

# Simplified Serum Neutralization Test Based on Enhanced Green Fluorescent Protein-Tagged Classical Swine Fever Virus

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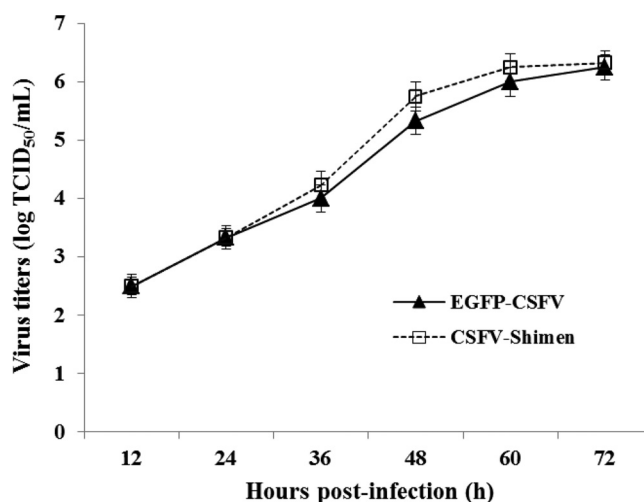
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**The neutralization immunofluorescence test (NIFT), currently used for detecting neutralizing antibodies (NABs) against classical swine fever virus (CSFV), is time-consuming. Here, a simplified neutralization test based on enhanced green fluorescent protein (EGFP)-tagged CSFV (EGFP-NT) was developed for direct detection of anti-CSFV NABs without immunostaining. The relative sensitivity and specificity between EGFP-NT and NIFT or blocking enzyme-linked immunosorbent assay (ELISA) were both 100%. The NAB titers by EGFP-NT and the blocking rates by blocking ELISA showed a good correlation.**

Classical swine fever (CSF) is an economically important disease of pigs worldwide caused by classical swine fever virus (CSFV), generally a noncytopathogenic virus (1, 2). A neutralization test (NT), either neutralization immunofluorescence test (NIFT) (3) or neutralization peroxidase-linked assay (NPLA) (4), is the gold standard for detection of neutralizing antibodies (NABs) against CSFV. NPLA can be used for serological discrimination of CSFV from other pestiviruses (5–7). However, these tests are labor-intensive and time-consuming due to the necessary incubation and staining procedures. It would be convenient to use CSFV tagged with a fluorescent molecule to detect NABs directly in unfixed cells. Therefore, the present study aimed to develop a simplified NT for rapid detection of anti-CSFV NABs in sera.

First, the enhanced green fluorescent protein (EGFP)-tagged CSFV, EGFP-CSFV, was generated from the pEGFP-CSFV, in which the EGFP gene was inserted between amino acids 13 and 14 of the N<sup>pro</sup> protein of CSFV in pBRCISM, a full-length infectious cDNA clone of the highly virulent CSFV strain Shimen (CSFV-Shimen) (8). The cDNA-derived virus was rescued and identified essentially as described previously (8). The observation of fluorescent foci and detection by the CSFV Antigen Test kit (Idexx, Switzerland), reverse transcription-PCR (RT-PCR), and indirect immunofluorescence assay (IFA) showed that the marker virus EGFP-CSFV was successfully rescued and the EGFP insertion in the recombinant virus was stable (data not shown). Importantly, the replication kinetics of EGFP-CSFV was similar to that of CSFV-Shimen (Fig. 1), indicating that insertion of the EGFP gene did not affect virus replication.

The NT based on EGFP-CSFV (EGFP-NT) was performed in 96-well flat-bottom nonpyrogenic polystyrene culture plates (Corning, NY, USA) as described previously (9). Unlike the NIFT based on CSFV-Shimen, EGFP-NT entailed direct examination under a fluorescence microscope (Nikon TE200; Nikon, Japan) and CSFV-specific NAB titers were determined rapidly. The CSFV-specific NAB titers were expressed as the reciprocal of the highest dilution that inhibited the infection of PK-15 cells in 50% of the culture wells. The NT results were validated using positive



**FIG 1** Single-step growth curves for EGFP-CSFV and CSFV-Shimen. PK-15 cells were infected with EGFP-CSFV or CSFV-Shimen (multiplicity of infection [MOI], 1) and harvested at 12, 24, 36, 48, 60, and 72 h postinfection. The titers of CSFV-Shimen were determined by IFA using the anti-E2 monoclonal antibody (MAB) 6E10, whereas the titers of EGFP-CSFV were obtained by direct observation of EGFP fluorescence and by IFA. Titers were expressed as log 50% tissue culture infective dose (TCID<sub>50</sub>)/ml. Data represent the means ± standard deviations (SD) from three independent experiments.

and negative reference sera. A back titration was mounted each time when performing NT.

A number of reference swine sera were used to evaluate the specificity and sensitivity of EGFP-NT. Experimental sera ( $n =$

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**TABLE 1** Agreement between CSFV-specific neutralizing antibody titers determined by EGFP-NT and NIFT

Sera <sup>a</sup>	No. of samples with:		Agreement (%)
	Identical titers	Different titers	
Experimental			
Positive	65	5	93.8
Negative	87	5	
Field			
Positive	18	2	96.2
Negative	32	0	

<sup>a</sup> Positive, titer >30; negative, titer <30.

