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Development of monoclonal antibody capture ELISA for the detection of antibodies against transmissible gastroenteritis virus

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Abstract

Transmissible gastroenteritis (TGE) is a disease confined to pigs of all ages, and can be a significant cause of economic loss in breeding herds, primarily because of the very high piglet mortality. The causative agent is a coronavirus, an enveloped positive strand RNA virus and closely related but non-enteropathogenic porcine respiratory coronavirus (PRCV). Although the TGEV has declined with its innocent relative, PRCV, further genome changes could not be excluded. Therefore, the herd-level immunity against this virus is important for the prevention of disease and should be carefully monitored. The aim of this study is to develop monoclonal antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) which can rapidly and accurately determine a large numbers of serum samples for surveillance purpose, and to compare the ELISA with a TGEV-specific serum neutralization test. The MAC-ELISA was sufficiently achieved, and the comparison with the virus-specific serum neutralization assays for 713 sera from pig farms showed a high correlation ($r^2=0.812$, $P<0.001$). The specificity and sensitivity of MAC-ELISA for the serum neutralization test 91.9% and 91.6%, respectively, which means that the antibody detected by the MAC-ELISA could be said to be protective antibodies. In conclusion, the developed MAC-ELISA would be very helpful in evaluating protective antibodies against TGEV.

Key words : Antibody detection, MAC-ELISA, Recombinant proteins, TGEV

INTRODUCTION

Transmissible gastroenteritis (TGE) is an acute enteric disease confined to pigs of all ages, and can be a significant cause of economic loss in breeding herds, primarily because of the very high piglet mortality. The causative agent is a coronavirus, an enveloped positive strand RNA virus, 60 to 220 nm in size, similar to coronaviruses affecting other domestic animals, including dogs and cats, with which it cross-reacts in a variety of serological tests (Dolye and Hutching, 1946; Siddel et al, 1983). The main clinical symptoms include watery diarrhea, vomiting and loss of appetite affecting all ages

of pigs. Sucking piglets are the most severely affected group. When a naïve herd is first infected, the mortality in piglets aged less than two weeks can reach 100% primarily due to dehydration (Saif and Wesley, 1999). This disease is on the list of diseases notifiable to the World Organisation for Animal Health (OIE).

Since TGEV was widespread in the mid 1980s, porcine respiratory coronavirus (PRCV) which is closely related but non-enteropathogenic and clinically inconsequential (Pensaert et al, 1986), has declined together with the TGEV. However, Pritchard et al. (1999) noted clinically silent seroconversion to TGEV in a number of herds in the UK and it means the TGEV or PRCV genomes could be possibly changed in the future. Therefore, the herd-level immunity against this virus are im-

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portant for the prevention of disease and should be carefully monitored.

The S protein of the TGEV known to be involved in cell adhesion is about 20 nm in size and highly glycosylated, which plays an important role to induce neutralizing antibodies in pigs (Suñé et al, 1990; Godet et al, 1994), containing at least five antigenic determinants (Delmas et al, 1986; Correa et al, 1988; Laude et al, 1990). It could effectively be used to diagnose antibodies to TGEV through those antigenic determinant sites (Gebauer et al, 1991). Paired serological tests between 2 and 6 months could inform when the maternally derived antibodies decay and the endemic TGEV or PRCV start to cause a problem in farms (Derbyshire et al, 1969; Saif and Wesley, 1999). Several serological assays found to be useful for the detection of antibodies to TGEV, including the serum neutralization test (Harada et al, 1967) and indirect fluorescent antibody assays (Benfield et al, 1978). However, the agar gel immunodiffusion test has been complained about discrepancies with a neutralizing antibody titration due to the too high specificity (Bohac and Derbyshire, 1976; Stone et al, 1976). Fluorescent antibody assays are more sensitive but the results could be subjective. Although the serum neutralization test is widely used as the most reliable method, it requires expertise and facilities, and it is difficult to test a large amount of serum.

The aim of this study is to develop the monoclonal antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) which can rapidly and accurately determine a large numbers of serum samples for surveillance purpose, and to compare the ELISA with a TGEV-specific serum neutralization test.

