

Susceptibility of Rats of Different Ages to Inoculation with Swine Haemagglutinating Encephalomyelitis Virus (a Coronavirus) by Various Routes

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Summary

Haemagglutinating encephalomyelitis virus, strain 67N, was used to inoculate 1-, 2-, 4- and 8-week-old rats by the intracerebral (i.c.), intranasal (i.n.), intraperitoneal (i.p.), subcutaneous (s.c.), intravenous (i.v.) and oral routes with graded doses. The routes of infection, in descending order of efficacy, were: i.c., i.n., s.c., i.p., i.v. and oral. Rats aged 1 and 2 weeks were generally similar in terms of mortality and mean time to death, regardless of inoculation route, except for the oral route, which had little effect. In comparison with the 1- and 2-week-old rats, the 4-week-old rats were less susceptible to the virus by all routes. Eight-week-old rats inoculated by the i.c., i.n. or s.c. routes died, but all those inoculated by other routes survived.

To follow the spread of virus in the central nervous system, 4-week-old rats inoculated by the i.c. route were examined. The virus was first detected in the brain on day 1 and in the spinal cord on day 2. The viral titres in both tissues reached a plateau of 10^7 plaque-forming units (PFU)/0.2 g by day 4, at which time clinical signs had developed. By immunohistochemical analysis, virus-specific antigen was found first in the pyramidal cells of the hippocampus and cerebral cortex, and later in the large-sized neurons of the pons and spinal cord. Still later (day 4) immunolabelling was found in Purkinje cells of the cerebellum, but not in the ependymal cells, choroid plexus or other glial cells.

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Introduction

Haemagglutinating encephalomyelitis virus (HEV), a coronavirus, causes a vomiting and wasting disease and encephalomyelitis in piglets (Andries *et al.*, 1978; Andries and Pensaert, 1980a, b; Siddell *et al.*, 1983). Infection was first recognized in Canada as a disease of nursing pigs, with high morbidity, vomiting, anorexia and constipation (Roe and Alexander, 1958). Greig and Girard (1963) isolated in primary cell culture of pig kidney a previously unrecognized viral pathogen with haemagglutinating properties from the brains of suckling pigs with encephalomyelitis. The disease

was produced experimentally in piglets by inoculation via the oronasal route (Alexander, 1962). In 1969, an antigenically similar virus was isolated in England from suckling pigs showing anorexia, depression and vomiting but not encephalomyelitis (Cartwright *et al.*, 1969). These isolates, which showed characteristic petal-shaped spikes on the viral envelope, were later identified as coronaviruses (Greig *et al.*, 1971; Phillip *et al.*, 1971). In America, Mengeling and Cutlip (1976) demonstrated that the two major clinical forms of the disease (i.e., vomiting and wasting, and encephalomyelitis) were due to the same field isolates.

HEV strain 67N has been adapted experimentally to grow in the brains of suckling mice (Hirai *et al.*, 1974; Kaye *et al.*, 1977). Yagami *et al.*

Table 1
Susceptibility of rats aged 1, 4 or 8 weeks to inoculation with the virus by different routes

Route	Dose (PFU)	Results* in rats aged (weeks)		
		1	4	8
Intracerebral	2×10^5	5/3·0 (3)	5/4·8 (4-5)	5/4·8 (4-6)
	2×10^4	ND	5/5·0 (4-6)	5/5·2 (5-6)
	2×10^3	5/3·2 (3-4)	ND	5/5·3 (5-6)
	2×10^2	ND	5/5·6 (4-6)	3/6·3 (5-8)
	2×10	5/4·0 (3-5)	3/6·3 (5-7)	ND
	2	5/4·0 (3-5)	ND	ND
Intranasal	2×10^5	5/4·0 (4)	5/7·8 (6-12)	5/7·4 (7-8)
	2×10^4	ND	5/9·0 (8-10)	3/8·3 (7-10)
	2×10^3	5/5·6 (4-6)	1/10 (10)	3/9·5 (9-10)
	2×10^2	5/6·0 (5-7)	ND	ND
	2×10	3/6·0 (5-7)	ND	ND
	2×10^6	5/3·6 (3-4)	5/6·8 (6-9)	0
Intraperitoneal	2×10^5	ND	1/10 (10)	0
	2×10^4	5/4·0 (4)	2/9·0 (8-10)	ND
	2×10^3	5/4·6 (4-6)	1/10 (10)	ND
	2×10^2	3/5·7 (4-7)	ND	ND
	2×10^6	5/3·8 (3-4)	5/5·2 (4-6)	5/5·0 (4-6)
	2×10^5	ND	5/5·4 (4-6)	5/6·0 (5-7)
Subcutaneous	2×10^4	5/4·4 (4-5)	5/5·0 (4-6)	3/9·0 (7-11)
	2×10^3	5/5·0 (4-6)	2/6·0 (6)	ND
	2×10^2	1/5 (5)	ND	ND
	2×10^6	ND	5/6·0 (6)	0
	2×10^5	ND	5/6·2 (6-7)	0
	2×10^4	ND	3/7·2 (6-8)	ND
Intravenous	2×10^3	ND	1/7 (7)	ND
	2×10^5	2/5 (5)	0	0
	2×10^4	0	0	0
Oral	2×10^5	2/5 (5)	0	0
	2×10^4	0	0	0

