

Micro-Indirect Hemagglutination Test for Detection of Antibody Against Transmissible Gastroenteritis Virus of Pigs

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A micro-indirect hemagglutination (IHA) test was developed for detecting antibody against transmissible gastroenteritis (TGE) virus of pigs. TGE virus propagated in swine kidney cell cultures was highly purified and concentrated by the combination of ammonium sulfate precipitation, treatment with fluorocarbon, and sucrose density gradient centrifugation. Tanned sheep erythrocytes were sensitized with purified virus for use in the IHA test. The results of testing 104 serum samples collected from pigs in the field indicated that the IHA antibody titers were approximately five times higher than those obtained by a serum neutralization test and that there was good correlation between the antibody titers determined by the two tests. High IHA antibody titers developed in pigs experimentally exposed to virulent TGE virus. Sensitized sheep erythrocytes were stable under long-term storage at 4°C (at least for 50 days). The conclusions made are that the IHA test described is more sensitive than the serum neutralization test for the detection of TGE antibody and may be of value for serodiagnosis of TGE.

Because transmissible gastroenteritis (TGE) of pigs is a highly contagious viral disease and usually spreads very rapidly to all susceptible pigs in a herd, rapid and reliable diagnosis is required for the control of the disease. Although several serological tests for detecting TGE antibody have been developed (2, 8, 14, 15), a serum neutralization (SN) test is the preferred method for serodiagnosis of TGE, especially if serum samples collected from pigs in both acute and convalescent stages of the disease are tested. The SN test gives reliable results and is of value for the detection and measurement of TGE antibody.

However, performing the SN test is time consuming and requires facilities for cell culture.

Indirect hemagglutination (IHA), which is a sensitive and rapid serological test, has been applied for the detection of antibodies against many viruses (4, 9, 10, 13).

Using erythrocytes sensitized with highly purified and concentrated TGE virus, an attempt was made to utilize the IHA test for detecting and measuring TGE antibody in pig sera.

The purpose of the present report is to describe the development of the IHA test for detecting and measuring TGE antibody and its applicability to serodiagnosis of TGE.

MATERIALS AND METHODS

Viruses. The TO strain of an attenuated TGE virus was used for the SN test and for production of the antigen used for sensitizing sheep erythrocytes (SRBC) in the IHA test. The strain had been serially passaged in swine kidney cell cultures (7) and was used at passage 168 for these experiments.

The Shizuoka strain (12), a virulent TGE virus, in the form of a 10% suspension of small intestine of infected piglet, was used for experimental inoculation of pigs.

Cell cultures. Monolayers of primary swine kidney cells grown in Roux bottles (150 by 220 mm) and test tubes (10 by 100 mm) were used for the production of antigen and for the SN test, respectively. The growth medium consisted of Earle balanced salt solution containing 0.5% lactalbumin hydrolysate (LE) supplemented with 10% heat-inactivated bovine serum, 0.021% sodium bicarbonate, 20 µg of kanamycin per ml, and 100 µg of streptomycin per ml. The maintenance medium was LE supplemented with 3% heat-inactivated bovine serum, 0.07% sodium bicarbonate, and the same antibiotics as in the growth medium.

Antigen for sensitization of SRBC. Confluent monolayers of swine kidney cells grown in Roux bottles were inoculated with 20 ml of viral suspension of the TO strain, containing $10^{6.0}$ median tissue culture infective doses (TCID₅₀)/ml. The virus was allowed to adsorb for 1 h at 37°C, and then 80 ml of the maintenance medium was added to the culture bottles. The cultures were incubated for 36 h at 37°C,