MATERIALS AND METHODS

Cells and pig sera

The TGEV (Pyeongtaek strain; Korean isolate) was maintained in a swine testicular (ST) cell line. The α -Minimum essential medium (α -MEM, GibcoBRL, NY, USA) supplemented with 5% fetal calf serum (FCS, Gemini, CA, USA), antibiotic-antimycotic (GibcoBRL,

NY, USA) was used as a cell-culture medium. The recombinant virus, pF6AH-bac (Tark, 1998), was proliferated in a *Spodoptera frugiperda* 9 (Sf9) cell line (Invitrogen, MA, USA). The Sf9 cell line was cultured in a Grace's medium at 28°C in a low-temperature incubator supplemented with 10% fetal calf serum and antibiotic-antimycotic. The pig serum used for the virus neutralization test and enzyme immunoassay was collected from a pig farm.

Concentration of recombinant TGEV spike protein

To prepare the antigen for ELISA, the recombinant S protein was prepared in pF6AH-bac by deleting transmembrane anchoring domain, rather including 6x histidine base toward C-terminal of the gene. The pF6AH-bac was inoculated on Sf9 cells and centrifuged for 3,000 rpm/20 min when the cytotoxic effect of 80~90% or more appeared after 5 days. After the supernatant was discarded, the recombinant protein was concentrated by modification of Zinc (Zn) acetate method used by Bohac et al. (1975). Briefly, 1 M Zn acetate was added to the supernatant to a final concentration of 0.01 M to 0.08 M, and the pH was adjusted to 1 N NaOH to a pH of 7.0~7.5. Then, it was centrifuged at 3,000 rpm for 20 min. After the supernatant was removed again, the precipitate was suspended by adding saturated EDTA (117 g EDTA-Na, 45 g Tris/L). Then, it was centrifuged again at the same condition and finally, the supernatant was dialyzed in phosphate buffered saline (PBS) at 4°C for overnight. The concentrated recombinant protein was subdivided into 0.5 mL portions and stored at -70°C for use as an antigen for ELISA.

Monoclonal antibody capture ELISA

The monoclonal antibody, 5C8 (Jang et al, 1998), specifically bound to TGEV Spike protein was diluted in 0.05 M carbonate/bicarbonate buffer to be sensitized to an ELISA plate (Maxi-sorp, Nunc, Denmark) at 37°C for 15~18 hours. The plate, then, was washed four times with a washing solution (0.01 M Tris pH 7.5, 0.05 M NaCl, 0.05% tween 20) and blocked with a blocking

buffer (0.01 M Tris pH 7.5, 0.15 M NaCl, 1% gelatin, 20% horse serum) at 37°C for one hour. Then, the plate was washed four times and the recombinant protein was incubated at 37°C for 3 hours after being diluted in a dilution buffer (0.01 M Tris pH 7.5, 0.15 M NaCl, 1% gelatin, 20% horse serum, 0.05% Tween 20). The plate was washed four times, and the serum samples were distributed after dilution, placed in a plate containing the recombinant protein, incubated at room temperature for 1 hour, and washed as above. Horseradish-peroxidase-conjugated goat anti-swine IgG (KPL, Gaithersburg, MD, USA) was diluted with dilution solution and added to the plate, followed by reaction at room temperature for 1 hour and then washing 4 times. Finally, 2,2'-azino-di-(3-ethyl benzothiazoline sulfonic acid) (ABTS) (KPL, Gaithersburg, MD, USA) was added to the plate and the result was revealed at 405 nm after 10 minutes.

Virus neutralization (VN) test

The VN test was carried out by modifying the method of Komaniwa et al. (1986). Neutralizing antibody titers in serum were expressed as reciprocal of serum dilution factor which could neutralize 100 TCID₅₀ Pyeongtaek strain by 50%.

Statistical processing

The statistical analysis was performed by Graph Pad

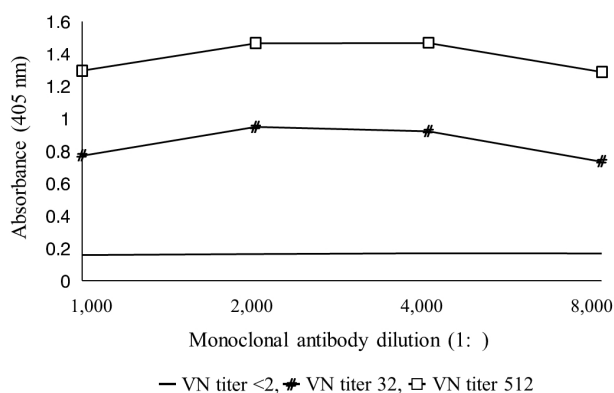


Fig. 1. Determination of the optimal monoclonal antibody (Mab) concentration. Dilution factors of each component are as follows: 1:100 for recombinant spike protein, 1:40 for porcine serum, 1:1,000 for conjugate.

