

Enzyme-Linked Immunosorbent Assay Using Glycoprotein and Monoclonal Antibody for Detecting Antibodies to Vesicular Stomatitis Virus Serotype New Jersey[∇]

Hyang-Sim Lee,^{1,2} Eun-Jeong Heo,¹ Hye-Young Jeoung,¹ Hyo-Rim Ko,¹ Chang-Hee Kweon,¹ Hee-Jeong Youn,² and Young-Joon Ko^{1*}

National Veterinary Research and Quarantine Service, Anyang, Gyeonggi-do 430-824,¹ and College of Veterinary Medicine, Seoul National University, Seoul 151-742,² Republic of Korea

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In this study, an enzyme-linked immunosorbent assay (ELISA) using glycoprotein and a monoclonal antibody (MAb) was developed for the detection of antibodies to vesicular stomatitis virus (VSV) serotype New Jersey (NJ). The glycoprotein to be used as a diagnostic antigen was extracted from partially purified VSV-NJ, and a neutralizing MAb specific to VSV-NJ was incorporated to compete with antibodies in a blocking ELISA using glycoprotein (GP ELISA). The cutoff of the GP ELISA was set at 40% inhibition, which corresponded to a virus neutralization test (VNT) titer of 32. With this threshold, the GP ELISA exhibited 99.6% specificity for naïve sera ($n = 3,005$) from cattle ($n = 1,040$), pigs ($n = 1,120$), and horses ($n = 845$) from domestic farms. The GP ELISA did not cross-react with sera positive for foot-and-mouth disease virus, swine vesicular disease virus, or VSV serotype Indiana. The GP ELISA was more compatible with the VNT than was the nucleocapsid-based ELISA for VSV-NJ-positive sera ($n = 19$). Taken together, this GP ELISA could be a useful tool as an alternative to the VNT for detecting antibodies specific to VSV-NJ.

Vesicular stomatitis (VS) is an infectious disease of cattle, swine, and horses occurring throughout the Americas (15, 20, 22). It causes significant economic and production losses of livestock due not only to veterinary costs but also to trade and animal movement restrictions (20). The causative agent of VS is vesicular stomatitis virus (VSV), a member of the genus *Vesiculovirus* in the family *Rhabdoviridae*. VSV has an 11-kb negative-sense single-stranded RNA that encodes five structural proteins: nucleocapsid (NC), phosphoprotein, matrix, glycoprotein (GP), and large polymerase (20). Of the two serotypes of VSV, the New Jersey (NJ) serotype is considered more important, since it is more pathogenic than the Indiana (IN) serotype and so accounts for the majority of clinical cases (16, 22).

Since the clinical signs of VS in cattle and pigs are indistinguishable from those of foot-and-mouth disease (FMD), with vesicular lesions on the mouth, tongue, and teats (8, 20), it is essential that VS be identified by rapid laboratory diagnostic methods. Whereas suspicious animals with clinical signs can simply be diagnosed through clinical surveillance or the detection of the virus, subclinically infected animals can be diagnosed only by serological surveillance. The virus neutralization test (VNT) is recognized as a standard method for the detection of anti-VSV antibodies by the Office Internationale des Epizooties. However, it is labor-intensive, requires cell culture facilities, and takes 2 to 3 days to complete. These aspects make it unsuitable for the purposes of mass serological surveillance. To address these drawbacks, several enzyme-linked

immunosorbent assay (ELISA) systems have been developed and used to measure antibody titers (1–5, 10, 13, 25, 27). Since VSV infects a broad spectrum of hosts (6), a serological method that can be performed irrespective of the susceptible species is preferable. In this regard, a blocking or competitive ELISA is more appropriate than an indirect ELISA. Previously, a competitive ELISA using recombinant NC (NC ELISA) instead of GP was reported (10). However, it showed low sensitivity relative to the VNT (5). Competitive ELISAs with inactivated VSV or GP have been developed as well (2, 4), but they employed polyclonal guinea pig serum as a competitor, which has inherent drawbacks. In contrast, the use of a monoclonal antibody (MAb) increases specificity and ensures unlimited quantity and consistent quality (26). Although an ELISA employing a MAb has been developed for VSV-IN (13), no ELISA for the detection of anti-VSV-NJ antibodies has been available yet. The aim of this study is to demonstrate that an ELISA using GP and a neutralizing MAb could be a robust assay to replace the VNT for the detection of antibodies specific to VSV-NJ.

