



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

## Antigenic Variation of Porcine Transmissible Gastroenteritis Virus Detected by Monoclonal Antibodies

T. HOHDATSU, Y. EIGUCHI, M. TSUCHIMOTO, S. IDE, H. YAMAGISHI and M. MATUMOTO<sup>1</sup>

*Research Center for Veterinary Science of Kitasato Institute, 1139-1 Matsugasaki, Kashiwa, Chiba 277 (Japan)*

<sup>1</sup>*Virology Department, Research Division, Kitasato Institute, Minato-ku, Tokyo 108 (Japan)*

(Accepted for publication 16 December 1986)

### ABSTRACT

Hohdatsu, T., Eiguchi, Y., Tsuchimoto, M., Ide, S., Yamagishi, H. and Matumoto, M., 1987. Antigenic variation of porcine transmissible gastroenteritis virus detected by monoclonal antibodies. *Vet. Microbiol.*, 14: 115-124.

Mouse myeloma cells (SP2/O) were fused with spleen cells from BALB/c mice immunized with detergent-solubilized antigen of purified virus, and 21 monoclonal (MC) antibodies reactive in enzyme-linked immunosorbent assay with the TO-163 strain of porcine transmissible gastroenteritis (TGE) virus were obtained. Of these MC antibodies, 14, 6 and 1 were IgG1, IgG2a and IgM, respectively. All of the MC antibodies contained light chains of the kappa type. Of these MC antibodies, 8 were found to have neutralization (NT) activity against the TO-163 strain. Comparison of 7 strains of TGE virus by NT tests using our panel of MC antibodies confirmed their close antigenic relationships, but also revealed the occurrence of distinct antigenic differences. These results suggest that there may be at least 6 different epitopes involved in NT reaction on the virion of the TO-163 strain. This notion was confirmed by the competitive binding assay.

### INTRODUCTION

Transmissible gastroenteritis (TGE) is a highly infectious viral disease of pigs, causing a high mortality rate in young piglets. Minor strain differences between TGE viruses have been shown by neutralization tests with polyclonal antibodies (Harada et al., 1967; Kemeny, 1976). Monoclonal (MC) antibodies have proved an extremely useful addition to the field of immunology (Köhler and Milstein, 1975; Blann, 1979): since they react with only one antigenic determinant (epitope) they are ideal reagents for studying antigenic structure and variation.

In the present study, MC antibodies were raised to TGE virus, strain TO-163, and used to examine antigenic variation of the virus.

## MATERIALS AND METHODS

### *Cell cultures*

The CPK cell line derived from swine kidney (Komaniwa et al., 1981) was grown in Eagle's minimum essential medium (MEM) containing 10% calf serum, 10% tryptose phosphate broth (TPB) (Difco, U.S.A.), 100 units ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> streptomycin. The maintenance medium was MEM containing 10% TPB, 0.11% bovine serum albumin and antibiotics as above. Primary swine kidney cells dispersed by trypsin were grown and maintained in the same culture media as used for CPK cells.

### *Viruses*

The TGE virus strains used were TO-163 (Harada et al., 1967; Furuuchi et al., 1975), h-5, Kanagawa (Harada et al., 1967), Purdue (Laude et al., 1981), Ukiha, Shizuoka (Harada et al., 1967) and Miller (Bohl and Cross, 1971). The TO-163, Kanagawa, Purdue, Shizuoka and Miller strains were provided by the National Institute of Animal Health of Japan. The h-5 and Ukiha strains were obtained from commercially available vaccines and had been maintained by serial passage in primary swine kidney cell cultures. After two or three passages in these cultures in our laboratory, the strains were stored at -80°C. Infectivity assay was carried out in microplate cultures of CPK cells by the method described previously (Yamagishi et al., 1981). The titer was expressed in 50% tissue culture infectious dose (TCID<sub>50</sub>).

### *Preparation of antiserum against TO-163 strain*

The antiserum was prepared in specific pathogen-free pigs, 2 months of age. The animals received two intramuscular doses of 10<sup>8</sup> TCID<sub>50</sub> each at intervals of 2 months. Serum was obtained 10 days after the second inoculation.

### *Preparation of virus antigen*

The antigen was prepared with the TO-163 strain grown in CPK cell cultures. Infectious culture fluid concentrated about 10-fold by ammonium sulfate precipitation was layered onto a discontinuous sucrose density gradient (20-60%) in a SW 28 rotor (Beckman) and centrifuged at 27 000 r.p.m. for 2 h. The virus bands formed were collected, mixed with an equal volume of fluorocarbon (Wako Pure Chemicals, Japan) and vigorously shaken for 2 min.