Prism (version 5.0). Summary statistics were calculated for all data to assess the overall quality, including normality. The values of the data from MAC-ELISA and virus neutralization test were analyzed for the correlation and significance. $P < 0.05$ was considered to be statistically significant. The sensitivity and specificity were compared between two methods according to the method of Cho and Bohac (1985).

RESULTS

Establishment of reaction conditions for setting-up MAC-ELISA

MAC-ELISA was established with the optimal dilution concentration of monoclonal antibody, 5C8, from 1:1,000 to 1:8,000. As a result, the best reactivity between positive and negative sera was observed at 1:2,000 and 1:4,000 (0.3~0.6 µg/mL) dilutions as shown in Fig. 1.

As shown in Fig. 2, Zn acetate at 0.01 M and 0.02 M in concentrations showed high absorbance, and it was confirmed that Zn acetate at a lower concentration performed effectively to concentrate the recombinant protein. To confirm the optimal antigen concentration, the concentrated recombinant spike protein was diluted in 1:5~1:640 times. As a result, the best response was obtained at a dilution of 1:20 times and the reaction was kept

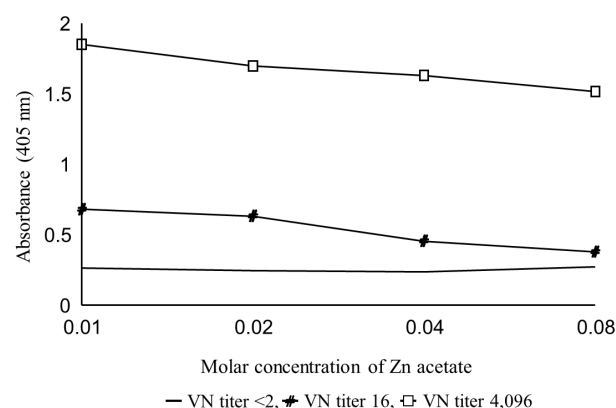


Fig. 2. Determination of the optimal molar concentration of Zn acetate for concentration of recombinant spike protein. Dilution factors of each component in MAC-ELISA for optimizing Zn acetate concentration are as follows: 1:4,000 for monoclonal antibody, 1:80 for recombinant spike protein, 1:40 for porcine serum, 1:1,000 conjugate.

constant up to 1:160 times (Fig. 3). Therefore, 1:100-fold (total protein amount: 5 µg/mL) was set to an appropriate dilution concentration of the antigen.

The optimal dilution factors were 1:40 for the serum and 1:1,000 for the conjugate (Fig. 4). To sum it up, the optimal dilution factor of each step was set at 1:4,000 (0.3 µg/mL) for monoclonal antibody, 1:100 (5 µg/mL) for recombinant protein antigen, 1:40 for test serum and 1:1,000 for conjugate.

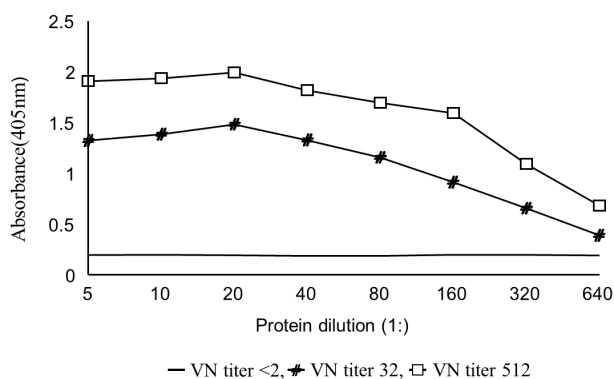


Fig. 3. Determination of the optimal antigen concentration. Dilution factors of each component in MAC-ELISA for optimizing recombinant spike protein are as follows: 1:4,000 for monoclonal antibody, 1:40 for porcine serum, 1:1,000 conjugate.