MATERIALS AND METHODS

Virus and cells. VSV-NJ was obtained from the National Veterinary Services Laboratory (NVSL), Ames, IA. BHK-21 cells were cultured with alpha-minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, CA) and an antibiotic-antimycotic solution (Invitrogen) in a 37°C incubator under 5% CO₂.

Extraction of GP. The GP was extracted from partially purified VSV-NJ by a minor modification of methods previously described (8, 18). BHK-21 cells were infected with VSV-NJ at a multiplicity of infection of 0.001. When maximal cytopathic effect was observed, the supernatant was harvested by centrifugation at 10,000 × *g* for 30 min. The virus in the supernatant was inactivated by the addition of 1 mM binary ethyleneimine (Sigma-Aldrich) at 37°C for 24 h, and the reaction was stopped by 10 mM sodium thiosulfate (Sigma-Aldrich) at 37°C for 1 h. The virus solution was concentrated with 7.5% polyethylene glycol 8000 (Sigma-Aldrich) at 4°C for 16 h, and the GP precipitate was collected by cen-

* Corresponding author. Mailing address: National Veterinary Research and Quarantine Service, Anyang-6-dong, Anyang, Gyeonggi-do 430-824, Republic of Korea. Phone: 82 31 467 1858. Fax: 82 31 449 5882. E-mail: kojy@nvrqs.go.kr.

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trifugation at $10,000 \times g$ for 30 min. The resulting precipitates were resuspended in 5% of the original volume of TEN buffer (50 mM Tris containing 1 mM EDTA and 0.1 M NaCl [pH 7.8]). The insoluble materials were removed by centrifugation at $3,500 \times g$ for 20 min. The supernatant was mixed with 0.03 M octyl- β -D-glucopyranoside (Sigma-Aldrich) at room temperature for 1 h in order to strip the GP from the virus particles, and the mixture was then centrifuged at $85,000 \times g$ for 2 h to sediment GP-free virus particles. The supernatant containing GP was dialyzed against TEN buffer and then stored at -20°C until use. The concentration of this GP was determined by a bicinchoninic acid protein assay (Thermo Fisher Scientific).

MABs. The hybridoma used to produce the MAb was generated by a minor modification of methods previously described (7). Mice (BALB/c) were immunized twice via the footpad, at an interval of 2 weeks, with 100 μg of the GP extracted as described above in a mixture of incomplete Freund's adjuvant. The lymphocytes derived from the immunized mice were fused with SP2/O myeloma cells. Hybridoma cells were screened by indirect ELISA, immunofluorescence assay, and VNT. The MAb, designated 1G11, was finally selected from several MABs by its capacity to compete with antibodies in antisera in the GP ELISA, and its isotype was determined as immunoglobulin G2b by MonoAb ID/SP kits (Zymed). The MAb was purified using the ImmunoPure IgG purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Sera. To examine the limit of detection of the GP ELISA, one bovine and two swine serum samples were employed. One bovine serum sample positive for VSV-NJ was obtained from the NVSL, Ames, IA. Two 60-day-old pigs were immunized twice intramuscularly with binary ethyleneimine-inactivated VSV-NJ plus IMS1313 adjuvant (Seppic, France) in a final volume of 3 ml at an interval of 2 weeks. They were bled 20 days after the second immunization.

Naïve sera ($n = 3,005$) from cattle ($n = 1,040$), pigs ($n = 1,120$), and horses ($n = 845$) were collected from domestic farms with no history of exposure to VS. Control sera, included in the liquid-phase blocking ELISA kits, that were strongly positive for FMD virus (FMDV) serotypes O, A, and Asia 1 (Pirbright Laboratory, Surrey, United Kingdom) were employed. A swine vesicular disease virus (SVDV)-positive serum (RS2), which is an international positive-control serum collected 21 days postinfection, was obtained from Pirbright Laboratory.

