouse colony. Tsuruse virus was lethal for suckling and weanling mice and hamsters inoculated ic, and chicken embryos inoculated in the yolk sac; mice and some hamsters developed CF antibodies after sc inoculation (Table IV). Plaques appeared in chicken embryonic cell cultures under agar overlay after 2–3 days at 37°, but only slight cytopathic effects were seen in CEC after 2 days of incubation at 37° with fluid medium. Tsuruse virus did not kill or produce antibodies in chickens nor did it cause cytopathic effects in primary human amnion, monkey and swine kidney, mouse embryo or continuous porcine kidney cells with fluid medium.

Because Tsuruse virus came from only one of six mice inoculated with Blue Magpie blood and reisolation was not attempted, there was a possibility that it was a common murine or avian virus. However, its size, apparent RNA content, sensitivity to lipid solvents, antigenicity and host range essentially eliminated its being one of these viruses and instead were compatible with its being considered a candidate arbovirus (Table V).

Growth of Tsuruse virus in A. aegypti following intrathoracic inoculation. Two experiments with *A. aegypti* which were fed Tsuruse virus did not establish viral multiplication unequivocally. Although virus disappeared by day 3 after ingestion and reappeared to significant titers by day 7 in some mosquitoes, it did not reappear consistently (Table VI). *A. aegypti* were therefore inoculated intrathoracically, and it was found that Tsuruse virus titers per mosquito increased, though not markedly, during the subsequent 14 days, whereas virus in test tubes held at the same temperature as mosquitoes

TABLE VI. Possible Growth of Tsuruse Virus in *Aedes aegypti* Infected by Feeding on a Blood–Virus Mixture.

Expt.	Number of mosquitoes tested per sample day	—log $SMicLD_{50}$/mosquito pool or single mosquito by days after feeding on virus suspension					
		0[a]	3	5	7	10	14, 17, and 21
I	pooled 10	3.3	<1.2		3.0	<1.2	<1.2
II	single 10	2.1–3.5	<1.2	<1.2			
	15				<1.2	<1.2	
	14				3.5	3.7	
	1						

[a] One hr after feeding.

became undetectable by 7 days (Fig. 1). Since no eclipse of virus was observed, these results did not clearly differentiate between viral multiplication and persistence in mosquitoes. Thus, an attempt was made to propagate Tsuruse virus serially in *A. aegypti* by intrathoracic inoculation. In this experiment, virus increased 10-fold during 7 days in passage 1, by about 10–1,000-fold during 14 days in passage 2, and by about 1,000-fold during 14 days in passage 3 (Table VII). Since the original inoculum underwent a dilution of $10^{-9.5}$ to $10^{-10.5}$ over a total of 35 days at 27°, unequivocal multiplication of Tsuruse virus in *A. aegypti* was demonstrated. It was subsequently found that *A. aegypti* were extremely sensitive to infection by Tsuruse virus. When virus was titrated simultaneously in 1–3 day-old mice inoculated ic and in *A. aegypti* inoculated intrathoracically, virus titers were $10^{-7.5}$ LD_{50}/ml and $10^{-8.0}$ ID_{50}/ml based on death of mice and isolation of virus from individual mosquitoes by ic inoculation of baby mice.

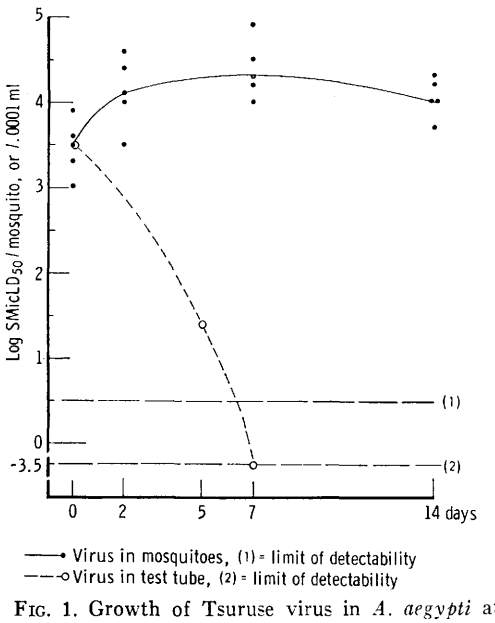

FIG. 1. Growth of Tsuruse virus in *A. aegypti* at 27° after intrathoracic inoculation. Virus decay in test tubes.

Discussion. Determination of virus growth in mosquitoes after intrathoracic inoculation was useful in characterizing Tsuruse virus as a likely arbovirus. However it should not be concluded that Tsuruse virus is transmitted in nature by the same mosquito species (*Aedes aegypti*) in which it grew in the laboratory. Unfortunately, common laboratory animals did not bcome infected after sc inoculation of small doses of virus, as might be

TABLE VII. Serial Passage of Tsuruse Virus in *Aedes aegypti* by Inoculation.

Passage number and (cumulative days)	Range of —log $SMicLD_{50}$ in each of 3–5 mosquitoes after	
	1 hr	7–14 days
1 (7)	3.0–3.9	4.0–4.9
2 (21)	<1.4–1.4	4.0–4.4
3 (35)	<1.4–1.4	3.5–4.5[a]

[a] Cumulated log dilution of virus after three passages = 9.5–10.5.

inoculated by mosquitoes during the act of feeding, and therefore, transmission studies with Tsuruse virus were not possible. Nevertheless, its ability to proliferate in mosquitoes and its physical, chemical and biologic properties were compatible with its classification as an arbovirus. It is hoped that this report will stimulate further studies of Tsuruse virus and other arboviruses to learn whether additional strains exist and whether the virus has public health significance.

Summary. Tsuruse virus, a candidate arbovirus, isolated in mice from bird (Blue Magpie) blood in Japan, grew unequivocally in *A. aegypti* after intrathoracic inoculation, though in only a rare mosquito after oral feeding. The estimated size of the virus was 50–100 mμ by filtration, and it was inactivated by lipid solvents, grew in the presence of iododeoxyuridine, produced plaques in cultures of primary chicken embryonic cells under agar and was lethal to suckling and weanling mice and weanling hamsters inoculated intracranially and to chicken embryos after yolk sac inoculation. No antigenic relationships were found between Tsuruse virus and 112 arboviruses.

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