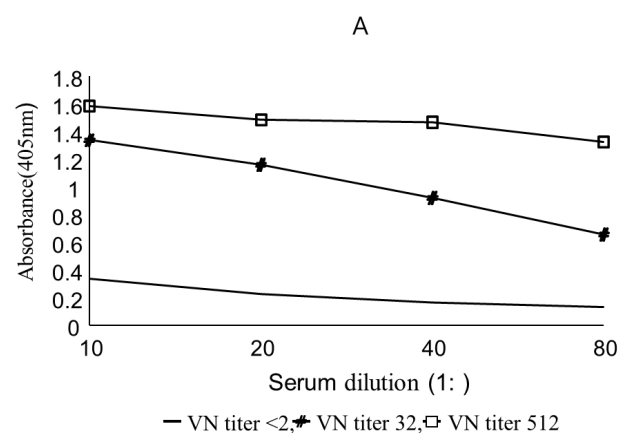


Fig. 4. Determination of the optimal serum and conjugate concentration. A; Dilution factors of each component in MAC-ELISA for optimizing serum concentration are as follows: 1:4,000 for monoclonal antibody, 1:100 for recombinant spike protein, 1:1,000 conjugate. B; Dilution factors of each component in MAC-ELISA for optimizing conjugate concentration are as follows: 1:4,000 for monoclonal antibody, 1:100 for recombinant spike protein, 1:40 for test serum.

Comparison of antibody detection efficiency between the VN and MAC-ELISA tests

To determine an appropriate cut-off value, 713 porcine sera were tested by paired ELISA established in this study and VN tests. The result suggested that when the P/N ratio (absorbance of the test serum/absorbance of the standard negative serum) was 2 or more, the cut-off value had the most appropriately high specificity and sensitivity when compared with the results of the VN test. The correlation between two assays were high and statistically significant ($r^2=0.812$, $P<0.0001$) (Fig. 5) as shown in Table 1.

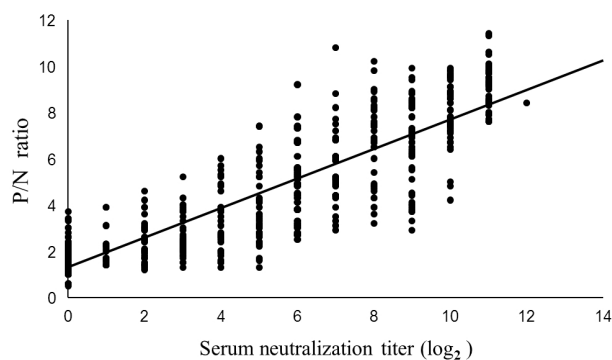


Fig. 5. Correlation between virus neutralization test and monoclonal antibody capture ELISA (MAC-ELISA). Dilution factors of each component in MAC-ELISA are as follows: 1:4,000 for monoclonal antibody, 1:100 for recombinant spike protein, 1:40 for porcine serum, 1:1,000 conjugate.

Table 1. Comparative analysis between the virus neutralization (VN) test and and MAC-ELISA to detect anti-TGEV antibodies in field porcine serum samples

MAC-ELISA	Virus neutralization test		
	Positives	Negatives	Total
Positives(P/N ratio ≥ 2)	381	24	405
Negatives(P/N ratio < 2)	35	273	308
Total	416	297	713
Sensitivity*		91.6%	
Specificity [†]		91.9%	

When the VN test resulted in 2 or more, the sample was regarded as positive. All serum samples were inactivated before test and all experiments were repeated three times.

*Sensitivity (%)=(No. of MAC-ELISA positive samples / all positive samples) $\times 100$

[†]Specificity (%)=(No. of MAC-ELISA negative samples / all negative samples) $\times 100$

Evaluation of the stability of MAC-ELISA

To confirm the long-term stability of the developed MAC-ELISA plate, it was completely dried at 37°C, sealed with a seal tape after air was removed, and was placed at -20°C. The reproducibility of the test by the MAC-ELISA was confirmed after 7 months of storage as shown in Fig. 6.

DISCUSSION

TGEV has an antigenic determinant site capable of producing a major neutralizing antibody in the spike protein among the N, M, and S proteins. ELISA test made only with S protein will present consistent results with the commonly used serum neutralization test will reduce confusion in applying the results. Therefore, in this study, the recombinant S protein of TGEV produced in the baculovirus expression system was used to develop the MAC-ELISA with high sensitivity and specificity compared with the serum neutralization test.