The sera that were positive for VSV-NJ by the VNT ($n = 19$) were derived from horses and were obtained from the NVSL, Ames, IA. The sera in the VSV neutralization test proficiency panel ($n = 20$), comprising bovine, equine, and swine sera, were also obtained from the NVSL, Ames, IA. These sera had been tested by the VNT and the NC ELISA, and the records were provided by the NVSL, Ames, IA.

GP ELISA. MaxiSorp ELISA plates (Nunc, Denmark) were coated with 1 $\mu\text{g}/\text{ml}$ of VSV-NJ GP in 0.05 M carbonate buffer (pH 9.6) overnight at 4°C . Plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and were then incubated with 50 μl of test sera diluted 1:5 in diluent (PBST containing 5% skim milk) at 37°C for 1 h. After a wash with PBST, 50 μl of 30-ng/ml anti-VSV-NJ GP MAb 1G11 in the diluent was added and incubated at 37°C for 1 h. After a wash with PBST, 50 μl of 160-ng/ml goat anti-mouse antibody conjugated with horseradish peroxidase (Thermo Fisher Scientific) in the diluent was added, and the plates were incubated at 37°C for 1 h. After the plates were washed five times, the colorimetric reaction was developed for 15 min by the addition of 0.6 mg/ml of *o*-phenylenediamine in 0.05 M citrate phosphate buffer (pH 5.0) plus 0.015% hydrogen peroxide. The reaction was stopped by the addition of 50 μl of 1.25 M sulfuric acid. The optical density (OD) was measured at 492 nm, and the OD value was converted to the percentage of inhibition (PI) by the following formula: $\text{PI} = 100 \times [1 - (\text{OD of test serum well}/\text{OD of control well})]$, where the control well is the well containing the MAb alone.

VNT. The VNT was carried out, using 96-well tissue culture microplates with flat-bottom wells seeded with 5×10^4 BHK-21 cells per well, according to the manual of standards for diagnostic tests and vaccines of the Office Internationale des Epizooties. In brief, a VSV-NJ suspension containing 1,000 tissue culture infective doses/well was distributed into each well. The VNT titer was expressed as the reciprocal of the final dilution of serum at which 50% of the cells in the wells were protected. Sera with VNT titers equal to or greater than 32 were considered positive.

RESULTS

Establishment of the GP ELISA. The GP that was used in the GP ELISA as a diagnostic antigen was extracted from partially purified VSV-NJ as usual. The selected MAb, 1G11, was specific to VSV-NJ, but not VSV-IN, by immunofluores-

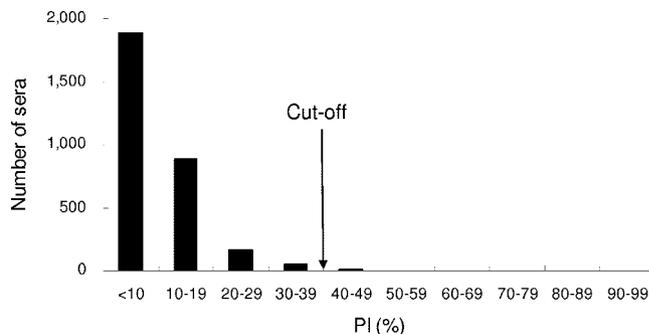


FIG. 1. Determination of the specificity of the GP ELISA. The frequency distribution of the PI by the GP ELISA was recorded for naïve sera ($n = 3,005$) from cattle ($n = 1,040$), pigs ($n = 1,120$), and horses ($n = 845$). The cutoff value (PI above background) was set at 40% inhibition.

cence assay and VNT (data not shown). Since the MAb did not bind to the GP by Western blotting, it was supposed to recognize the conformational epitope of VSV-NJ GP.