The water phase was separated by centrifugation and layered onto a linear sucrose density gradient (20–60%) in a SW 28 rotor and centrifuged at 27 000 r.p.m. for 4 h. Fractions with densities of 1.18–1.20 g ml<sup>-1</sup> were pooled and centrifuged at 77 000×g for 1 h. The pellets were resuspended in a 1/500 volume of NTE buffer (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA). The suspension was treated with 1% Triton X-100 for 1 h at 4 °C and dialyzed against phosphate buffered saline (PBS) (0.1 M NaCl, 0.01 M phosphate buffer, pH 7.2). The resulting material was used for mouse immunization and enzyme-linked immunosorbent assay (see below).

#### *Production of antibody-secreting hybridomas*

BALB/c mice, about 5 weeks of age, were inoculated intraperitoneally with a mixture of 10 µg of the viral antigen prepared as above and 10<sup>9</sup> cells of pertussis adjuvant. Four or 6 weeks later the mice received an intravenous booster dose of 10 µg of viral antigen, and spleen cells were obtained for fusion 3 days later. The fusion was carried out using essentially the same method as that described by Köhler and Milstein (1975). Polyethyleneglycol-1000 (Wako Pure Chemicals, Japan) was used as a fusing agent and the ratio of mouse spleen cells and mouse myeloma cells (SP2/O) was 10:1. The selective medium contained hypoxanthine (10<sup>-4</sup> M), aminopterin (4×10<sup>-7</sup> M) and thymidine (1.6×10<sup>-5</sup> M). The fused cells at a concentration of 3.5×10<sup>6</sup> spleen cells per ml were dispensed in 100 µl volumes into wells of 96-well, flat-bottomed microplates (Corning Glass Works, Corning, NY) and incubated at 37 °C in a humid atmosphere containing 5% CO<sub>2</sub>. After incubation for 2 weeks, the wells were examined and those which contained hybridoma cultures were tested for TGE specific antibody by an enzyme-linked immunosorbent assay (see below). The colonies in antibody positive wells were passaged in 24-well multiplates (Corning Glass Works, Corning, NY) and incubated in medium containing hypoxanthine (10<sup>-4</sup> M) and thymidine (1.6×10<sup>-5</sup> M). The cells were then cloned twice by the soft agar method.

#### *Production of MC antibodies in ascitic fluids*

BALB/c mice were injected intraperitoneally with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane) (Aldrich, U.S.A.) 14 days before intraperitoneal inoculation of 2×10<sup>6</sup> hybridoma cells. Ten days later the ascitic fluids were collected, clarified by centrifugation and stored at -80 °C until use.

#### *Determination of antibody class and subclass*

The supernatant fluids of antibody-secreting hybridoma cultures were concentrated 10-fold by 50% saturation of ammonium sulfate and used for deter-

mination of antibody class and subclass by double diffusion in a 1% agar gel containing 0.1%  $\text{NaN}_3$ . Rabbit antisera against mouse immunoglobulins, IgG1, IgG2a, IgG2b, IgG3, IgM and IgA, and kappa and lambda chains (Miles Laboratories, U.S.A.) were placed in the center wells and test samples were added to adjacent wells. The plates were incubated overnight at room temperature in a humidified chamber.

#### *Enzyme-linked immunosorbent assay (ELISA)*

Virus antigen (see above) was appropriately diluted with carbonate buffer (0.05 M, pH 9.6) and delivered in 100  $\mu\text{l}$  volumes into wells of 96-well, flat-bottomed Microelisa plates (Dynatech. Lab., U.S.A.). The plates were allowed to stand overnight at 4 °C, washed with 0.85% NaCl solution containing 0.02% Tween-20, and 100  $\mu\text{l}$  of the test sample was added to each well. Horseradish peroxidase-conjugated rabbit antibodies against mouse immunoglobulins were diluted to the optimal concentration with PBS containing 10% calf serum and 0.05% Tween-20 and 100  $\mu\text{l}$  of the dilution was added to each well of the plates. After incubation at 37 °C for 30 min, each well received 100  $\mu\text{l}$  of substrate solution and incubated at 25 °C for 20 min in a dark room. The substrate solution was prepared by dissolving O-phenylenediamine dihydrochloride at a concentration of 0.4 mg ml<sup>-1</sup> in 0.1 M citric acid-0.2 M  $\text{Na}_2\text{HPO}_4$  buffer (pH 4.8) and adding 0.2  $\mu\text{l}$  ml<sup>-1</sup> of 30%  $\text{H}_2\text{O}_2$ . After completion of the incubation, the reaction was stopped with 3 N  $\text{H}_2\text{SO}_4$  solution and the optical density (OD) at 492 nm was determined.