* Deaths in groups of 5/mean number of days (and range) to death, See text for comparable results in rats aged 2 weeks. ND, not done.

(1986) studied the pathogenicity of this strain for mice of different ages by various routes of inoculation. After inoculation by the intracerebral (i.c.) route, 35-day-old mice died from encephalitis, with central nervous system (CNS) signs. However, mice aged 20 days or more proved to be resistant to intranasal (i.n.), intraperitoneal (i.p.) or subcutaneous (s.c.) inoculation.

Hirano *et al.* (1990), who reported successful propagation and plaque assay of HEV strain 67N in an established swine cell line (SK-K cell culture), found that the mortality from encephalitis in 4-week-old mice inoculated by the i.c. route with SK-K-grown virus was strongly dose-related. Rats aged 4 weeks also died showing CNS signs after i.c., i.n., s.c., i.p. or intravenous (i.v.) inoculation (Hirano *et al.*, 1995). However, rats infected by the s.c. route died a few days earlier than those inoculated with comparable doses by the i.p. or i.v. route. By direct inoculation into the sciatic nerve or footpad, the virus was first isolated from

the lumbar spinal cord and later from the brain. In accordance with these results, antigen-positive neurons were first detected in the lumbar spinal cord and later in the cerebral cortex, hippocampus, pons and cerebellum (Hirano *et al.*, 1998). These findings suggested that the virus spread to the CNS by neural routes rather than via the blood-stream.

The purpose of the present study was to examine (1) the susceptibility to HEV 67N of rats of different ages inoculated by various routes, and (2) the spread of virus in the CNS after i.c. inoculation, as demonstrated by immunohistochemical methods.

Materials and Methods

Rats

Pregnant specific pathogen-free (SPF) Wistar rats, serologically negative for murine coronavirus infections, were obtained from SLC (Hamamatsu, Japan) to supply offspring aged 1, 2, 4 or 8 weeks

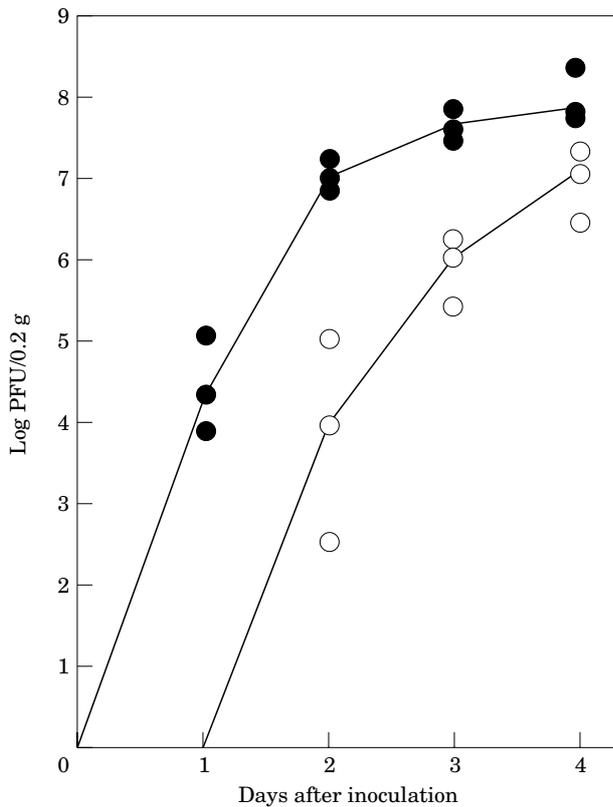


Fig. 1. HEV 67N growth in the brain and spinal cord of rats after intracerebral inoculation with 2×10^5 PFU. ●—●, Brain; ○—○, spinal cord.

