ゲタウイルスKanagawa株のプラックサイズと乳のみマウスに対する病原性

誌名	Japanese journal of veterinary science
ISSN	00215295
著者	宝達, 勉 高橋, 伸和 井出, 誠弥
巻/号	52巻3号
掲載ページ	p. 519-526
発行年月	1990年6月

The Relation between Pathogenicity and Plaque Size of Getah Virus Kanagawa Strain in Suckling Mice

Tsutomu HOHDATSU, Nobukazu TAKAHASI[1)], Seiya IDE[2)], Hiroo YAMAGISHI[2)], Hirosi SAITO, Yujiro FUJISAKI[2)], and Hiroyuki KOYAMA[1)]

Department of Veterinary Microbiology, [1)]Veterinary Infectious Diseases, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034, and [2)]Research Center for Veterinary Science, The Kitasato Institute, Kashiwa, Chiba 277, Japan

(Received 4 September 1989/Accepted 16 January 1990)

ABSTRACT. The relation among biological properties, particularly pathogenicity for suckling mice, and plaque size was studied in four virus strains: Getah virus strain Kanagawa; two strains obtained by plaque cloning of the Kanagawa strain, Getah Kanagawa SP (G-K-SP) strain which forms small plaques (SP) only and strain G-K-LP which forms large plaques (LP) only; and strain Haruna which forms SP only. There were no marked differences among the four strains in serological properties, growth curves and sensitivity to pH, trypsin and temperature. Strain G-K-LP showed higher pathogenicity for suckling mice than strain G-K-SP. However, the pathogenicity of strain Haruna, which forms SP only, was as high as that of strain G-K-LP. Some of the clones in SP of strain Kanagawa kill all mice in 5 to 6 days after inoculation while the others required 9 to 11 days or longer before causing death. The present study showed that the pathogenicity of Getah viruses shortly after being isolated from the field, such as the Kanagawa strain, is different between large and small plaques, and even among small plaques, at least in suckling mice, and that the pathogenicity has no relation to plaque size.—KEY WORDS: Getah virus, pathogenicity, plaque size.

Jpn. J. Vet. Sci. 52(3): 519–526, 1990

Antibodies to Getah viruses have been detected in various animals [3, 4, 15, 18]. Reports on pathogenicity or infectivity of the viruses, however, have been limited to horses [8, 11, 16] and mice [12, 17]. More recently, Getah viruses have been reported to be pathogenic for newborn piglets [9, 13, 19] and possibly to induce abortion or stillbirth in pregnant sows [7].

It is also noted that Getah viruses form large plaques (LP) and small plaques (SP) by spontaneous variation [1, 10]. In our experiment to induce plaque formation in the Kanagawa strain (brain emulsion subcultured in HmLu-1 cells for three passages) isolated from the brain of a newborn piglet which died with depression, tremor and yellowish-brown diarrhea, LP and SP appeared at a ratio of 1:100. Isolation of LP and SP was then undertaken by plaque cloning. Although it has not been clarified whether there is any difference in pathogenicity between LP and SP in horses and pigs, LP-forming strains have been reported to be more pathogenic for suckling mice than SP-forming strains [1, 10].

In previous reports [2, 5, 6] on viruses such as TGE virus, correlation was found between pathogenicity and *in vitro* properties in the virulent and attenuated TGE viruses. High-passage, cell-culture-attenuated TGE virus strain had higher sensitivity to trypsin and to acidity than the low-passage, virulent virus.

In the present study, the authors examined the pathogenicity of the isolated LP and SP strains, the original Kanagawa strain and the Haruna strain [14], which forms small plaques only, in suckling mice, and further investigated the relation between

pathogenicity and plaque size. The pathogenicity for suckling mice was also examined in relation to biological properties other than plaque size (serological properties, growth curve and sensitivity to pH, trypsin and temperature).

