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Identification of Tettnang Virus ('Possible Arbovirus') as Mouse Hepatitis Virus[1]

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Summary. Virus isolation attempts in specific-pathogen-free suckling mice (strain ICR) yielded 21 virus strains initially shown to be closely related to or identical with Tettnang virus. Subsequent studies showed them, as well as standard strains of Tettnang virus, to be closely related to or identical with mouse hepatitis virus. Tettnang virus is not an arbovirus.

In the summer of 1977, virus isolation attempts in specific-pathogen-free (SPF) suckling mice (strain ICR) were performed with heparinized bloods and cerebrospinal fluids from patients with fever of unknown origin or central nervous system syndromes. During this study, many apparently identical virus strains were isolated and were identified initially as Tettnang virus, recently classified as a 'possible arbovirus' [1]. We describe here our definitive characterization of these isolates and of various strains of Tettnang virus.

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Materials and Methods

Counterimmunoelectrophoresis (CIEP)

Precipitin antigens from the isolated strains were prepared by intracerebral (i.c.) inoculation of 1- to 2-day-old SPF suckling mice (strain ICR) with a 5% dilution of seed virus in phosphate-buffered saline (PBS) clarified by low-speed centrifugation. From the brains of dead and exsanguinated sick mice (usually 72–96 h after infection) a 50% suspension was prepared in cold Tris-buffered saline at pH 8.0 and was clarified by centrifugation at 27,000 *g* for 30 min. The supernatant fluid was stored in aliquots of 0.3 ml at −65°. Tahyňa virus antigen was similarly prepared after inoculation of suckling mice with a 1% virus suspension and harvesting of the brains at 44–72 h. The optimum dilution of antigens for the tests was determined by checkerboard titration in CIEP. The optimum was considered that dilution of antigen which detected the lowest concentration of antibody.

Immune Sera

Immune sera were prepared by intraperitoneal (i.p.) inoculation (0.2 ml) of a 10% mouse brain sus-

Table I. Mortality in suckling SPF mice (strain ICR) inoculated i.c. with blood or cerebrospinal fluid

Date	Animal farm	Number of specimens	Number of dead after 48 h/ number inoculated	Number of isolated strains	% dead[1]
6/28/77–8/2/77	Šumice	59	9/490	0	1.8 ± 0.6
8/5/77–9/30/77	Černý Vůl	77	155/750	21 (11)[2]	20.7 ± 1.5

[1] $p < 0.001$.
[2] Reisolated.

Table II. Biologic properties of the isolated strain Z/60

Passages	Suckling mice			Young mice	CPE in PS and Vero cells	Reduction of titer ($-\log_{10}$ LD_{50}/ml)	
	incubation time (days) after i.c. inoculation	titer ($-\log_{10}$ LD_{50}/ml)		titer ($-\log_{10}$ LD_{50}/ml) i.c.		chloro-form	ether
		i.c.	s.c.				
Original	6–20[1]						
First	4–7						
Third	4–5	5.1	3.4	< 1.0	negative	0.9	2.0
Fifth	3–4						

[1] 66% died within 10–16 days.

pension (25,000 i.c. suckling mouse LD_{50}) of the isolated strains in weanling SPF mice (strain ICR) 6–10 times at weekly intervals. 1 week after the last inoculation, the mice were exsanguinated. The sera were stored at $-25°$.

CIEP System

The electrophoresis equipment consisted of a Tesla BS 452E power pack and electrophoresis bath. 14.4 ml of 0.75% agarose (Fluka, catalogue No.05070, series No.17764-25) in Tris-buffered saline (pH 8.0) was poured into 85 × 85 mm glass plates. Wells were 3 mm in diameter, with their centers 5 mm apart. CIEP was performed at room temperature in an electrophoresis chamber for 3 h at 25 mA. The buffer in the chamber was 0.04 *M* barbital acetate in 0.1 *M* sodium chloride (pH 8.2). Reaction patterns were graded from 0 to 4 according to the precipitin arc intensity.

Microcomplement Fixation (CF)

CF was performed by the standard procedure described by *Kapikian* [2].

Results

From June 28 to August 2, 1977, 59 specimens were inoculated i.c. into 59 litters of mice from the animal farm in Šumice (table I). Cannibalism or death after 48 h was observed in 9 of 490 inoculated mice (1.8%). No virus was isolated from the brains of these dead mice.