old for the experiments. In most of the experiments male offspring only were used. Animals were given unlimited access to commercial pellets and water, but those aged 2 weeks or less were nursed by their dams. Rats were kept in lamina-flow units throughout the experiments, which were performed

in accordance with the guidelines of animal experimentation of Iwate University.

Viral Assay

The 67N strain of HEV (Mengeling *et al.*, 1972) was propagated and assayed by the plaque method in SK-K cell culture, as described by Hirano *et al.* (1990), the titres being expressed as plaque-forming units (PFU). For viral assay of brain and spinal cord (see below), 10% (w/v) homogenates were prepared in Eagle's minimum essential medium, and the supernates were subjected to the plaque method after centrifugation at 2000 rpm (1000 *g*) at 4°C for 10 min.

Inoculation of Rats

In a group of experiments designed to examine the effects of age and route of inoculation on susceptibility of rats to infection, rats ($n = 400$) aged 1, 2, 4 or 8 weeks were inoculated by the i.c., i.n., i.v., i.p., s.c. or oral routes with various doses. The dose volume was 0.02 ml for i.c. and i.n. inoculation. For inoculation by the i.p., i.v., s.c. and oral routes it was 0.2 ml, except that rats inoculated orally at the age of 2 weeks or less received a volume of only 0.02 ml. As control, several rats were given non-infected fluid from SK-K cell cultures in each individual experiment.

In a further experiment to examine the growth of virus in the brain and spinal cord by viral assay and immunohistochemical examination, 20 rats aged 4 weeks were inoculated with 2×10^6 PFU by the i.c. route and killed in groups of five, 1, 2, 3 and four days later. On each day, three rats were

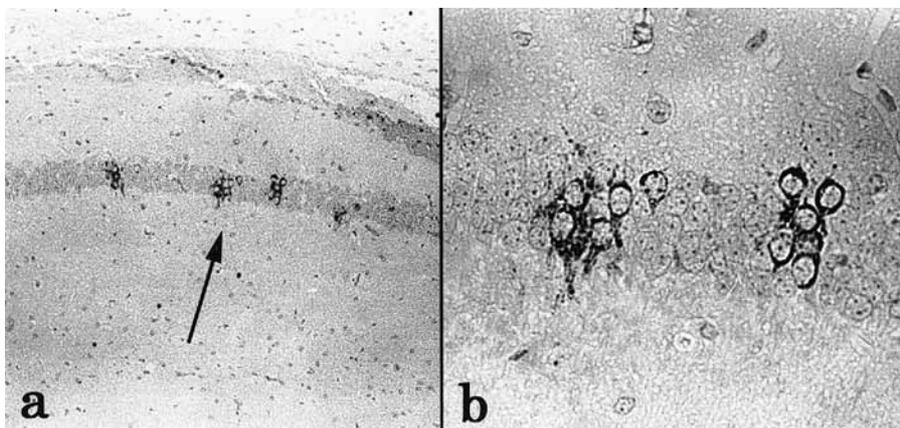


Fig. 2. Viral antigen-positive neurons in the hippocampus of rat on day 1 after i.c. inoculation. ABC. a, $\times 50$; b, $\times 250$.