MATERIALS AND METHODS

Cell cultures: The HmLu-1 cell line (derived from hamster lung tissues) and the BHK-21 cell line (derived from hamster kidney tissues) were grown in Eagle's minimum essential medium (MEM) containing 10% newborn calf serum, 0.3% tryptose phosphate broth (TPB of Difco, Laboratory, Detroit, U.S.A.), 100 units/m*l* penicillin and 100 μg/m*l* streptomycin. The maintenance medium was MEM containing 2% newborn calf serum, 3% TPB and antibiotics as above.

Virus: The viruses used were strain Kanagawa [19], which was isolated from the brain of dead piglet and strain Haruna [14], which was isolated from the blood of a spontaneously infected swine. The Kanagawa strain (brain homogenate of dead piglet), provided by Kanagawa Livestock Diagnostic Laboratory of Japan, was passaged 3 times in HmLu-1 cell cultures in our laboratory. The Haruna strain provided by National Institute of Health of Japan, at the level of 7th passage in suckling mouse brain, was further passaged 3 times in HmLu-1 cell cultures in our laboratory. These viruses were stored at −80°C until use.

Preparation of antisera: This was preformed by the method described previously [10]. For the preparation of immune sera, mice and guinea pigs were immunized by four intraperitoneal injections at weekly intervals with 10^5 plaque forming units (PFU)/m*l* of each virus in the infected culture fluid. Animals were bled by cardiac puncture 14 days after the last injection.

Plaque assay and virus titration: Confluent BHK-21 cell monolayers in 60-mm plastic petri dishes (Corning Glass Works, Corning, U.S.A.) were inoculated with 0.2 m*l* of each virus dilution (serial tenfold dilutions) in maintenance medium employing 4 dishes per dilution. After virus adsorption at 37°C for 60 min, the inoculated cultures were covered with 5 m*l* of agar overlay medium which was consisted of 1% Bacto agar (Difco Laboratories, Detroit, U.S.A.) in meintenance medium. The cultures were incubated in a CO_2 incubator at 37°C for 3 days, and stained by incubating at 37°C for 12 hrs under a second overlay medium containing 0.01% neutral red. The plaque size was measured by slide calipers.

The virus titration was performed by the plaque method, and the infectious titer was expressed in plaque-forming units (PFU).

Growth studies at different temperatures: Monolayer cultures of HmLu-1 cells in 24-well multiplates (Corning Glass Works, Conring, U.S.A.) from which the growth medium had been removed were inoculated with viruses at a multiplicity of infection of approximately 2.0. After incubation at 37°C for 1 hr, they were washed 3 times with phosphate buffered saline (PBS-). To each monolayer was then added 1 m*l* of maintenance medium and incubated at a given temperature (32, 37 or 41°C). At various intervals thereafter, specimens of fluid were harvested from three wells, pooled, and centrifuged at 1,500 g for 10 min. The supernatant fluids were stored at −80°C, and assayed for infectivity at the completion of the growth curve.

Treatment of trypsin: This was performed by the method described previously [5]. Trypsin (Difco, 1:250) was made to 1.0% solution in PBS-. The virus was diluted 1:9 with PBS-. Equal volume of the diluted virus and trypsin solution was mixed, shaken, and incubated at 37°C for 2 hr. The sample was mixed with an equal volume of culture medium containing 50% fetal calf

serum, and then titrated for infectivity.

Sensitivity to pH: This was performed by the method described previously [5]. Tris (hydroxymethyl)-aminomethan was added to a tissue culture medium without serum to give a final concentration of 0.01M. The pH was adjusted to 3.0, 5.0 and 7.0 by the addition of 1N HCl. The virus culture fluid was diluted 1:10 in the medium adjusted to the respective pH. These samples were adjusted again to pH 3.0, 5.0 and 7.0, respectively and allowed to stand at 22°C for 3 hr. The pH was then adjusted to neutrality, and titrated for infectivity.