#### *Neutralization (NT) test*

NT tests were carried out by the antibody dilution method in CPK cell cultures prepared in flat-bottomed microplates (Corning Glass Works, Corning, NY). 25  $\mu\text{l}$  of each of serial 2-fold dilutions of the antibody inactivated at 56 °C for 30 min were mixed with 25  $\mu\text{l}$  of maintenance medium containing 200 TCID<sub>50</sub> of virus in wells of a transfer plate. Four wells were employed for each antibody dilution. The virus-antibody mixtures were incubated at 37 °C for 60 min, transferred into wells of a microplate containing CPK cell cultures, and incubated in an atmosphere of 5%  $\text{CO}_2$  in air at 37 °C for 5 days. The antibody titer was expressed as the reciprocal of the antibody dilution showing 50% NT calculated by the method of Kärber.

#### *Peroxidase-labeled MC antibody*

This was prepared by the method of Nakane and Kawaoi (1974). MC antibodies from ascitic fluid were purified by 3 cycles of precipitation with  $(\text{NH}_4)_2\text{SO}_4$  at 35% saturation. Ten mg of the purified MC antibody was mixed

with 4 mg of horseradish peroxidase oxidized with 0.1 M NaIO<sub>4</sub> in 0.01 M carbonate buffer (pH 9.6) at room temperature for 2 h. After addition of 4 mg NaBH<sub>4</sub> solution and incubation at 4 °C for 2 h, the mixture was dialyzed against 0.01 M PBS (pH 7.2). The enzyme conjugated antibody was separated from the enzyme and unconjugated antibody on a Sephadex G-200 column.

### *Competitive binding assay*

This was carried out by a modified ELISA (Kimura-Kuroda and Yasui, 1983). One hundred  $\mu$ l of serial 10-fold dilutions of each competing MC antibody from ascitic fluid were added to wells of a flat-bottomed Microelisa plate coated with virus antigen (see above), and incubated at 37 °C for 60 min. After three washings with 0.85% NaCl solution containing 0.02% Tween-20, 100  $\mu$ l of peroxidase-labeled MC antibody (see above) diluted to an OD of 1.0 was added. The competitive binding was estimated from the ODs in the presence or absence of unlabeled competing antibodies. The percentage of competition was determined by the formula:  $100(A - n)/(A - B)$ , where  $A$  is the OD in the absence of competing antibody,  $B$  is the OD in the presence of homologous antibody ( $10^4$  ELISA units), and  $n$  is the OD in the presence of competitor.

## RESULTS

### *Isolation and characterization of MC antibodies*

In two fusion experiments, hybridoma cultures were obtained in 152 of 632 inoculated wells. Antibody-positive colonies were cloned twice in soft agar, and 21 clones secreting MC antibody were finally obtained. Of these cloned cells, 14, six and one produced IgG1, IgG2a, and IgM antibodies, respectively. All of these MC antibodies had light chains of the kappa type (Table I).

### *Reactivity of MC antibodies to the TO-163 strain*

The reactivity of the MC antibodies to the TO-163 strain of TGE virus was examined by NT and ELISA using the mouse ascitic fluid and the culture fluid concentration 10-fold by 50% saturation of ammonium sulfate. As shown in Table I, eight of the 21 MC antibodies had NT activity. Of these, three antibodies (4-4, 75-4, 66-A) had high antibody titers in both the NT and ELISA tests, and four (55-2, 16-C, 38-A, 39-7) had high ELISA titers, but low NT titers, 10-fold to 100-fold lower than those of 4-4, 75-4 and 66-A MC antibodies, and one (9-C) had rather low ELISA and NT titers. The remaining 13 MC antibodies showed positive ELISA titers but no NT activity. Culture fluid of mouse myeloma cells (SP2/O) concentrated 10-fold by ammonium sulfate was negative in ELISA and NT.