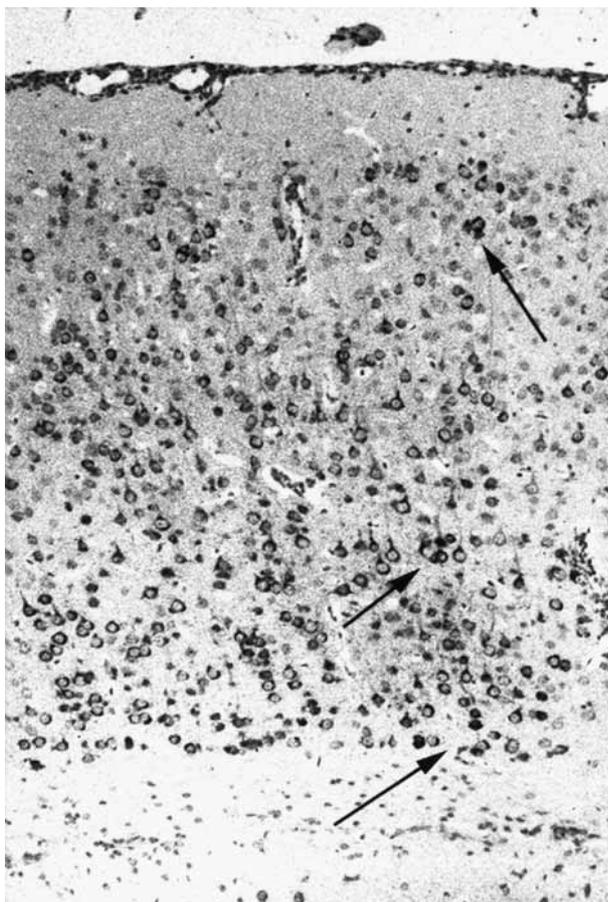


Fig. 3. Antigen-positive pyramidal cells (arrows) in the cerebral cortex on day 2 after i.c. inoculation. ABC. $\times 100$.

used for viral assay and two for immunohistochemistry (IHC).

IHC

The infected rats were perfused with phosphate-buffered saline (PBS) and Bouin's solution without acetic acid. Multiple sections of the brain and spinal cord were cut and embedded in paraffin wax. The virus-specific antigen was detected in sections by the avidin-biotin peroxidase complex (ABC) method, with a commercial ABC kit (Vector Laboratories, Burlingame, CA, USA). The sections were treated sequentially with normal goat serum, anti-HEV mouse serum (1 in 1000), biotinylated goat anti-mouse IgG, ABC reagent, and 3,3'-diaminobenzidine tetrahydrochloride 0.05% and H_2O_2 0.1% in Tris buffer, pH 7.6. The sections were counterstained with haematoxylin. The anti-HEV 67N mouse serum showed an antibody titre of 1028 by the haemagglutination inhibition test (Hirano *et al.*, 1990).

Results

Effect of Age and Route of Inoculation

The experimental design and results in rats aged 1, 4 and 8 weeks are shown in Table 1. In animals that survived until the experiment was terminated (14 days), no virus was detected in the brain by tissue culture or IHC. Control rats remained normal throughout.

Animals aged 1 week given doses ranging from 2×10^3 to 2×10^6 PFU by all routes except the oral died. (The i.v. route was not tested in rats of this age.) Doses ranging from 2 to 2×10^2 PFU usually resulted in death in the majority of animals inoculated. By the oral route, however, even 2×10^5 PFU produced only two deaths in five rats, and 2×10^4 PFU produced none. All deaths occurred within 3 to 7 days of inoculation.

The results in 2-week-old rats (not shown in Table 1) were generally similar to those described above. Doses of 2×10^3 PFU by the i.v. route killed three of five rats, but i.v. doses of 200 PFU were without effect. No deaths occurred in rats given 2×10^6 PFU by the oral route.

Rats aged 4 weeks appeared somewhat less susceptible. For example, doses of 2×10^3 PFU or less by the i.n., i.p., s.c. or i.v. route killed only a minority of animals. Moreover, the time to death ranged from 4 to 12 days.

Rats aged 8 weeks were still less susceptible. Thus, doses as large as 2×10^6 PFU by the i.p. and i.v. routes were without apparent effect, and doses of 2×10^4 PFU by the i.n. and s.c. routes killed only a proportion of rats. By the i.c. route, a dose of 2×10^3 PFU was still capable of killing all rats, but 200 PFU resulted in the death of only three of five animals. Deaths occurred within 4 to 11 days of inoculation, but the mean time to death was greater in rats inoculated by the i.n. route than in those given s.c. or i.c. inoculation.

Growth of Virus in Brain and Spinal Cord

On the basis of the results of the previous experiment, 4-week-old rats were inoculated by the i.c. route with 2×10^5 PFU of the virus. As shown in Fig. 1, the virus was first detected in the brain on day 1 after inoculation. The viral titres in the brain increased exponentially, reaching a maximum of more than 10^7 PFU/0.2 g on day 3 to 4, at which time CNS signs developed. The virus was first isolated from the spinal cord on day 2, and the infectivity titre had reached that of the brain by day 4. No virus was detectable in the liver, spleen or blood of rats examined on days 1 to 4.

