

Development of a blocking ELISA for screening antibodies to porcine rubulavirus, La Piedad Michoacan Virus

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Abstract. A blocking enzyme-linked immunosorbent assay (ELISA) was developed to detect antibodies to porcine rubulavirus (La Piedad Michoacan Virus [LPMV]) in serum samples from pigs. The test, based on a monoclonal antibody against the LPMV hemagglutinin-neuraminidase glycoprotein, had a sensitivity of 99% and a specificity of 97%. The results of this test were in agreement with those obtained by an indirect ELISA and hemagglutination inhibition, indirect immunofluorescence, and virus neutralization tests. The blocking ELISA is considered the most suitable test for routine screening for antibodies against LPMV.

The family Paramyxoviridae is classified into 4 genera: *Paramyxovirus*, *Rubulavirus*, *Morbillivirus*, and *Pneumovirus*.¹³ These viruses have been identified as the causative agents of a variety of diseases in both humans and animals. Some of these viruses, such as Newcastle disease virus in chickens,³ canine distemper virus in dogs,¹ and measles and mumps viruses in humans,^{5,15} can affect the central nervous system. The porcine rubulavirus was originally isolated from the brain of a piglet showing signs of a central nervous system disorder, pneumonia, and corneal opacity.⁹ The disease was first observed in 1980 during an outbreak of encephalitis in piglets on farms around the town La Piedad, district of Michoacan, Mexico. Since the report of the initial outbreak of the disease, the La Piedad Michoacan virus (LPMV) has spread throughout Mexico and is now endemic in that country.¹⁶ Because of the variability in symptoms associated with LPMV infection, it is difficult to diagnose the disease clinically. Therefore, an accurate and rapid laboratory diagnosis of the infection is particularly important to confirm LPMV as the causal agent of a disease outbreak. Procedures currently in use for the diagnosis of LPMV infection in Mexico are limited to retrospective demonstration of seroconversion to LPMV and/or isolation of the virus in cell culture and subsequent serologic typing. For serologic diagnosis, the most commonly used test is the hemagglutination inhibition (HI)

test. Recently, a comparative study was performed⁸ using 4 different techniques for screening antibodies to LPMV, i.e., an indirect ELISA and the HI, indirect immunofluorescence (IIF), and the virus neutralization (VN) tests. All 4 tests were satisfactory for the detection of antibodies to LPMV. However, there is a need for a specific, more sensitive, and rapid test for screening large numbers of samples. Therefore, the aim of the present study was to develop a blocking ELISA (b-ELISA) based on a monoclonal antibody (MAb) against the hemagglutinin-neuraminidase (HN) glycoprotein of the LPMV and to evaluate its accuracy in the diagnosis of LPMV infection.

Materials and methods

Cells and virus

The pig kidney cell line PK-15 or IB-RS-2 (cell line derived from a kidney of a 3-mo-old pig) grown in Eagle's minimum essential medium (EMEM) with Earle's salts was used for propagation of the LPMV-85 strain. This strain was isolated in 1985 from an outbreak of the disease in Mexico.⁹ The stock lyophilized virus of the third passage was titrated in microtiter plates^a and thereafter used throughout this study.

Virus purification

Supernatants of virus-infected cell cultures were clarified by low-speed centrifugation. The virus in the supernatants was concentrated by pelleting in a centrifuge^b at 16,000 × *g* for 4 hr. The pelleted virus was resuspended in TEN buffer (10 mM Tris-HCl, pH 7.5, 1 M ethylenediaminetetraacetic acid [EDTA], and 0.1 M NaCl), layered on a linear 20–60% sucrose gradient, and centrifuged at 100,000 × *g* for 3 hr. The virus band was collected and pelleted through a 30% sucrose cushion. The virus titer was determined by a hemagglutination test using chicken erythrocytes in a microsystem. Protein concentration also was determined.² This partially purified virus was used as antigen in the b-ELISA.

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Sera from Mexican pigs

Serum samples collected from pig herds in different parts of Central Mexico (states of Guanajuato, Michoacan, and Querétaro) were inactivated at 56 C for 30 min. Samples were submitted to the diagnostic laboratories at the Faculty of Veterinary Medicine, Mexico City, and the Association of Pig Producers, Irapuato, to be screened for antibodies to LPMV by an HI test. For further analysis, 600 randomly selected serum samples were sent to SVANOVA Biotech (Uppsala, Sweden).