Temperature sensitivity: This was performed by the method described previously [11]. Virus culture fluid was incubated at 37°C and 56°C for 1 hr in a stirring water bath and then titrated for infectivity.

Virus neutralization (VN) test: Virus neutralization test was carried out in the presence of complement by the microtiter method. In wells of a transfer plate, 25 μl of each of serial twofold dilutions of the antiserum inactivated at 56°C for 30 min was mixed with 25 μl of maintenance medium containing 200 tissue culture infectious dose $(TCID)_{50}$ of virus and 25 μl of fresh guinea pig serum diluted 1:5. Two wells were employed for each antiserum dilution. The mixtures were incubated at 37° for 60 min, transferred into wells of a microplate containing BHK-21 cell cultures, and incubated in an atmosphere of 5% CO_2 in air at 37°C for 5 days. The antibody titer was expressed as the reciprocal of the serum dilution showing no cytopathic effect in at least one of the two wells.

Virus inoculation of suckling mice: One or two day-old suckling mice (ddY) were used to assay for virulence. Serial 10-fold dilutions of the viruses were prepared and inoculated intracerebrally into suckling mice. One litter of suckling mice was used for each virus dilution, with each mouse receiving 0.025 m*l* of each suspension. The suckling mice were observed for 14 days for signs of disease and death.

RESULTS

Plaque cloning to obtain LP and SP strains from strain Kanagawa: In plaque formation following inoculation of strain Kanagawa into BHK-21 cells, LP (about 4.0 mm in diameter) and SP (about 1.0 mm in diameter) appeared at a ratio of about 1:100. This ratio remained unchanged after seven-generation subculture in HmLu-1 cells. LP-forming or SP-forming clones were separately obtained by three clonings of the collected plaques of each type. The clones which formed LP only were designated as strain G-K-LP, and those forming SP only as strain G-K-SP. The G-K-LP and G-K-SP strains did not change in their plaque size even after subculture for at least three passages *in vitro* following three plaque clonings.

Growth curves of Getah viruses at different temperatures: Growth curves of the Kanagawa, G-K-LP, G-K-SP and SP-forming Haruna strains were compared at cultivation temperatures of 32°C, 37°C and 41°C. The virus titer of the four strains reached the maximum level in 12 to 24 hr, and gradually decreased thereafter (Fig. 1). All strains showed similar curves at all temperatures.

Sensitivity of Getah viruses to pH, trypsin and temperature: Table 1 shows the sensitivity of each strain to pH, trypsin and temperature. The titers of all strains were reduced by treatment with trypsin or by heating at 56°C. There were no marked differences among the strains. In the test for sensitivity to pH 3.0, strain G-K-LP was found to be more resistant than the other three strains. The difference, however, was not statistically significant.