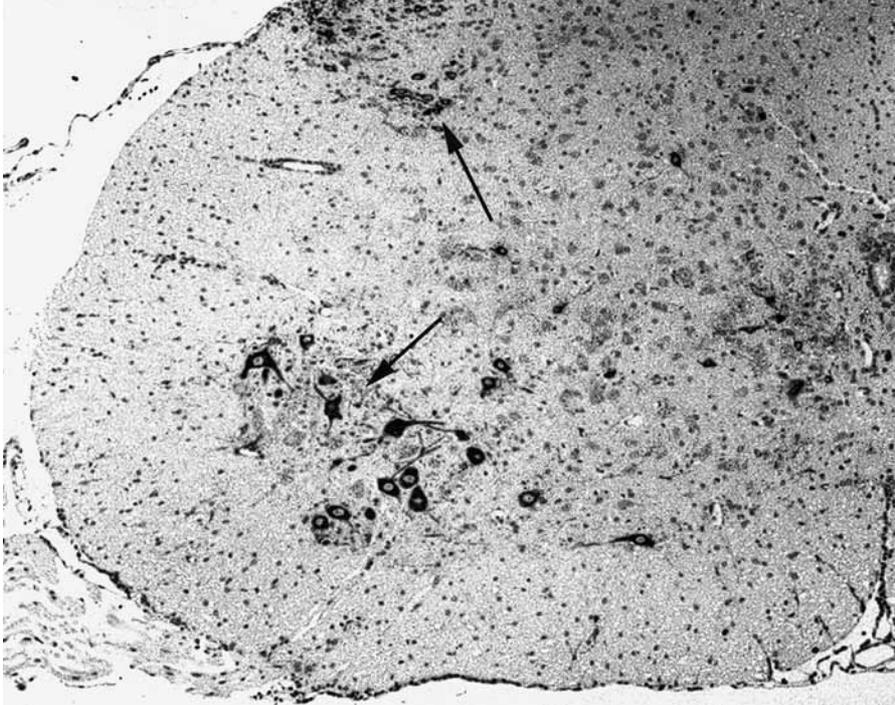


Fig. 4. Antigen-positive neurons (arrows) in the ventral and dorsal horns of the cervical spinal cord on day 3 after i.c. inoculation. ABC. $\times 150$.

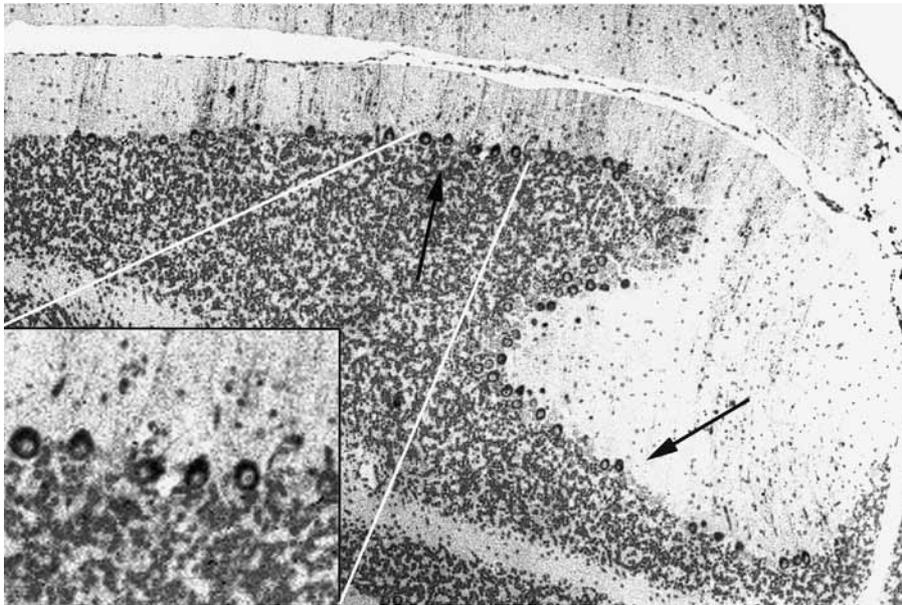


Fig. 5. Antigen-positive Purkinje cells (arrows) in the cerebellum on day 4 after i.c. inoculation. ABC. $\times 100$. Inset: Higher magnification of Purkinje cells. ABC. $\times 250$.