then culture fluid was harvested and clarified by low-speed centrifugation at $1,000 \times g$ for 10 min, and the virus was purified and concentrated as follows. Solid ammonium sulfate was added to the harvested fluid to 45% saturation. The mixture was stirred for 3 h at 4°C and then centrifuged at $1,500 \times g$ for 20 min. The precipitate was resuspended in phosphate-buffered saline (PBS; pH 6.4) to approximately 1/25 of the original volume. The viral suspension was then mixed with one-third volume of fluorocarbon (Diflon solvent S3, Daikin Kogyo Co., Osaka, Japan), shaken vigorously for 1 min, and centrifuged at $1,000 \times g$ for 5 min. The aqueous phase was collected and centrifuged at $56,000 \times g$ for 2 h. Virus pellets were resuspended in approximately 1/100 volume of PBS. Then, 1 ml of concentrated viral suspension was layered on top of a linear 10 to 50% sucrose density gradient prepared in a cellulose nitrate tube and centrifuged at $85,000 \times g$ for 3 h in a Spinco SW27.1 rotor. The virus band formed was collected, dialyzed against three changes of PBS, and used for sensitization of SRBC.

Preparation of sensitized SRBC. SRBC in 8% suspension were mixed with an equal volume of 3% Formalin solution in PBS, incubated at 37°C for 18 h, washed three times with distilled water, and suspended to a 10% suspension in PBS. The Formalin-treated SRBC were treated with an equal volume of 0.005% tannic acid solution in PBS at 37°C for 15 min, washed twice, and suspended in PBS. The Formalin-treated, tanned SRBC were then sensitized with TGE antigen by mixing 1 volume of 10% cell suspension and 5 volumes of a suspension of purified TGE virus and incubating at 37°C for 30 min. The sensitized cells were washed twice with diluent medium consisting of PBS (pH 7.2) supplemented with 0.1% gelatin and 1% heat-inactivated normal rabbit serum, which had been previously adsorbed with 50% Formalin-treated, tanned SRBC. Finally, the cells were suspended in the diluent medium to a 1% suspension and used in the IHA test.

Sera. Serum samples were collected from 104 pigs raised in the field farms. The pigs had been exposed to the virulent field strain of TGE virus or inoculated with the TO-163 vaccine strain (5). All sera were inactivated at 56°C for 30 min and tested for TGE antibody by both the IHA and SN tests.

Exposure of experimental pigs to the virulent TGE virus. Fifteen Yorkshire pigs weighing 15 to 25 kg, which had been serologically negative for TGE antibody, were exposed orally to 10^3 median pig infective doses of the virulent Shizuoka strain of TGE virus. Sera were collected before and after exposure and tested for both IHA and SN antibodies.

IHA test. Heat-inactivated sera were diluted 1:5 with the diluent medium and adsorbed with 2 volumes of a 50% suspension of Formalin-treated, tanned SRBC for 1 h at 37°C with occasional shaking. The supernatant fluids were considered to represent a 1:10 serum dilution and were used for the IHA test.

The test was carried out by the micromethod, using plastic microtrays with V-shaped cups. Serial twofold dilutions of sera were made in a volume of 0.025 ml with microdiluters, and 0.025 ml of sensitized SRBC suspension was added to each dilution. The microtrays were shaken and incubated overnight at 4°C . Settling patterns of SRBC were read as 100% agglutination when uniformly agglutinated cells had coated the bottom of the wells. The IHA antibody titer was expressed as the reciprocal of the highest dilution of serum that showed 50% agglutination (Fig. 1).

SN test. The SN test was performed according to the method of Harada et al. (8). Briefly, serial twofold dilutions of sera were incubated with an equal volume of viral suspension containing 200 TCID₅₀/0.1 ml of the TO strain at 37°C for 1 h. Two tubes of swine kidney cell cultures were inoculated with 0.1 ml of each serum-virus mixture. All cultures received 0.5 ml of maintenance medium and were incubated in a roller drum at 37°C for 4 days. The occurrence of cytopathic effect was determined, and