TABLE I

Enzyme-linked immunosorbent assay (ELISA) and neutralization (NT) titers of monoclonal (MC) antibodies to TO-163 strain

MC antibody	Immunoglobulin class	ELISA titer		NT titer	
		C.F. <sup>a</sup>	A.F. <sup>b</sup>	C.F.	A.F.
4-4	IgG2a	25 600	5 120 000	3 200	102 400
75-4	IgG1	12 800	2 560 000	800	204 800
66-A	IgG2a	12 800	1 280 000	1 600	204 800
55-2	IgG2a	1 024	1 024 000	64	25 600
16-C	IgG2a	1 024	2 048 000	16	2 560
38-A	IgG1	3 200	256 000	32	640
39-7	IgG1	1 600	2 048 000	2	2 560
9-C	IgG1	32	5 120	2	512
65-8	IgG1	64 000	10 240 000	<2	<2
53-B	IgG1	32 000	1 280 000	<2	<2
69-D	IgG1	25 600	1 280 000	<2	<2
40-A	IgG1	12 800	320 000	<2	<2
34-A	IgG1	3 200	1 024 000	<2	<2
28-F	IgG2a	2 560	256 000	<2	ND <sup>c</sup>
18-7	IgG2a	12 800	ND	<2	ND
71-C	IgG1	1 600	128 000	<2	<2
19-A	IgG1	160	204 800	<2	<2
25-8	IgG1	320	12 800	<2	<2
44-7	IgG1	1 280	16 000	<2	<2
11-A	IgG1	32	5 120	<2	<2
45-1	IgM	2	128	<2	<2

<sup>a</sup>Tissue culture fluid concentrated 10-fold by 50% saturation of ammonium sulfate.

<sup>b</sup>Ascitic fluid from mice inoculated with antibody-secreting hybridoma.

<sup>c</sup>Not done.

#### *Variation in NT activity of MC antibodies to TGE virus strains*

Seven strains of TGE virus were examined for their antigenicity by NT tests using the eight MC antibodies with NT activity to the TO-163 strain. The results are summarized in Table II. Based on these results the seven virus strains could be classified into four groups: the TO-163, h-5 and Kanagawa strains in Group I, the Purdue strain in Group II, the Ukiha strain in Group III, and the Shizuoka and Miller strains in Group IV.

MC antibodies 4-4, 55-2 and 38-A, demonstrated NT activity to all of the virus strains tested, although the 38-A antibody showed low titers compared with those of 4-4 and 55-2. The 16-C antibody neutralized the six virus strain in Groups I, III and IV, but not the Purdue strain of Group II, while the 9-C antibody neutralized all the strains except the Ukiha strain in Group III. The 66-A and 75-4 antibodies showed high NT activities to the strains of Group I,

TABLE II

Neutralization (NT) titers against seven strains of transmissible gastroenteritis virus of eight monoclonal (MC) antibodies with neutralization activity against TO-163 strain

MC <sup>a</sup> antibody	Group I			Group II	Group III	Group IV	
	TO-163 <sup>c</sup>	h-5	KA	Purdue	Ukiha	SH	Miller
4-4	102 400 <sup>d</sup>	409 600	51 200	102 400	409 600	409 600	51 200
55-2	25 600	12 800	12 800	12 800	52 200	102 400	12 800
38-A	640	128	64	8	8	32	16
16-C	2 560	512	64	<2	512	1 024	256
9-C	256	32	8	8	<2	8	2
66-A	204 800	409 600	409 600	4	<2	<2	<2
75-4	204 800	25 600	204 800	4	<2	<2	<2
39-7	2 560	512	512	<2	<2	<2	<2
Polyclonal <sup>b</sup> antibody	6 400	6 400	1 600	5 120	6 400	3 200	1 600

<sup>a</sup>Mouse ascitic fluid.

<sup>b</sup>Prepared in pig against the TO-163 strain.

<sup>c</sup>The virus strains used in NT tests. The number of passages in swine kidney cells was 176, 122, 51, 163, 92, 21 and 30 for TO-163, h-5, KA (Kanagawa), Purdue, Ukiha, SH (Shizuoka) and Miller strains.

<sup>d</sup> NT titer.

but low NT activity to the Purdue strain of Group II, and no NT activity to the strains of Group III and Group IV. The 39-7 antibody exhibited NT activity only to the Group I strains, but none to the other four strains in Groups II, III and IV. The polyclonal antiserum to the TO-163 strain prepared in a pig showed essentially the same NT titers to all the virus strains and could not differentiate between them.