The GP ELISA was designed in a blocking format in which MAb 1G11 competes with antibodies in test sera. The optimal concentrations of GP antigen (1 $\mu\text{g}/\text{ml}$) and MAb 1G11 (30 ng/ml) were determined by checkerboard titration. The optimal serum dilution turned out to be fivefold in the experiment with serially diluted rabbit sera (data not shown).

To determine the cutoff level for the GP ELISA, naïve sera ($n = 3,005$) derived from domestic farms with no history of exposure to VSV were employed (Fig. 1). They included the main susceptible species: cattle ($n = 1,040$), pigs ($n = 1,120$), and horses ($n = 845$). Since the mean PI was 8.0% and the standard deviation was 9.8%, the cutoff was set at a PI of 40% by calculating the mean plus three times the standard deviation [$8.0 + (3 \times 9.8) = 37.4$] to secure a high specificity irrespective of the species. With this cutoff level, there were four false-positive reactions (0.4%) for bovine sera, seven for swine sera (0.6%), and one for equine sera (0.1%). In total, only 12 sera (0.4%) were interpreted as nonspecific positive, resulting in a specificity of 99.6%.

Limit of detection of the GP ELISA. To examine the limit of detection of the GP ELISA in relation to the VNT, one bovine serum sample obtained from the NVSL that tested positive by the NC ELISA and two sera from swine immunized with binary ethyleneimine-inactivated VSV-NJ were employed (Fig. 2). The positive sera serially diluted in negative sera were assessed by the GP ELISA and the VNT. Based on the cutoff of 40% inhibition for the GP ELISA and the cutoff of a titer of 32 for the VNT, the last dilutions of sera that were scored positive by both assays were a 64-fold-diluted serum sample for cattle (Fig. 2A) and 400-fold-diluted sera for pigs (Fig. 2B). This indicates that the detection limit of the GP ELISA corresponds to the VNT titer of 32.

Evaluation of the GP ELISA. The GP ELISA was evaluated with equine sera ($n = 19$) that were all positive by the VNT for VSV-NJ (Table 1). The panel consisted of sera with high titers by the VNT; 14 out of 19 sera showed VNT titers above 512. While the GP ELISA correctly scored all the sera as positive, the NC ELISA from the NVSL gave negative results for five sera, which had relatively low VNT titers except for one serum