Cross neutralization test with Haruna, Kanagawa, G-K-LP and G-K-SP strains of

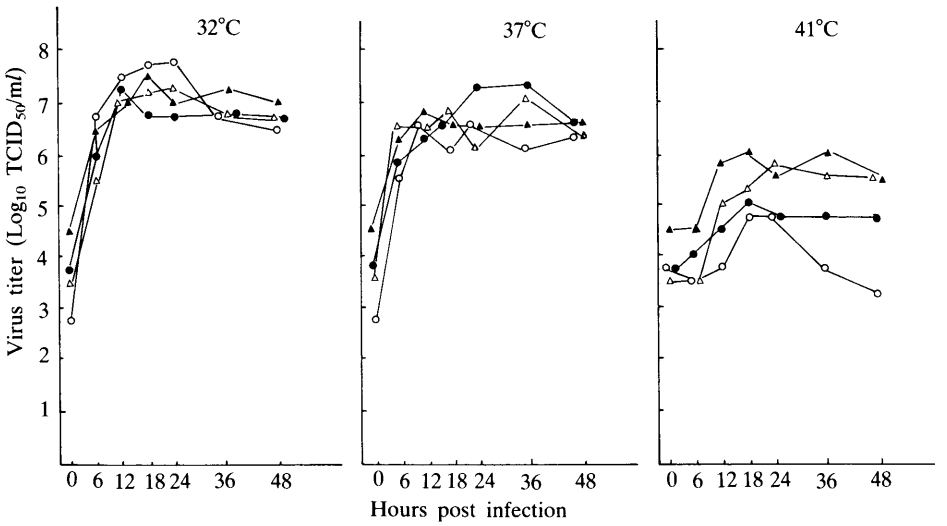

Fig. 1. Growth curves of Getah viruses at different temperatures.
○—○: Strain Haruna
●—●: Strain Kanagawa
△—△: Strain G-K-LP
▲—▲: Strain G-K-SP

Table 1. Sensitivity to pH, trypsin and temperature of Getah viruses

Experiment		Virus strain							
		Haruna		Kanagawa		G-K-LP		G-K-SP	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated
Sensitivity to pH[a)]	pH 7.0	6.00[d)]	5.25	5.75	5.75	6.25	6.00	6.25	5.75
	pH 5.0	6.00	4.25	5.75	5.50	6.25	5.75	6.25	4.50
	pH 3.0	6.00	2.50	5.75	3.25	6.25	4.50	6.25	3.25
Treatment of trypsin[b)]		5.00	2.50	5.25	2.00	5.25	3.25	6.00	2.75
Sensitivity to temperature[c)]	37°C	6.75	6.75	7.00	6.50	6.75	6.50	6.50	6.50
	56°C	6.75	2.00	7.00	2.00	6.75	1.75	6.50	2.75

a) Incubated at 22°C for 3 hr.
b) Incubated at 37°C for 2 hr.
c) Incubated for 1 hr.
d) Virus titer (Log $TCID_{50}$/m*l*).

Getah virus: To determine antigenic differences in the four strains, a cross neutralization test was performed with immune sera of guinea pigs or mice in the presence of complement. As shown in Table 2, the homologous neutralization titers were 2 to 4 times greater than the heterologous titers, suggesting serological cross reaction.

Pathogenicity of Getah viruses for suckling mice: Virus suspensions of each strain were prepared at the levels of 10^5 PFU/m*l* to 10^{-1} PFU/m*l* and inoculated into the brains of suckling mice. The results are shown in Table 3. Inoculation of the SP-forming

Table 2. Cross neutralization test with Haruna, Kanagawa, G-K-LP and G-K-SP strains of Getah virus

Antiserum		Virus strain			
		Haruna	Kanagawa	G-K-LP	G-K-SP
Haruna	Guinea pig	640[a)]	160	80	160
	Mouse	160	160	80	160
Kanagawa	Guinea pig	640	1280	640	640
	Mouse	160	320	160	160
G-K-LP	Mouse	80	320	160	160
G-K-SP	Mouse	40	160	40	160

a) Neutralization titer.

Table 3. Pathogenicity of Getah viruses for suckling mouse

Virus strain	PFU/ml (Log_{10})							Mean death time (day)
	5	4	3	2	1	0	−1	
Haruna (1.0mm)[a)]	ND[b)]	ND	12/12[c)]	9/10	6/15	3/14	0/12	5.3
Kanagawa LP : SP=1 : 100 (LP=4.1mm) (SP=1.0mm)	ND	ND	11/11	6/6	2/7	1/14	0/4	10.5
G-K-LP (4.9mm)	11/11	15/15	15/15	19/19	9/11	0/8	0/11	3.4
G-K-SP (1.0mm)	7/13	0/15	0/9	0/13	0/15	ND	ND	12.6

a) Plaque size.
b) ND=not done.
c) Number of dead mice/number of inoculated.