For unknown reasons, the farm stopped delivering mice, and so we obtained the same

strain of mice from another farm in Černý Vůl. We inoculated 77 specimens into these mice. Again, cannibalism and death were observed, but the mortality was higher: of 750 inoculated suckling mice, 155 died (20.7%). 21 strains of a virus were isolated, and 11 were reisolated from the brains of mice dying during a 21-day observation period. Sick and dying mice were found prostrate and had spastic movements of their extremities. In some, we observed a peculiar gait with extremities out-stretched.

Since all isolated strains caused similar signs of illness in suckling mice, one strain (Z/60) was chosen for further studies (table II). The incubation period in mice inoculated i.c. with the original material was 6–20 days. In the next few passages this was shortened first to 4–7 days and then to 3–4 days. The LD_{50} titer reached $10^{-5.1}$/ml by i.c. inoculation and $10^{-3.4}$/ml by s.c. inoculation; older mice survived i.c. infection. CPE was not observed in PS and Vero cells, and chloroform and ether reduced the log of the infectious titer by 4.2 and 3.1 LD_{50}/ml, respectively.

Our first impression was that we had isolated an arbovirus (Tettnang virus). However, since no neutralizing antibodies to strain Z/60 were detected in the convalescent serum of the patient (i.c. test in suckling mice), we suspected that we had actually isolated a virus from the brain of enzootically infected suckling mice. To clarify this matter, the following studies were undertaken (table III).

72 suckling mice of the same age, from the Černý Vůl farm, were inoculated i.c. with PBS. 24 died more than 48 h after inoculation, all within a similar period of time and with a similar clinical picture as seen previously, strengthening our suspicion that we were dealing with an enzootic virus infection. One strain was chosen for further study and was designated PBS2. We kept 4 uninoculated litters in a separate room. Death was observed in these uninoculated mice under similar conditions of housing and care and with similar clinical symptoms. One strain of virus from these uninoculated mice, designated 03, was chosen for study. In addition, 6 of 88 (6.8%) nursing mothers died during the 3-week observation period. A review of the virus surveillance program of the mouse colony revealed that only reovirus infection had been detected, even though the battery of antigens used consisted of a number of murine viruses, including mouse hepatitis virus (MHV).

Table III. Test for a possible spontaneous infection in suckling mice

Inoculum i.c.	Number tested	Number of deaths	Incubation period, days
PBS	72	24	7–17
None	37	27	6–18

Since biologic properties of the isolated strains resembled those of Tettnang (TET) virus, we requested the investigators [3–5] who had described the isolation of this virus to send us immune sera to TET virus. However, we deliberately did not ask for TET virus strains in order to avoid possible contamination in our laboratory.

By CIEP the three antigens demonstrated precipitating activity against the immune anti-TET sera (table IV). To determine the specificity of these results, we performed another CIEP test according to *Ibrahim* [6]; these results confirmed the previous ones (table V).

Two of our isolates were tested by CF using one of the immune sera received [3] (table VI). The results again confirmed the previously obtained evidence that our strains were closely related to or identical with TET virus.

Table IV. Identification of the isolates in CIEP

Sera	Antigens					
	Z/60	PBS2	03	NMB[1]	TAH-'236'[2]	Tris
Hyperimmune mice sera						
AK 63	3	3	3	0	0	0
K 247	4	2	3	0	0	0
M 63	3	3	4	0	0	0
TAH-'P6b' (1:4)[3]	0	0	0	0	4	0
NMS[4]	0	0	0	0	0	0
Tris	0	0	0	0	0	0

1 Normal mouse brain.
2 Ťahyňa virus (strain '236').
3 Anti-Ťahyňa hyperimmune rabbit serum.
4 Negative mouse serum.

Table V. Confirmation of the identification of the isolates in CIEP by an absorption method

Sera	Antigen: Z/60 Absorbing antigens					Antigen: PBS2 Absorbing antigens					Antigen: 03 Absorbing antigens				
	Z/60	PBS2	03	NMB[1]	TAH-'236'[2]	Z/60	PBS2	03	NMB	TAH-'236'	Z/60	PBS2	03	NMB	TAH-'236'
Hyperimmune mice sera[3]															
K 247	0	0	0	4	4	0	0	0	3	2	0	0	0	3	3
M 63	0	0	0	3	4	0	0	0	3	3	0	0	0	3	3
TAH-'P6b'	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NMS[4]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

1 Normal mouse brain.
2 Ťahyňa virus (strain '236').
3 Serum AK 63 was no longer available.
4 Negative mouse serum.

At this point, we stopped working with these strains for a few months for the following reasons. First, TET virus is registered in the International Catalogue of Arboviruses as a 'possible arbovirus' [1], and we did not have sufficient reason to doubt its identity. Second, routine serological tests of sera of retired breeder mice, using MHV antigen, had given consistently negative results. Third, HAN-NMRI mice used in the Federal Republic of Germany for the isolation of prototype TET virus are well-known and of acknowledged good quality.