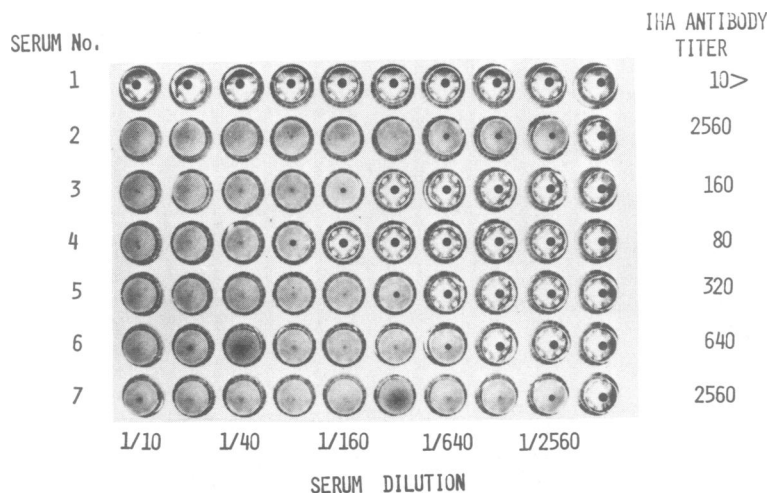


FIG. 1. Hemagglutinating patterns in the IHA test for TGE antibody. IHA antibody titers are indicated in the right column.

the SN antibody titer was expressed as the reciprocal of the highest dilution of serum that completely inhibited cytopathic effect in the test.

RESULTS

Virus purification and concentration. The recovery of infectious virus during the purification and concentration procedures is shown for a representative experiment in Table 1. Some loss of infectious virus occurred at each step of the procedure.

The virus was efficiently precipitated by ammonium sulfate (recovery of 78%), as reported by Garwes and Pocok (6).

Treatment with fluorocarbon did not greatly reduce virus infectivity, indicating that fluorocarbon treatment is one of the useful methods for removing contaminating protein from the crude viral suspension.

However, a great loss of infectious virus occurred with sucrose density gradient centrifugation. This may be due to incomplete recovery of the virus rather than degradation of viral particles during the centrifugation.

Fifteen percent of infectious virus were finally recovered in the experiment shown in Table 1.

Determination of optimum concentration of virus for sensitizing SRBC. To examine the effect of the virus concentration used for sensitizing SRBC on the IHA antibody titers, purified virus containing $10^{9.0}$ TCID₅₀/ml was serially diluted in twofold steps and used to sensitize SRBC. The results of tests on five sera are shown in Table 2. It is evident that the IHA antibody titers obtained were dependent on the virus concentration used for sensitizing SRBC. When SRBC coated with $10^{8.1}$ TCID₅₀ of virus per ml were used in the test, the IHA antibody titers obtained were extremely lower in two (sera 3 and 4) of four positive sera as compared with those obtained with more than $10^{8.4}$ TCID₅₀ of the virus per ml. SRBC sensitized with $10^{7.8}$ TCID₅₀ of the virus per ml gave negative results in three positive sera (sera 2, 3, and 4). More consistent results were obtained when the serum samples were tested with SRBC sensitized with more than $10^{8.4}$ TCID₅₀ of the virus per ml. Therefore, SRBC sensitized with virus titering more than $10^{8.5}$ TCID₅₀/ml were prepared and used in subsequent experiments.

Typical IHA patterns are shown in Fig. 1.

Relationship between IHA and SN antibody titers. Sera collected from 104 pigs in the field were tested for antibodies by both the IHA and SN tests to compare the sensitivity of these tests for detecting TGE antibody and to examine the correlation between antibody titers ob-

TABLE 1. Recovery of virus during purification and concentration procedures

Virus sample	Vol (ml)	Infectivity (TCID ₅₀ /ml)	Recovery (%)
Cell culture fluid	5,000	$10^{8.7}$	100
Ammonium sulfate precipitate	195	$10^{8.0}$	78
After treatment with fluorocarbon	171	$10^{8.0}$	68
Pelleted virus by high-speed centrifugation	2	$10^{8.8}$	50
Virus band in 10 to 50% sucrose density gradient	3	$10^{9.1}$	15

TABLE 2. Effect of virus concentration used for sensitizing SRBC on the IHA test

Virus concn (TCID ₅₀ /ml)	IHA antibody titer				
	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5
$10^{9.0}$	80	160	320	320	<10
$10^{8.7}$	160	160	160	320	<10
$10^{8.4}$	80	80	160	320	<10
$10^{8.1}$	80	80	40	80	<10
$10^{7.8}$	40	<10	<10	<10	<10