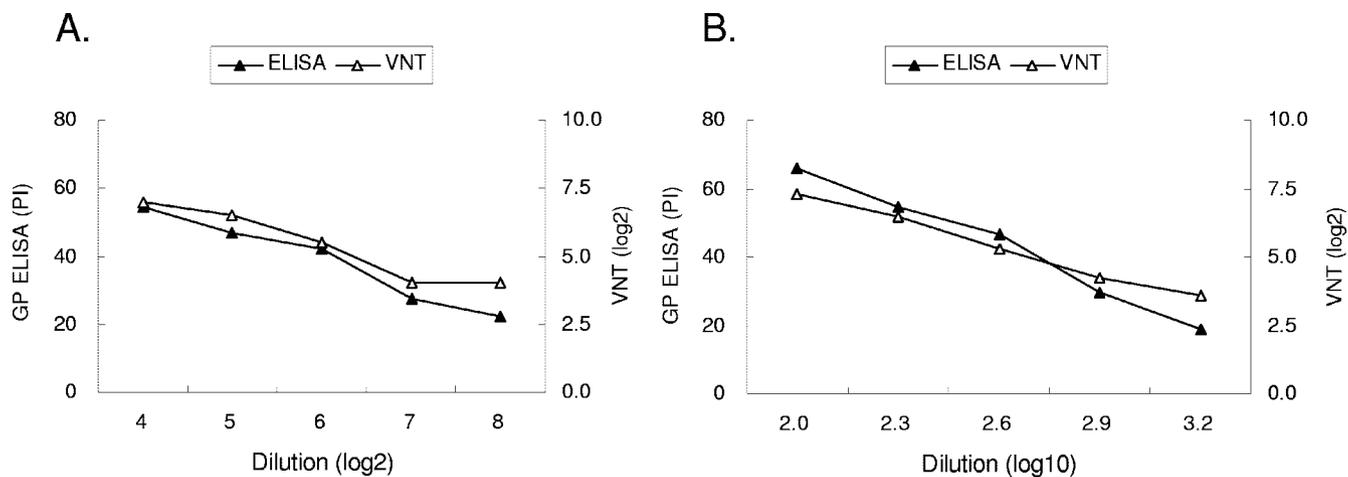


FIG. 2. Defining the limit of detection of the GP ELISA in relation to that of the VNT. One positive bovine serum sample infected with VSV-NJ (A) and two swine sera immunized with binary ethylenimine-inactivated VSV-NJ (B) were serially diluted with the respective VSV-negative sera and tested by the GP ELISA and the VNT. The results were analyzed comparatively by identifying the last serum dilution defined as positive by each assay, based on the cutoff of 40% inhibition for the GP ELISA and a titer of 32 for the VNT. The results in panel B are means for two swine sera.

sample (serum sample 2). Taken together, the GP ELISA could detect antibodies with VNT titers above 64, a finding consistent with the results shown in Fig. 2.

To assess the sensitivity of the GP ELISA more definitely in relation to the VNT, a panel of sera from the NVSL with titers bordering on the VNT cutoff level was employed (Table 2). The panel was composed of bovine, equine, and swine sera, including negative sera; the VNT titers ranged from 16 to 128. The panel included six VSV-NJ-positive sera and six VSV-IN-positive sera. The GP ELISA scored five out of six VSV-NJ-

positive sera in the panel correctly; it scored one sample (serum sample 12) as negative, just below the cutoff value. Although serum sample 12, with a VNT titer of 64, was negative by the GP ELISA, the other sera with the same VNT titers (Table 1, serum sample 11; Table 2, serum samples 4 and 13) were defined as positive by the GP ELISA, and even the serum sample with a VNT titer of 32 (Table 2, serum sample 16) was also positive by the GP ELISA. It was notable that serum sample 6 (Table 2), which had a VNT titer of 16 for VSV-NJ, was negative by the GP ELISA, demonstrating that

TABLE 1. Relative sensitivities of the GP ELISA and the NC ELISA for VSV-NJ-positive sera

Serum sample	VNT titer ^a	PI ^b (%) by:	
		NC ELISA	GP ELISA
1	128	36	57 ± 0.9
2	>512	49	94 ± 1.6
3	>512	64	83 ± 1.5
4	>512	56	90 ± 0.4
5	>512	57	94 ± 1.7
6	>512	64	94 ± 1.3
7	>512	63	82 ± 1.2
8	256	67	79 ± 3.2
9	>512	52	74 ± 0.8
10	>512	63	87 ± 2.8
11	64	44	69 ± 2.7
12	>512	76	78 ± 0.6
13	128	44	80 ± 0.2
14	>512	59	93 ± 2.5
15	>512	70	89 ± 1.5
16	>512	80	88 ± 0.1
17	>512	76	88 ± 0.3
18	256	34	63 ± 3.0
19	>512	76	68 ± 18.2

^a From the records of the NVSL, Ames, IA.

^b The NC ELISA results were provided by the NVSL, Ames, IA. The GP ELISA results were obtained in this study and are means ± standard deviations (n = 3). Boldface numbers indicate positive results by each assay, based on a cutoff of 50% inhibition for the NC ELISA and 40% inhibition for the GP ELISA.