When we then closely examined published

Table VI. Identification of two of the isolated strains: CF test 1

Sera	Antigens[1]			
	Z/60	03[2]	LCM[3]	Adeno[3]
AK 63	4	8	<4	<4
LCM	<4	<4	64	<4
Adeno	<4	<4	<4	80

[1] Reciprocal titers.
[2] Crude 10% suspension clarified by centrifugation.
[3] LCM (lymphocytic choriomeningitis) virus and adenovirus were obtained from Microbiological Associates (Bethesda, Md.).

Table VII. Preliminary identification: CF test 2

Sera	Antigens[1]		
	MHV (USA)	Z/60	K 247
MHV (1976), 1:5	≥40	≥20	≥10
MHV (1978), 1:5	≥40	≥10	≥20
03/9	6	≥ 8	ND[2]
NMS[3]	AC[4]	AC	ND

[1] Reciprocal titers.
[2] ND = Not done.
[3] Negative mouse serum.
[4] AC = Anti-complementary.

electron micrographs of TET virus [4], we realized that there was not sufficient evidence to exclude TET virus as a coronavirus-like agent. Therefore, we resumed our studies and tested our strains by CF, using MHV antigen and MHV immune serum.

The results provided the first evidence that our strains were closely related to MHV (table VII). The preliminary CF test was repeated in a checkerboard titration using two MHV antigens from different suppliers. These results confirmed the previous ones (table VIII).

Table VIII. Identification: CF test 3

Sera	Antigens				
	MHV (USA)	MHV (JAP)	Z/60	03	NMB[1]
MHV (1:5)	8/40[2]	8/40	8/20	8/40	<8/<10
03/9	8/8	16/4	16/16	16/16	<8/<8
NMS[3]	<2/<8	<2/<8	<2/<8	<2/<8	<8/<8

[1] Normal mouse brain.
[2] Titer of antigen/titer of serum.
[3] Negative mouse serum.

Next, we compared two of our isolates in a CIEP test with the immune anti-TET mouse serum used by Dr. *J. Casals* and MHV antigen and MHV immune serum simultaneously. The results confirmed those of the CF test (table IX). Succeeding CF tests also confirmed that TET virus is closely related to MHV. In these tests, immune sera of two of our strains not used in the previous tests fixed complement in the presence of MHV antigen (table X).

The high spontaneous mortality in our SPF mice (strain ICR) appeared to indicate that our mice had no previous experience with this virus. We tried, therefore, to determine any connection between our mice and those NMRI mice used for isolation of TET virus in Köln (FRG) [3]. Mice from Hannover (FRG) were sent to Czechoslovakia on October 9 and 11, 1972 (fig. 1). These mice were also

Table IX. Identification of Z/60 and 03 strains in a cross CIEP test

Sera	Antigens					
	Z/60	03	63 Casals	247 K	MHV (USA)	NMB[1]
03/10	4	4	4	4	0	0
63 Casals	2	3	2	4	1	0
63 AK	2	3	3	3	2	0
MHV (1:5)	0	0	0	0	1	0
NMS[2]	0	0	0	0	0	0

[1] Normal mouse brain.
[2] Negative mouse serum.

Table X. Identification: CF test 4

Sera	Antigens	
	MHV (USA)	MHV (USA) control
63 Casals	8	<4
PBS2[1]	4	<4
Z/60[1]	8	<4

[1] 6×0.2 ml i.p. of 25,000 suckling mouse LD_{50} at weekly intervals.

Table XI. The origin of NMRI mice[1]

NMRI	Inbr(Lac): 20 Genet: c. Origin: noninbred Swiss mice from Lynch to Poiley in 1937. Inbred by Pl, known as NIH/Pl. To US Naval Medical Research Institute at F 51, known as NMRI. Maintained by: Lac. Note: Many colonies of NMRI, particularly European, are random – or penbred.