Haruna strain killed about 40% of the mice at approximately 10 PFU/m*l*, and the mean death time was 5.3 days. The Kanagawa strain killed about 30% at 10 PFU/m*l*, and the mean death time was 10.5 days, about twice as long as that for animals injected with strain Haruna. About 80% of mice were killed by inoculation with strain G-K-LP at 10 PFU/m*l*. The mean death time was 3.4 days. Strain G-K-SP required a virus level of 10^5 PFU/m*l* to kill about 50% of the inoculated mice, and the mean death time was about 12.6 days. Strain G-K-SP was less pathogenic for suckling mice than the other three strains, being similar to strain Kanagawa in terms of death time.

Pathogenicity of LP and SP clones of Getah virus for suckling mice: The pathogenicity of strains G-K-LP and G-K-SP, which were obtained from the Kanagawa strain by plaque cloning, for suckling mice was consistent with findings reported previously [10, 12, 14, 17]. Strain LP showed higher pathogenicity than SP. However, the Haruna strain, which formed SP only, was also found to be highly

Table 4. Pathogenicity of LP and SP clones of Getah virus for suckling mouse

Virus strain	Plaque form	Clone NO	Days after inoculation 2	3	4	5	6	7	8	9	10	11	12	13	14
Haruna	Small	H-2	0/6[a)]	4/6	6/6										
		H-3	0/5	0/5	5/5										
		H-5	0/12	0/12	12/12										
		H-7	0/9	0/9	4/9	9/9									
		H-8	0/9	0/9	0/9										
		H-9	0/9	0/9	9/9										
Kanagawa	Large	L-3	0/7	1/7	4/7	7/7									
		L-6	0/7	0/7	0/7	2/7	7/7								
		L-8	0/8	1/8	7/8	8/8									
	Small	S-1	0/9	0/9	0/9	0/9	0/9	2/9	6/9	6/9	8/9	9/9			
		S-2	0/8	0/8	6/8	8/8									
		S-3	0/11	0/11	3/11	7/11	11/11								
		S-5	0/11	0/11	0/11	0/11	1/11	6/11	6/11	7/11	8/11	9/11	9/11	9/11	10/11
		S-9	0/9	0/9	0/9	0/9	1/9	2/9	2/9	5/9	8/9	9/9			
		S-9	0/9	0/9	0/9	0/9	0/9	0/9	4/9	9/9					

a) Number of dead mice/number of inoculated.

pathogenic, inducing an acute infection with shorter mean death time (Table 3). In an attempt to explain this discrepancy, the following experiment was carried out.

Several plaques each from the Haruna strain (SP) and the Kanagawa strain (SP and LP) were subjected to plaque cloning once and subcultured in BHK-21 cells. After confirming that the resulting clone viruses formed SP or LP only, clones of each type were inoculated into the brains of suckling mice at 1×10^5 PFU/m*l*. The animals were observed until death for differences in pathogenicity. As shown in Table 4, all of the SP clones from strain Haruna and LP clones from strain Kanagawa required 4 to 6 days to kill the inoculated mice, and no difference was found among these clones. In contrast, SP clones from strain Kanagawa showed variations in pathogenicity, falling into two groups: clones requiring 9 to 11 days or longer to kill all the animals and the others taking only 5 to 6 days. The death times for the latter were comparable to those for LP clones of the same strain and SP clones of the Haruna strain.

DISCUSSION

The Kanagawa strain, the G-K-SP and G-K-LP strains were compared for biological properties, particularly pathogenicity for suckling mice. Chanas *et al.* [1] and Kimura and Ueba [10] reported that LP of Getah virus has higher pathogenicity for suckling mice than SP. In the present study, strain Haruna, which formed SP only and was thought to be less pathogenic, was used as a control in addition to the above three strains. Contrary to our expectation, strain Haruna was found to be highly pathogenic for suckling mice, causing an acute infection after inoculation. As reported previously [1, 10], strain G-K-LP which was obtained from cloning of strain Kanagawa showed high pathogenicity, while strain G-K-SP was less pathogenic than the other strains (Table 3). Since strain G-K-SP used in this study may have incidentally consisted of less pathogenic clones of the Kanagawa strain, several plaques each from the Kanagawa (SP and LP) and Haruna (SP) strains were further subjected to single cloning, and their