TABLE 2. Serotype specificity of the GP ELISA versus the VNT using a panel of sera (n = 20)

Serum sample	VNT titer ^a for:		PI (%) by GP ELISA ^b
	VSV-IN	VSV-NJ	
1	<8	<8	11 ± 13.8
2	<8	<8	1 ± 2.7
3	<8	<8	1 ± 0.7
4	<8	64	56 ± 2.8
5	64	<8	5 ± 3.0
6	<8	16	18 ± 3.6
7	32	<8	5 ± 3.9
8	16	<8	7 ± 4.1
9	64	<8	4 ± 4.4
10	32	<8	3 ± 1.5
11	<8	128	83 ± 4.4
12	<8	64	32 ± 9.4
13	<8	64	73 ± 1.9
14	128	<8	2 ± 2.3
15	64	<8	1 ± 1.3
16	<8	32	48 ± 3.1
17	<8	<8	3 ± 3.4
18	<8	128	41 ± 6.0
19	<8	<8	1 ± 1.5
20	<8	<8	2 ± 2.4

^a Provided by the NVSL, Ames, IA. Boldface numbers indicate positive results by each assay, based on a cutoff titer of 32 for the VNT (for VSV-NJ and VSV-IN) and a cutoff PI of 40% for the GP ELISA.

^b Determined in this study. Results are means ± standard deviations (n = 3).

the cutoff level of 40% inhibition established for the GP ELISA corresponded exactly to the cutoff level for VNT titers. In contrast, the GP ELISA scored all six VSV-IN-positive sera as negative, with PIs far below the cutoff value, regardless of the VNT titers.

To investigate whether the GP ELISA could also distinguish VSV-NJ infection from other, similar diseases, sera strongly positive for FMDV (serotypes O, A, and Asia 1) that were included in the LPB ELISA kits and a reference serum (RS2) strongly positive for SVDV were employed. The PIs of sera positive for FMDV serotypes O, A, and Asia 1 and of the SVDV-positive serum RS2 were $6\% \pm 1.7\%$, $4\% \pm 3.0\%$, $8\% \pm 2.1\%$, and $8\% \pm 2.1\%$, respectively, equivalent to the mean PIs of naïve sera as shown in Fig. 1.

DISCUSSION

In this study, we established an ELISA using the GP extracted from VSV-NJ and a MAb specific to VSV-NJ, and we explored its feasibility in relation to the VNT for the detection of VSV-NJ antibodies. VSV GP is the sole protein anchored in the envelope and plays a critical role in the early stage of virus infection (9, 16, 21, 23). Therefore, the epitopes involved in virus neutralization are clustered on the GP (12). An ELISA with a similar format using recombinant GP and a MAb for VSV-IN has been reported previously (13). Since VSV-NJ causes more severe pathogenicity than VSV-IN (16, 22), it was essential to develop a rapid assay for the detection of antibodies specific to VSV-NJ.

The GP was successfully extracted from the partially purified VSV-NJ. The yield was sufficient to test 30 plates per ml of culture volume. However, in an effort to avoid exposure to VSV in the process of manufacturing the diagnostic antigens, the production of genetically engineered recombinant GP for VSV-NJ is now under way.

Even though several MAbs had higher VNT titers than 1G11, none were comparable to 1G11 in competing with anti-VSV-NJ antibodies in susceptible animal sera by the GP ELISA (data not shown). This suggests that the ability of a MAb to neutralize VSV-NJ does not always parallel its usefulness as a diagnostic competitor in a blocking format of a GP ELISA for VSV-NJ. In addition, whereas some other MAbs showed variable competitive reactivities depending on the species, the competition of 1G11 with serum antibodies was consistent irrespective of the species (data not shown); this was the key determinant in selecting 1G11 as a detector in the blocking format of the GP ELISA. Since 1G11 did not show reactivity to the GP by Western blotting, it is considered to recognize a conformational epitope. The exact epitope of 1G11 needs to be defined in order to elucidate the importance of this locus in relation to other, previously reported epitopes (11, 14, 19).