The TO-163 and Shizuoka strains at low and high passage levels in swine kidney cell cultures were tested against the eight MC antibodies with NT activity against the TO-163 strains. As shown in Table III, the low-passage TO strain showed an NT titer pattern quite different from that of the high-passage TO strain. The high-passage TO strain (176 passages) showed the NT titer pattern of Group I, whereas the low-passage TO strain (17 passages) demonstrated an NT titer pattern similar to that of Group IV (Tables II and III). On the other hand, the Shizuoka strain showed no difference in NT titer pattern between the low-passage (22 passages) and high-passage virus (163 passages), the NT titer pattern being that of Group IV (Tables II and III).

#### *Results of competitive binding assay*

Table IV summarizes the results of the competitive binding assay. Enzyme-labeled 4-4 and 55-2 MC antibodies were blocked completely by either one of these antibodies, and similar results were obtained with MC antibodies, 66-A



TABLE III

Neutralization (NT) test of TO and Shizuoka (SH) strains at low and high passage levels with monoclonal (MC) antibodies positive for NT activity to TO-163 strain

Strain	Passage level	MC antibodies <sup>a</sup>							
		4-4	55-2	16-C	38-A	9-C	66-A	75-4	39-7
TO	17th	409 600 <sup>b</sup>	25 600	2 048	1 024	128	<2	<2	<2
	176th	102 400	25 600	2 560	640	256	204 800	204 800	2 560
SH	21st	409 600	12 800	1 024	32	8	<2	<2	<2
	163rd	409 600	51 200	1 024	64	8	<2	<2	<2

<sup>a</sup>Mouse ascitic fluid.

<sup>b</sup>NT titer.

and 75-4. MC antibodies 38-A, 16-C, 9-C and 39-7 completely blocked only homologous enzyme-labeled antibody. MC antibodies 66-A and 75-4, partially blocked enzyme-labeled antibodies 4-4, 55-2, 38-A, 16-C, 9-C and 39-7.

## DISCUSSION

Three major structural proteins have been demonstrated in the virion of TGE virus. They include phosphorylated nucleocapsid protein (N), glycosylated envelope protein (E1) and glycosylated peplomer protein (E2) (Jacobs et al., 1986; Laude et al., 1986). Garwes et al. (1979) reported that glycoprotein VP1 (E2) was the structural component of virus surface projections and that a preparation of virus surface projections produced NT antibody in animals.

TABLE IV

Results of competitive binding assay

Competitor	% inhibition of binding with enzyme-labeled MC antibody <sup>a</sup>							
	4-4	55-2	38-A	16-C	9-C	66-A	75-4	39-7
4-4	+	+	-	-	-	-	-	-
55-2	+	+	-	-	-	-	-	-
38-A	-	-	+	-	-	-	-	-
16-C	-	-	-	+	-	-	-	-
9-C	-	-	-	-	+	-	-	-
66-A	36%	28%	29%	37%	30%	+	+	23%
75-4	37%	30%	33%	31%	28%	+	+	24%
39-7	-	-	-	-	-	-	-	+

<sup>a</sup>+, ≥80% inhibition of binding.

-, ≤20% inhibition of binding.

Laude et al. (1986) raised MC antibodies against structural proteins of TGE virus and showed all major NT-mediating determinants to be carried by E2 peplomer protein. The epitopes recognized by our panel of MC antibodies with NT activity may also be present on E2 peplomer protein. MC antibodies reacting in ELISA but having no NT activity may recognize epitopes on N or E1 proteins. Further studies are required to elucidate the epitopes for these MC antibodies by Western immunoblotting, immunoprecipitation and other methods.

Comparison of seven TGE strains by NT tests using our panel of MC antibodies confirmed their close antigenic relationships, but also revealed the occurrence of distinct antigenic differences (Table II). Similar results were reported by Laude et al. (1986). The results of NT tests on the seven TGE strains (Table II) suggest that there may be at least six NT-mediating determinants on the virion of the TO-163 strain. These six epitopes are as follows: (1) the epitope recognized by MC antibodies, 4-4 and 55-2, which showed high NT activity for all the virus strains; (2) the epitope recognized by MC antibody, 38-A, which showed low NT activity for all the virus strains; (3) the epitope recognized by MC antibody 16-C, which showed NT activity for all the virus strains except the Purdue strain; (4) the epitope recognized by MC antibody 9-C, which showed NT activity for all the virus strains except the Ukiha strain; (5) the epitope recognized by MC antibodies, 66-A and 75-4, which showed high NT activity for the TO-163, h-5 and Kanagawa strains, very low NT activity for the Purdue strain and no NT activity for the Ukiha, Shizuoka and Miller strains; (6) the epitope recognized by MC antibody 39-7, which showed NT activity for the TO-163, h-5 and Kanagawa strains, but no NT activity for the other four virus strains.