[1] Taken from *Staats* [7].

used in Köln. Between 1973 and 1978 these mice, under the name HAN:NMRI, were also sent to other European countries and in 1976 to Egypt (fig. 2). Since the Egyptian TET strain was isolated in 1970, we can assume that MHV was present also in mice of other countries [1]. The Hannoverian mice actually came from Tübingen (FRG), where the NMRI mice had been imported earlier. They were sent for the first time to Hannover in 1960. It is possible that TET virus was isolated in Köln from these two different suppliers, Tübingen and Hannover, because both have the same NMRI strain of mice.

Mice delivered to the animal farm in Černý Vůl, Czechoslovakia, in October 1972 were used 2 months later as nursing mothers for suckling mice obtained by Cesarean section of SPF mice (strain ICR) received from the farm in Šumice (fig. 1). These mice could now be designated as ICR:HAN:NMRI. These first had been delivered to the institutes in Prague and Bratislava in 1974 and 1975, respectively. The first TET virus strains were isolated at these institutes in 1976 and 1977, respectively [4, 5].

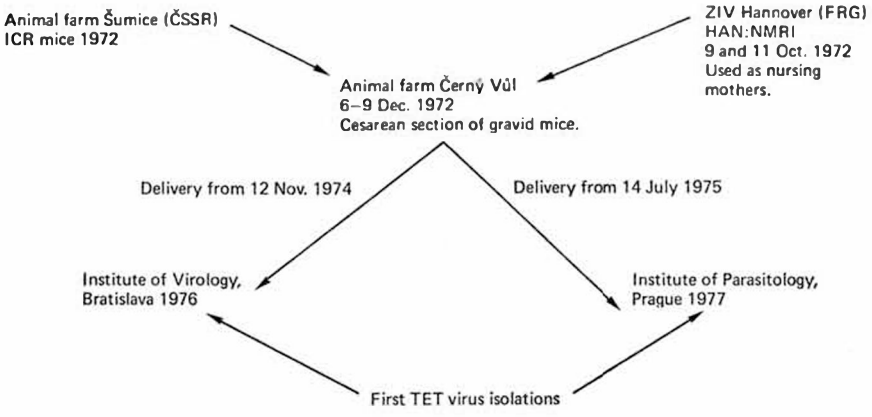

Fig. 1. Handling of HAN:NMRI mice in Czechoslovakia. ZIV = Zentral-Institut für Versuchstierzucht.

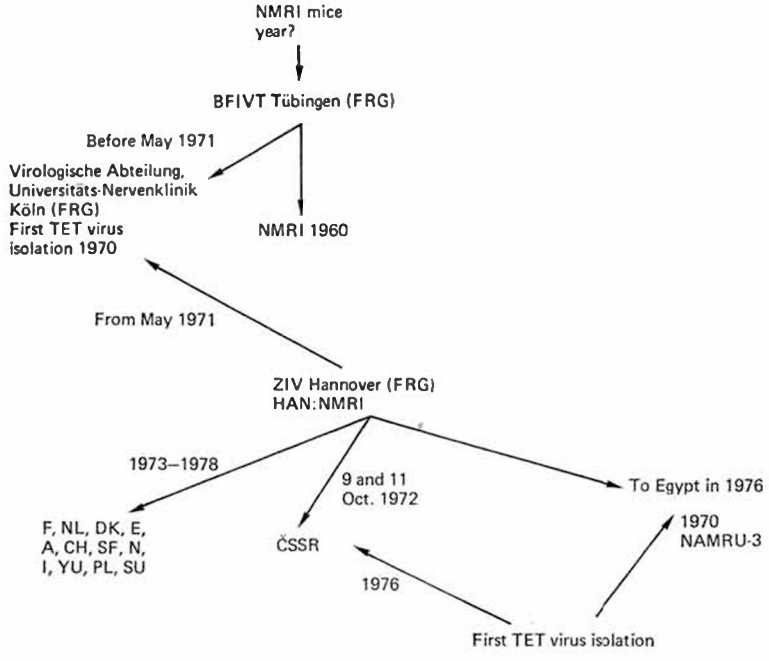

Fig. 2. Tracing of MHV infection in NMRI mice. BFIVT = Bundesforschungsanstalt für Viruskrankheiten der Tiere; ZIV = Zentral-Institut für Versuchstierzucht; F = France; NL = Netherlands; DK = Denmark; E = Spain; A = Austria; CH = Switzerland; SF = Finland; N = Norway; I = Italy; YU = Yugoslavia; PL = Poland; SU = Union of Socialist Soviet Republics; NAMRU-3 = Naval Medical Research Unit Number Three, Cairo, Egypt.