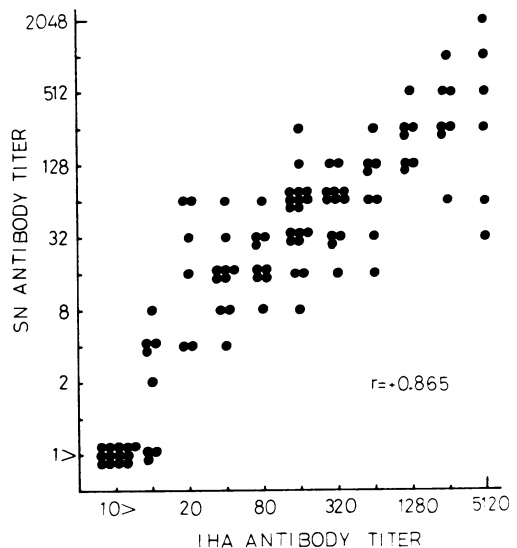


FIG. 2. Correlation between IHA and SN antibody titers.

tained by each test (see Fig. 2). The average antibody titers obtained by the IHA and SN tests were 125 and 25, respectively.

Although with a few exceptions the IHA antibody titers were higher (approximately five times) than SN antibody titers, antibody titers obtained by the two tests showed good correlation. The correlation coefficient was +0.865 and was statistically significant ($P < 0.001$).

Antibody responses of pigs experimentally exposed to virulent TGE virus. Fifteen pigs negative for TGE antibody were orally exposed to $10^{3.0}$ median pig infective doses of the virulent Shizuoka strain of TGE virus.

Sera obtained before and after exposure were tested for antibodies by both the IHA and SN tests.

All pigs exposed showed severe diarrhea after incubation periods of 1 to 3 days, which continued for 2 to 4 days.

TGE antibody was demonstrated by both IHA and SN tests in the convalescent sera collected from all infected pigs on days 7 and 14 of infection (Table 3). Antibodies detectable by both tests gradually increased in titer with the lapse of time and reached titers of 640 and 128 in the IHA and SN tests, respectively, 14 days after exposure. Again, the IHA antibody titers were higher (approximately five times) than those of SN antibody in this experiment.

Stability of sensitized SRBC upon long-term storage. A batch of sensitized SRBC was prepared, preserved at 4°C, and tested for reactivity in the IHA test after various times to examine the stability of the cells upon storage.

TABLE 3. *Antibody responses of pigs experimentally exposed to virulent TGE virus*

Pig no.	Pre-exposure ^a		Postexposure		Collection of postexposure serum (day)
	SN	IHA	SN	IHA	
1	<1	<10	2	10	7
2	<1	<10	4	10	7
3	<1	<10	4	20	7
4	<1	<10	4	40	9
5	<1	<10	4	40	10
6	<1	<10	8	40	10
7	<1	<10	8	40	10
8	<1	<10	8	80	10
9	<1	<10	16	80	10
10	<1	<10	16	80	11
11	<1	<10	16	160	14
12	<1	<10	32	80	14
13	<1	<10	32	80	14
14	<1	<10	128	320	14
15	<1	<10	128	640	14

^a SN, SN antibody titer; IHA, IHA antibody titer.

TABLE 4. *Stability of sensitized SRBC upon storage at 4°C*

Storage period (days)	IHA antibody titer				
	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5
0	160	160	160	320	<10
15	320	160	80	640	<10
30	160	160	80	320	<10
50	160	80	160	160	<10

The results of tests on five sera are shown in Table 4. Sera used for testing the ability of the sensitized SRBC to measure TGE antibody were the same through the experiment.

No marked changes in antibody titer detected by the IHA test occurred during 50 days of storage of the cells at 4°C. Furthermore, serum free from SN antibody (serum 5) did not agglutinate the sensitized SRBC to any extent, even at the termination of storage (50 days). This would indicate that sensitized SRBC are stable upon storage at 4°C for at least 50 days.