162) were obtained from pigs immunized with CSF vaccines and challenged with CSFV-Shimen in a previous study (10). Field serum samples ( $n = 52$ ) were collected from several pig farms in China. CSFV-negative sera ( $n = 40$ ) originated from healthy, unvaccinated pigs. Reference swine antisera against CSFV, pseudorabies virus (PrV), porcine reproductive and respiratory syndrome virus (PRRSV), transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), or porcine rotavirus (PRV) ( $n = 3$  for each) were available in our institute. The sera showed different blocking rates in the Idexx CSFV antibody enzyme-linked immunosorbent assay (ELISA) (Idexx-ELISA) (positive,  $\geq 40\%$  blocking; negative,  $\leq 30\%$  blocking).

All sera were tested in parallel by EGFP-NT and NIFT. Receiver operating characteristic (ROC) analysis was performed with MedCalc software version 12.1.0 (Mariakerke, Belgium) to determine the cutoff value of EGFP-NT and the relative specificity and sensitivity between EGFP-NT and Idexx-ELISA or NIFT. The ROC analysis showed that the cutoff value of EGFP-NT was 30, and the relative sensitivity and specificity of EGFP-NT were both 100% compared to Idexx-ELISA or NIFT.

Next, we examined whether the NAb titers determined by EGFP-NT were consistent with those by NIFT. For the experimental sera, EGFP-NT and NIFT measured identical NAb titers in 87 of 92 negative sera and in 65 of 70 positive sera. The agreement between EGFP-NT and NIFT was 93.8% (152/162). For the field sera, the accordance between EGFP-NT and NIFT was 96.2% (50/52) (Table 1). These results indicated that the antibody titers measured in EGFP-NT were comparable with those from NIFT, suggesting that EGFP-NT could be used to detect CSFV-specific NAbs.

Antisera against non-CSFV swine viruses, including PrV, PRRSV, PEDV, TGEV, or PRV, were tested by EGFP-NT to evaluate their cross-reactivity. These sera all scored negative (with NAb titers of <16) in EGFP-NT and NIFT, indicating a high specificity of EGFP-NT.

Based on the results of 144 sera (of 162, excluding 18 sera with titers of >512) tested, a scatter chart and correlation between the NAb titers measured by EGFP-NT and the blocking rates determined by a blocking ELISA (Idexx, USA) were performed with Microsoft Excel software. The NAb titers correlated with the blocking rates, showing a clear regression equation [blocking rates =  $0.1397 \ln(\text{NAb titers}) - 0.1313$ ;  $R^2 = 0.928$ ] (Fig. 2). The peak antibody titers were >384, which corresponded to a blocking rate of 70 to 80%, consistent with the data reported by Sun et al. (11).

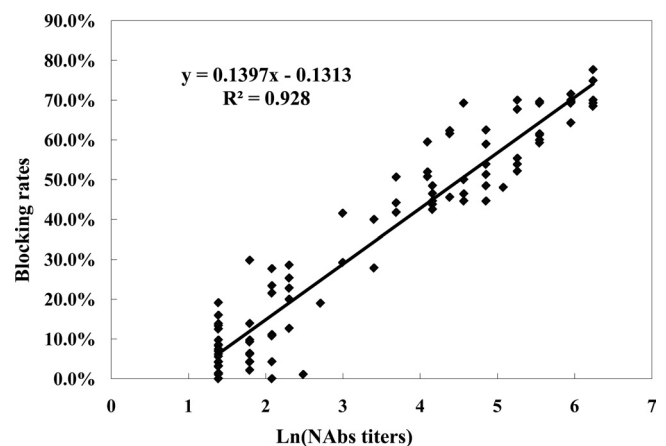
It has been reported that the insertion of the EGFP gene into the NSP2 gene of PRRSV resulted in an unstable mutant (12). In this study, however, the EGFP gene was stably inserted and expressed in the CSFV genome. Fortunately, we found that the in-frame insertion of EGFP into N<sup>pro</sup> did not affect the virus replication, which was consistent with the findings of a previous study (13).

The threshold dilution for a positive NPLA remains unabated at 25 and has been applied in developing ELISAs (4, 14). In this study, the cutoff value of 30 was determined based on ROC analysis. This value was slightly higher than that reported in previous studies (4, 15) but very close to the NAb titer of  $\geq 32$ , which was believed to provide an adequate protection both to the individual animal and to the herd (14).

EGFP-NT was consistent with NIFT in the qualitative analysis, with a slight difference in the quantitative analysis (93.8% and 96.2% agreement for experimental and field sera, respectively). This discrepancy was due to the inclusive fluorescence signals in some cases in NIFT. In contrast, EGFP-NT is more definitive even for less-experienced personnel. EGFP-NT requires no additional experimental steps after the initial incubation, making it more convenient than NPLA or NIFT. Therefore, EGFP-NT is simple, reliable, time saving, and cost-effective.

A previous report showed that the percent inhibition of serum samples in complex-trapping-blocking (CTB)-ELISA was either highly variable or extremely high depending on the titers of the sera determined in NPLA (16). In this study, we found that the anti-CSFV antibody titers measured in EGFP-NT correlate well with the blocking rates measured by Idexx-ELISA. However, unlike ELISAs, NT assays are not suitable for high-throughput analysis. The next task will be to automate the NT assay so that it can be used to detect large numbers of serum samples for the presence of anti-CSFV antibodies.

In summary, EGFP-NT is easier to perform and less time-consuming than NIFT, with comparable specificity and sensitivity, and represents an improvement over conventional NT methods for detection of CSFV.



**FIG 2** Correlation between EGFP-NT and Idexx-ELISA. The relationship between the blocking rates and the logarithms of the NAb titers was analyzed based on the data from 144 serum samples (of 162, excluding 18 sera with titers of >512) obtained from animals vaccinated with CSF vaccines and challenged with CSFV strain Shimen.

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