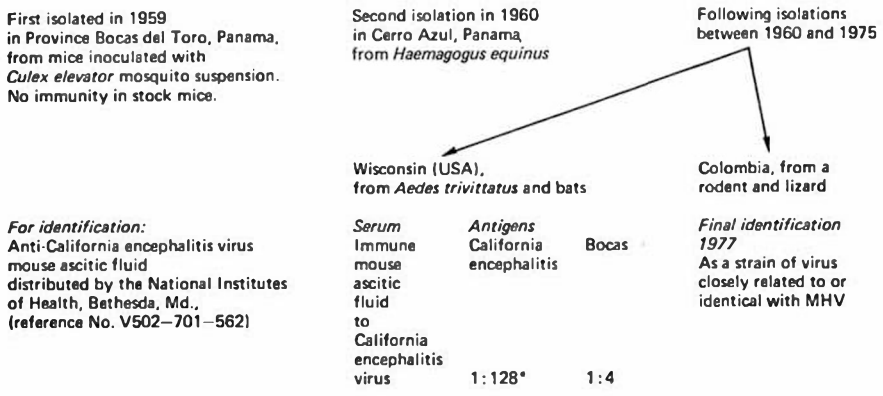

Fig. 3. History of isolation of Bocas (date of registration, 1971) and closely related virus strains. * = Complement fixation titer.

Since the Egyptian strain of TET virus was not isolated in mice from Germany, we were interested in the genealogy of the NMRI mice [7] (table XI). These mice, previously known as NIH/Pl, were obtained by the US Naval Medical Research Institute at the F 51 generation and have been known since then as NMRI mice. Since TET virus was isolated at NAMRU-3 in Cairo, Egypt, it is assumed NMRI mice were used for the isolation experiments. It is possible that at the time these mice were sent to Tübingen they were already infected. An example of a similar occurrence is the history of 'Bocas virus' [1] (fig. 3). The first strain of 'Bocas virus' was isolated in 1959 in Panama from mice inoculated with *Culex elevator* mosquito suspension. Since these mosquitoes feed principally on reptiles, the authors tested their breeding mice for immunity to this isolate; they found no such antibody. A second strain of Bocas virus was isolated in Panama in 1960; between 1960 and 1975 similar strains were isolated from mosquitoes and bats in Wisconsin and from a rodent and a lizard in Colombia [1] (fig. 3). Prototype Bocas virus reacted with anti-California encephalitis virus mouse immune ascitic fluid distributed by the National Institutes of Health and therefore was assumed to be a variant of California encephalitis virus, California serogroup. In 1977, Bocas was identified as a virus closely related to or identical with MHV [8]. It is presumed that the apparent isolations of Bocas virus previously described were actually only recoveries of enzootic MHV from the inoculated mice.

Discussion

We do not consider our study closed; further work with the neurotropic variant (JHM strain) of MHV is necessary. When new, spontaneously infected animals are imported to refresh the gene pool, a high mortality among susceptible animals often occurs. In spite of our findings, we feel that NMRI:HAN: NMRI or ICR:HAN:NMRI mice are of good quality. This has been confirmed by many workers for many years and in many areas

of the world where these mice have been used. The many excellent pathogenetic studies in these mice indicate their usefulness. However, it must be emphasized that, although immune sera and antigens prepared in these mice need not be discarded, the specificity of these products must be determined regarding evidence of indigenous murine and other agents, particularly MHV. Further safeguards are needed to assure that apparently new arboviruses are not actually indigenous murine viruses.

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Addendum

Upon returning to the USA from the Symposium at Smolenice, Dr. *Ch. Calisher* informed Dr. *J. Casals* of the content of the lecture. Dr. *J. Casals* performed CF tests on July 12–13, 1979, and wrote to Dr. *Ch. Calisher:* 'Tettnang (Köln 63) indeed is a mouse hepatitis virus, or at least is mixed with it, in our laboratory'. The strains obtained in our laboratory directly from Köln and from two virological laboratories in Czechoslovakia were identical with the strain obtained from Dr. *J. Casals*. Thus, it is justified to assume that all are strains of mouse hepatitis virus.

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