DISCUSSION

Although several serological tests, such as the SN (8), bentonite agglutination (14), complement fixation (15), and agar immunodiffusion (2, 15), have been developed by the previous workers for detecting TGE antibody, they possess certain disadvantages for diagnostic use.

The SN test is a sensitive and reliable method for serodiagnosis of TGE; however, it is time consuming and requires facilities for cell culture.

The bentonite agglutination test reported by Sibinovic et al. (14), in which TGE antibody agglutinates bentonite particles coated with TGE virus, may be of limited value for diagnosis of TGE since results do not correlate with those obtained in the SN test (3).

The complement fixation and immunodiffusion tests are limited in their sensitivity for detecting TGE antibody. Only sera that possess high SN antibody, i.e., hyperimmune sera, give positive results, whereas sera obtained from TGE convalescent pigs usually show negative reactions in both tests (15).

Bohac and Derbyshire (1, 2) have found that certain antigens demonstrable by the immunodiffusion test are present in digestive tracts of pigs infected with TGE virus, and that TGE convalescent sera can react with those antigens. However, results do not correlate with those obtained in the SN test for some sera (2).

Therefore, the IHA test was explored as a more rapid, sensitive, and reliable method for detecting and measuring TGE antibody.

TGE antibody was successfully demonstrated by the IHA test, using SRBC coated with highly purified and concentrated TGE virus. This indicates that viral particles are the antigens reactive in the IHA test.

Although antibody titers determined by the IHA test were approximately five times higher than those determined by the SN test, there was good correlation ($r = +0.865$, $P < 0.001$) between titers obtained in the two tests. Conva-

lescent sera collected from pigs experimentally exposed to virulent TGE virus were all positive for IHA antibody as well as SN antibody. Further, sensitized SRBC used were prepared by coating SRBC with highly purified virus. Thus, it appears that the IHA test is specific and more sensitive than the SN test for detecting TGE antibody; this suggests that the IHA test may be of value for serodiagnosis of TGE.

An antigenic relationship between TGE virus and hemagglutinating encephalomyelitis virus, another coronavirus, has been demonstrated by the immunodiffusion test (11). Sera examined in this study were not tested for antibody against hemagglutinating encephalomyelitis virus, and it remains to be established whether hemagglutinating encephalomyelitis antibody agglutinates SRBC sensitized with TGE virus.

Sensitized SRBC were stable upon storage at 4°C for at least 50 days, which is advantageous in using the IHA test for diagnostic purposes.

During these studies, it was noticed that some sera negative for SN antibody agglutinated SRBC sensitized with crude or partially purified virus. Distinct and specific hemagglutination occurred only when SRBC were sensitized with highly purified virus. Further, more consistent antibody titers were obtained when SRBC were sensitized with virus titrating more than $10^{8.4}$ TCID₅₀/ml. These findings indicate that SRBC should be sensitized with purified and high-titered virus to obtain reproducible results.

In the experiment shown in Table 1, 3 ml of purified virus, with a titer of $10^{9.1}$ TCID₅₀/ml, was obtained from 5,000 ml of infected cell culture fluid, and the recovery of infectious virus was only 15%. The reason for the loss of infectious virus during the purification and concentration procedures is not apparent. If SRBC are sensitized with virus titrating $10^{8.8}$ TCID₅₀/ml, only 12 ml of sensitized SRBC suspension can be prepared from 3 ml of the purified virus of $10^{9.1}$ TCID₅₀/ml, or from 5,000 ml of infected cell culture fluid. This severely limits the practical application of the IHA test. Methods for mass production and purification of TGE viral antigen must be devised to permit application of the IHA test to serodiagnosis of TGE. A roller bottle culture system for mass production

of TGE virus is now being used, and virus titrating more than $10^{8.0}$ TCID₅₀/ml can be easily and regularly obtained. Therefore, the drawback of the IHA test mentioned above will probably be overcome in the near future.

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