Nelson and Kelling (1984) attempted to develop an ELISA using purified TGEV but did not obtain highly sensitive results. On the other hand, Rukhadze et al. (1989) performed ELISA after isolating whole TGEV protein and S, M, N protein separately. As a result, when M and N proteins were used as antigens, they

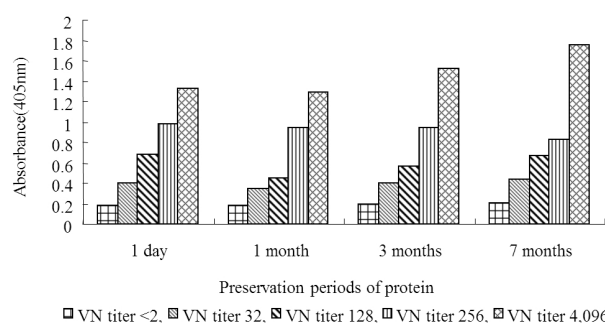


Fig. 6. Stability of the monoclonal antibody capture ELISA kit containing plate and other components during 7 months.

showed the lowest reactivity (absorbance) to antibodies to TGEV, and even the total TGEV protein was also lower than that of S protein. It also was reported that there was a high correlation ($r=0.97$) between serum neutralization assays and ELISA using S protein as an antigen. Based on the above results, S protein was considered to be the most useful protein for TGEV serological assay, and the author produced recombinant S protein from newly isolated TGEV (Korean isolated NVRI 48 strain) in order to more easily obtain S protein. When the recombinant baculovirus produced by cell culture is used as an antigen for ELISA, the cellular component can induce a nonspecific reaction (Cho et al, 1991). Therefore, the transmembrane anchor domain portion of the recombinant S gene is removed, the half of the amino terminus containing the antibody production epitope was expressed. Therefore, the recombinant S protein is secreted out of the cell so that the cell component is minimized when it is used as an antigen for ELISA. For the production of antigens for ELISA, only the cell culture supernatant was collected without any a conventional purification method such as sucrose gradient or ultracentrifugation steps (Nelson and Kelling, 1984; Hohdatsu et al, 1987; Rukhadze et al, 1989) and treated with 0.1M Zn acetate followed by concentration to 1/20. Lanza et al. (1993) showed a very low specificity (58%) when comparing the results of indirect ELISA and serum neutralizing antibody test. Therefore, in order to overcome the disadvantages of indirect ELISA, the ELISA antigen was prepared by ultracentrifugation of TGEV cultured on swine testicle cells and then attached to an ELISA plate using two kinds of monoclonal anti-

bodies specifically binding to S protein and then subjected to the same procedure as indirect ELISA. As a result, the same effect as indirect purification of the antigen was obtained, and the nonspecific reaction was remarkably eliminated, and the specificity (99%) of the test method was also increased. In this study, in order to reduce nonspecific reactions, MAC-ELISA was performed by immobilizing the recombinant S protein on the plate by coating an ELISA plate with a monoclonal antibody, 5C8, which specifically binds to the spike protein, according to the method of Lanza et al. (1993). Antibody tests of TGEV against 713 pig sera collected from farms using established MAC-ELISA showed a high correlation with serum neutralization test (Fig. 6, Table 1). However, about 8.2% (59/713 sera) of serum did not match the results of the serum neutralization. These discrepancies may include 1) the conjugate (HRP conjugated goat anti-swine IgG) that was used in the experiment did not react with the porcine IgM antibody produced early in the disease, 2) the presence of antibody to the cell nucleus or cellular components in serum, and 3) a lot of antibodies against the antigenic site that does not have the ability to produce neutralizing antibodies (Reynolds, 1982; Cesbron et al, 1985). In order to put the established ELISA into practical use, the ELISA plate containing the capture monoclonal antibody and the recombinant S protein was stored at -20°C and evaluated for a period of 7 months at a constant interval. As a result of the test, the established MACELISA showed a stable preservation and the possibility of practical use was confirmed. In conclusion, the established MACELISA can be used to detect antibody levels to TGEV in pig herd, which may be useful for serological screening for TGEV.

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