IHC

Virus-specific antigen was first detected as cytoplasmic labelling in a few pyramidal cells of the cerebral cortex and of the hippocampus on day 1 after inoculation. In the hippocampus, positive

neuron groups were found in patches as shown in Fig. 2. On day 2, viral antigen was found in the large-sized neurons in the pons. On days 2 to 3, the antigen-positive neurons increased in number and were distributed widely in the cerebral cortex and hippocampus. Fig. 3 shows many pyramidal

cells with viral antigen in the cerebral cortex on day 2. In the spinal cord, antigen-positive neurons were first found in ventral and dorsal horns in the cervical region. On day 3, antigen-positive cells were found in the thoracic and lumbar spinal cord, as well as in the cervical spinal cord (Fig. 4). On day 4, Purkinje cells in the cerebellum were also antigen-positive (Fig. 5). Virus-specific antigen was not detected in the glial cells, ependymal cells or choroid plexus, or in the liver or spleen.

Discussion

HEV infection in pigs has three possible consequences, namely, fatal encephalomyelitis in neonatal piglets (Mitchell, 1963), vomiting and wasting disease in piglets under 2 weeks old (Alexander and Saunders, 1969; Cartwright *et al.*, 1969; Kershaw, 1969) and symptomless infection in adults (Appel *et al.*, 1965). This suggests that host age plays a crucial role in the susceptibility of pigs to HEV infection, and in the manifestation of infection.

Yagami *et al.* (1986) demonstrated experimentally that mice aged 20 days or more survived after inoculation by the i.n., i.p. and s.c. routes, whereas 35-day-old mice succumbed to i.c. inoculation.

The present study in 1-, 2-, 4- or 8-week-old rats showed that susceptibility to infection by various routes (i.c., i.n., i.p., s.c., i.v. and oral) declined with age. The i.c. route was the most successful in producing disease, and the oral route the least. The age-related decline in susceptibility to infection by the oral route was similar to that observed by Hirano *et al.* (1975) with another murine coronavirus, mouse hepatitis virus (MHV). The explanation of this phenomenon may lie in the decrease in pH of the mouse stomach from 5.5 to 2.0 during the first 2 weeks of life.

After i.c. inoculation, HEV antigen was detected immunohistochemically mainly in the large-sized neurons in the brain and spinal cord but not in ependymal cells, choroid plexus or other glial cells, suggesting that viral spread in the CNS was not established via the central canal or the bloodstream.

In a previous study to define HEV 67N spread by neural pathways or the bloodstream from peripheral tissues to the CNS, rats were inoculated in the right hind footpad; fatal infection was aborted by cutting the right sciatic nerve within 6 h but not after 12 h (Hirano *et al.*, 1994). In addition, virus inoculated directly into the right sciatic nerve was recovered from the caudal half of the spinal cord on day 2, and from the rostral half of the spinal cord as well as the brain on day 3. In accordance with the results of viral isolation, HEV antigens

were found in neurons of the lumbar dorsal root ganglion and in the right side of the spinal cord at an early stage of infection; at a late stage of infection, specific antigen was found in the neurons of the cerebral cortex, hippocampus and brainstem, and in Purkinje cells of the cerebellum, but not in any glial cells, including ependymal cells and cells of the choroid plexus (Hirano *et al.*, 1998).

Our observations in rats indicate that, after i.c., footpad or sciatic nerve inoculation, HEV infects neurons of the CNS via neural pathways. Andries and Pensaert (1980a) detected HEV antigens in the respiratory tract and neurons of piglets after oro-nasal inoculation. Narita *et al.* (1989) also demonstrated HEV antigens in the neurons of the midbrain, thalamus and pons, and in the trigeminal ganglion, of piglets infected orally or intranasally.

The present and previous studies suggest that rats would be useful for studying the pathogenesis of HEV in pigs. Moreover, the neurotropic properties of HEV 67N indicate that this virus might be useful as a transsynaptic tracer for analysing neuronal connections in the CNS of rats, in the field of neuroscience.

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