The presence of six epitopes was confirmed by the competitive binding assay (Table IV): MC antibodies 4-4 and 55-2 recognized one epitope and 66-A and 75-4 recognized another, while antibodies 38-A, 16-C, 9-C and 39-7 each recognized one distinct epitope. These results are in good agreement with those of NT tests (Table II). MC antibodies 66-A and 75-4 blocked enzyme-labeled antibodies 4-4, 55-2, 38-A, 16-C, 9-C and 39-7 to some extent but the reason for this is obscure.

As shown in Table II, the seven TGE strains were similarly neutralized with polyclonal antibodies against the TO-163 strain. This finding suggests that subunit vaccines containing E2 protein of the TO-163 strain may have protective efficacy against other strains. MC antibodies 4-4 and 55-2 may be useful for purification of E2 protein.

#### ACKNOWLEDGEMENT

The authors thank Dr. H. Koyama of the University of Kitasato, Japan, for useful suggestions in the development of this manuscript.

## REFERENCES

- Blann, A.D., 1979. Cell hybrids: an important new source of antibody production. *Med. Lab. Sci.*, 36: 329-338.
- Bohl, E.H. and Cross, R.F., 1971. Clinical and pathological differences in enteric infection in pigs caused by *Escherichia coli* and by transmissible gastroenteritis virus. *Ann. N.Y. Acad. Sci.*, 176: 150-161.
- Furuuchi, S., Shimizu, Y. and Kumagai, T., 1975. Comparison between virulent and attenuated strains of transmissible gastroenteritis virus. *Natl. Inst. Anim. Health Q.*, 15: 159-164.
- Garwes, D.J., Lucas, M.H., Higgins, D.A., Pike, B.V. and Cartwright, S.F., 1979. Antigenicity of structural components from porcine transmissible gastroenteritis virus. *Vet. Microbiol.*, 3: 179-190.
- Harada, K., Kumagai, T. and Sasahara, J., 1967. Studies on transmissible gastroenteritis in pigs. III. Isolation of cytopathogenic virus and its use for serological investigation. *Natl. Inst. Anim. Health Q.*, 7: 127-137.
- Jacobs, L., Van Der Zeijst, B.A.M. and Horzinek, M.C., 1986. Characterization and translation of transmissible gastroenteritis virus mRNAs. *J. Virol.*, 57: 1010-1015.
- Kemeny, L.J., 1976. Antibody response in pigs inoculated with transmissible gastroenteritis virus and cross reactions among ten isolates. *Can. J. Comp. Med.*, 40: 209-214.
- Kimura-Kuroda, J. and Yasui, K., 1983. Topographical analysis of antigenic determinants on envelope glycoprotein V<sub>3</sub>(E) of Japanese encephalitis virus, using monoclonal antibodies. *J. Virol.*, 45: 124-132.
- Köhler, G. and Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256: 495-497.
- Komaniwa, H., Fukusho, A. and Shimizu, Y., 1981. Micromethod for performing titration and neutralization test of hog cholera virus using established porcine kidney cell strain. *Natl. Inst. Anim. Health Q.*, 21: 153-158.
- Laude, H., Gelfi, J. and Aynaud, J.M., 1981. In vitro properties of low- and high-passaged strains of transmissible gastroenteritis coronavirus of swine. *Am. J. Vet. Res.*, 42: 447-449.
- Laude, H., Chapsal, J.M., Gelfi, J., Labiau, S. and Grosclaude, J., 1986. Antigenic structure of transmissible gastroenteritis virus. I. Properties of monoclonal antibodies directed against virion proteins. *J. Gen. Virol.*, 67: 119-130.
- Nakane, P.K. and Kawaoi, A., 1974. Peroxidase-labeled antibody: a new method of conjugation. *J. Histochem. Cytochem.*, 22: 1084-1091.
- Yamagishi, H., Nagamine, T., Shimoda, N., Ide, S., Igarashi, Y., Yoshioka, I. and Matumoto, M., 1981. Infectivity assay and neutralization test for equine influenza virus in microplate cell cultures. *Vet. Microbiol.*, 6: 309-315.