pathogenicity was compared. As shown in Table 4, some SP clones of the Kanagawa strain induced a course similar to those induced by LP clones, although SP clones mostly showed low pathogenicity. Both SP clones of strain Haruna and LP clones of strain Kanagawa consistently showed high pathogenicity. The above results suggest that pathogenicity for suckling mice is not necessarily correlated with plaque size and that there are a number of SP that show as high pathogenicity as LP.

In contrast to previous reports [2, 5, 6] on viruses such as TGE virus, no correlation was found between pathogenicity and growth curve or sensitivity to pH and trypsin in the four strains of Getah virus used for the present study. There was no marked difference in serological properties as well.

Death time of animals inoculated with strain Kanagawa was similar to that after strain G-K-SP inoculation. This may be due to the large population of SP (LP/SP ratio, 1:100) in the Kanagawa strain. In the Haruna strain, which is a Getah virus derived from swine, as well as strain Kanagawa, the LP/SP ratio shortly after isolation has not been determined yet. It has been reported that a mixture of LP and SP is also present in strain Haruna [1]. The possible mechanism involved in development of a strain to form highly pathogenic SP alone is an interesting question. During the process of virus passage by intracerebral inoculation in suckling mice, the highly pathogenic clones, such as S-2 and S-3, in SP of the Kanagawa strain (Table 4) may have been selectively maintained.

In the reports on the pathogenicity of Getah viruses in horses, mice and pigs, there is no statement about plaque size or LP/SP ratio of the viruses used. The LP/SP ratio of Getah viruses in the field remains unknown, and possible changes in pathogenicity with changes in the ratio are yet to be demonstrated. It was shown that in the viruses soon after isolation from the field, such as the Kanagawa strain, pathogenicity at least for suckling mice differed between large plaques and small plaques, and even among small plaques.

REFERENCES

1. Chanas, A. C., Johnson, B. K., and Simpson, D. I. H. 1977. A comparative study of related Alphaviruses-a naturally occurring model of antigenic variation in the Getah sub-group. *J. Gen. Virol.* 35: 455–462.
2. Chen, K. S. 1985. Enzymatic and acidic sensitivity profiles of selected virulent and attenuated transmissible gastroenteritis viruses of swine. *Am. J. Vet. Res.* 46: 632–636.
3. Doherty, R. L. 1972. Arboviruses of Australia. *Aust. Vet. J.* 48: 172–180.
4. Doherty, R. L., Gorman, B. M., Whitehead, R. H., and Carley, J. G. 1966. Studies of arthropod-borne virus infections in man and other animals. *Aust. J. Exp. Biol. Med. Sci.* 44: 365–378.
5. Furuuchi, S., Shimizu, Y., and Kumagai, T. 1975. Comparison of properties between virulent and attenuated strains of transmissible gastroenteritis virus. *Natl. Inst. Anim. Health Q. (Jpn.)* 15: 159–164.
6. Hess, R. G. and Bachmann, P. A. 1976. In vitro differentiation and pH sensitivity of field and cell culture-attenuated strains of transmissible gastroenteritis virus. *Infect. Immun.* 13: 1642–1646.
7. Izumida, A., Takuma, H., Inagaki, S., Kubota, M.,Hirahara, T., Kodama, K., and Sasaki, N. 1988. Experimental infection of Getah virus in swine. *Jpn. J. Vet. Sci.* 50: 679–684.
8. Kamada, M., Ando, Y., Fukunaga, Y., Imagawa, H., Wada, R., Kumanomido, T., Tabuchi, E., Hirasawa, K., and Akiyama, Y. 1981. Studies on Getah virus-pathogenicity of the virus for horses. *Bull. Equine Res. Inst.* 18: 84–93.
9. Kawamura, H., Yago, K., Narita, M., Imada, T., Nishimori, T., and Haritani, M. 1987. A fatal case in newborn piglets with Getah virus infection: pathogenicity of the isoalte. *Jpn. J. Vet. Sci.* 49: 1003–1007.
10. Kimura, T. and Ueba, N. 1987. Some biological and serological properties of large and small plaque variants of Getah virus. *Arch. Virol.* 57: 221–229.
11. Kono, Y., Sentsui, H., and Ito, Y. 1980. An epidemic of Getah virus infection among racehorses: properties of the virus. *Res. Vet. Sci.* 29: 162–167.
12. Kumanomido, T., Wada, R., Kanemaru, T.,