The advantage of the NC ELISA is that it distinguishes between animals vaccinated with VSV GP and VSV-infected animals (3). However, considering that no VSV vaccine is currently available, it was more important to develop a rapid assay to detect anti-VSV-NJ antibodies in animals expressing early-stage infection than to distinguish between infected animals and those that might be vaccinated. In this regard, the ELISA using the GP and an anti-GP MAb was established in a blocking format to be applied regardless of species. The

detection limit of the GP ELISA was determined as the point corresponding to a VNT titer of 32, the cutoff level for bovine and swine sera. Even though the detection limit remains to be determined with more sera, this result suggests that the GP ELISA is feasible as a surrogate assay in place of the VNT for bovine and swine sera. This was expected, because the GP ELISA and the VNT target the same GP, even though the numbers of epitopes on the GP recognized by the GP ELISA and the VNT are different. It has been reported that more than one epitope on the antigen surface can be recognized directly by a competitor MAb or blocked by steric hindrance or conformational changes induced by antibodies binding to other epitopes (24). These phenomena could compensate for the small number of epitopes recognized by 1G11 in the GP ELISA compared to the VNT. There is also another possibility, that the 1G11 epitope may be the predominant antigenic site on the GP of VSV-NJ.

Even though we did not use the sequential sera post-VSV infection to investigate how early the GP ELISA could detect VSV-NJ antibodies in comparison to the NC ELISA or the VNT, evidence that the sensitivity of the GP ELISA is comparable to that of the VNT was provided by the data showing that the GP ELISA was more sensitive than the NC ELISA in detecting antibodies to VSV-NJ in sera ($n = 19$) that were positive by the VNT (Table 1). This result was in accordance with previous reports that discrepant results between the NC ELISA and the VNT may be due to different antibodies in the NC ELISA and the VNT (5, 10). Actually, the GP ELISA and the VNT should be equivalent, because they recognize the same neutralizing antibodies.

Among the proficiency panel sera employed to examine the correlation between the GP ELISA and the VNT, the negative result for one serum sample (serum sample 12) for which the PI was $32\% \pm 9.4\%$ could be explained by certain individual differences between sera. The PI of the serum sample (Table 2, serum sample 16) with a VNT titer of 32 was $48\% \pm 3.1\%$, demonstrating that the cutoff of the GP ELISA is at least comparable to that of the VNT. This suggestion was corroborated by the result that serum sample 6 in Table 2, with a VNT titer of 16, was negative by the GP ELISA.

The GP ELISA could be a useful tool to distinguish VSV-NJ from VSV-IN, in contrast to the NC ELISA, as shown in Tables 1 and 2. This is reflected in the previous report that the NC ELISA could not replace the VNT for serotyping purposes, because the NC ELISA titers were less serotype specific than the titers measured by the VNT (10). This may be explained by the fact that the amino acid similarity of the NC of VSV-NJ to the NC of VSV-IN is 69%, as opposed to 50% for the GP (3, 10, 16, 17). Since the amino acid similarity between the GPs of VSV-NJ and VSV-IN amounts to 50%, it was speculated that there would be some degree of cross-reactivity between the serotypes by the GP ELISA. Contrary to our expectation, there was no cross-reactivity toward VSV-IN, even for the serum sample with a VNT titer of 128. This may be due to the use of a MAb instead of polyclonal sera as a competitor in the GP ELISA. In particular, the PIs of sera positive for VSV-IN were equivalent to the mean PI of naïve sera, suggesting low chances that VSV-IN-positive sera could cause interference with the interpretation of results for VSV-NJ by the GP ELISA. Of course, this GP ELISA should

be further examined with sera with VSV-IN neutralization titers above 128 in the future.

Since VS is clinically indistinguishable from other vesicular diseases, it is essential to differentiate VS from other vesicular diseases, such as FMD and SVD, by a rapid serological assay. Particularly in South America, where VS and FMD occur frequently, most cattle are vaccinated annually with FMDV vaccines (4). The GP ELISA was found to be specific to VSV-NJ and did not react with sera that were strongly positive for FMD or SVD. In particular, this serotype specificity of the GP ELISA should be useful for epidemiological analysis in VS outbreak regions.

Taken together, this is the first report to demonstrate that an ELISA using the GP and an anti-GP MAb could replace the VNT for the detection of VSV-NJ antibodies. This GP ELISA is a rapid and robust assay that could be coupled with the previously developed VSV-IN GP ELISA (13) for efficient serological surveillance of anti-VSV antibodies.

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