Kamada, M., Akiyama, Y., and Matumoto, M. 1988. Transplacental infection in mice inoculated with Getah virus. *Vet. Microbiol.* 16: 129–136.
13. Kumanomido, T., Wada, R., Kanemaru, T., Kamada, M., Hirasawa, K., and Akiyama, Y. 1988. Clinical and virological observations on swine experimentally infected with Getah virus. *Vet. Microbiol.* 16: 295–301.
14. Matsuyama, T., Nakamura, T., Isahai, K., Oya, A., and Kobayashi, M. 1968. Haruna virus a group A arbovirus isolated from swine in Japan. *Virus* 18: 11–14 (in Japanese with English summary).
15. Sanderson, C. J. 1969. A serologic servey of Queensland cattle for evidence of arbovirus infection. *Am. J. Trop. Med. Hyg.* 18: 433–439.
16. Sentsui, H. and Kono, Y. 1980. An epidemic of Getah virus infection among racehorses: isolation of the virus. *Res. Vet. Sci.* 29: 157–161.
17. Sentsui, H. and Kono, Y. 1981. Pathogenicity of Getah virus for mice. *Natl. Inst. Anim. Health Q. (Jpn.)* 21: 7–13.
18. Stanly, N. F. and Choo, S. B. 1964. Studies of arboviruses in Western Australia. Serdogical epidemiology. *Bull. WHO* 30: 221–226.
19. Yago, K., Hagiwara, S., Kawamura, H., and Narita, M. 1987. A fatal case in newborn piglets with Getah virus infection: isolation of the virus. *Jpn. J. Vet. Sci.* 49: 989–994.

要　約

ゲタウイルス Kanagawa 株のプラックサイズと乳のみマウスに対する病原性：宝達　勉・高橋伸和[1]・井出誠弥[2]・山岸郭郎[2]・斉藤　博・藤崎優次郎[2]・小山弘之[1]（北里大学獣医畜産学部微生物学講座，[1]伝染病学講座，[2]北里研究所附属家畜衛生研究所）――ゲタウイルス Kanagawa 株，Kanagawa 株からプラッククローニングして得た SP のみを形成するゲタ Kanagawa SP (G-K-SP) 株，LP のみを形成するG-K-LP 株および SP だけを形成する Haruna 株の 4 株について，それぞれの生物学的性状なかでも特に乳のみマウスに対する病原性とプラックサイズとの関係を調べた．プラックサイズあるいは乳のみマウスに対する病原性と血清学的性状，増殖曲線ならびに pH，トリプシン，温度に対する感受性には顕著な差はなかった．乳のみマウスに対して G-K-LP 株はG-K-SP 株に比べ強い病原性を示した．しかし，SP のみを形成するにもかかわらずHaruna株の病原性はG-K-LP株と同様に強かった．Kanagawa 株の SP の中にも 5 日から 6 日ですべてのマウスを死亡させるクローンと，9 日から14日以上を必要とするクローンが存在した．野外から分離されてまもない Kanagawa 株のようなゲタウイルスでは，少なくとも乳のみマウスに対する病原性は LP と SP あるいは SP 間でも差があり，プラックサイズとは無関係であることが明らかとなった．