Sera from experimentally infected pigs

Sera from experimentally infected piglets were also included in this study. In an experiment carried out in Belfast,⁸ 3 pigs (3 days old) were inoculated with 10⁷ TCID₅₀ of LPMV-85 strain. This inoculum was administered in a 10-ml volume given as 2 4.5-ml doses by the intranasal route and 2 0.5-ml doses by the intraconjunctival route, 6 hr apart. Blood was sampled on days 0, 5, 8, 11, and 14 after inoculation, and serum was separated for the use in serologic studies.

In a second experiment designed in Mexico¹² for studies of the reproductive tract, 4 9-mo-old boars were inoculated intranasally and intramuscularly with 10⁷ TCID₅₀ of porcine rubulavirus strain PARC-3. This inoculum was administered in a 4-ml volume for each route. Blood was sampled on days 0, 9, 12, 15, 19, 32, and 38 after inoculation, and selected serum samples from 3 boars were tested by the b-ELISA.

Serologic studies

The HI and VN tests on sera from experimentally infected piglets were performed according to protocols described previously. For the HI tests the sera were pretreated with heparin/MgCl₂ and titrated by serial 2-fold dilutions in phosphate-buffered saline (PBS) and assayed using a 3-volume HI test (50 µl serum, 50 µl 1% guinea pig erythrocytes, 50 µl antigen) against 4 hemagglutination units of LPMV antigen in V-well^a plates. End points were read by erythrocyte streaming, i.e., the last serum dilution that completely inhibited virus hemagglutination. For the VN test, serial 2-fold dilutions of the sera were prepared, and a 100-µl volume of each dilution was aliquoted into duplicate wells of a flat-bottom microtiter plate. A 100-µl volume of LPMV containing 100 TCID₅₀ was added, and the plate was incubated at 37 C for 2 hr. Finally, 50 µl of freshly trypsinized PK-15 cells suspended in EMEM (10⁴ cells/well) was added and the cells were incubated for 48–72 hr at 37 C. The VN titers were expressed as the reciprocal of the highest dilution of serum that completely neutralized the test virus.

The indirect ELISA⁸ was performed in microtiter immunoplates.^a Plates were coated with 100 µl of detergent-disrupted, partially purified whole virus particles overnight at 4 C at a protein concentration of 3 µg/ml in 50 mM carbonate buffer, pH 9.6. Blocking was done with 2% bovine serum albumin (BSA) in PBS for 1 hr at room temperature. Serum samples diluted in PBS containing 0.2% Tween 20 (PBST) were added, and the plates were incubated for an additional 1 hr at 37 C. Horseradish peroxidase (HRP)-conjugated anti-swine immunoglobulins^c were diluted 1:1,000 in

PBST and allowed to react for 1 hr at room temperature. Substrate used was 3,5,3-tetramethylbenzidine and 3.5 mM H₂O₂ in a substrate buffer (0.1 M sodium acetate, pH 6.0). The enzyme reaction was stopped after 10 min by adding 50 µl of 5 M H₂SO₄, and the optical density was read at 450 nm with a spectrophotometer.^d

Preparation and conjugation of MAbs

Six Balb/C mice were immunized subcutaneously twice, 6 wk apart, with 70 µg of purified LPMV mixed with an equal volume of Freund's complete or incomplete adjuvant. The animals were euthanized 4 days after the second immunization. Spleen cells were collected and fused with the myeloma cell line SP 2/0.⁶ Supernatants from the hybridoma cell clones were tested by an indirect ELISA. Bound antibodies were detected by an HRP anti-mouse conjugate^e diluted 1:1,000. Positive clones were subcloned several times, retested by ELISA and characterized by IIF and radioimmunoprecipitation tests. Subclones were further tested for their capacity to be blocked by positive LPMV sera. The subclone 2:64:159:9:6, hereinafter referred to as 2:64, was selected for further testing and was conjugated to NaIO₄-activated HRP¹¹ and stored in 50% glycerol at -20 C.

Characterization of the MAb

Indirect immunofluorescence. PK-15 cells infected with LPMV and uninfected cells grown on a glass coverslips were fixed with 4% paraformaldehyde, washed and permeabilized with 0.2% Triton X-100 for 2 min at room temperature. The fixed cells were then incubated with MAb 2:64 (cell culture supernatant diluted 1/50 in PBS) for 45 min, washed, incubated with fluorescein isothiocyanate-labeled secondary anti-mouse antibodies^e for an additional 45 min. Thereafter the cells were washed, overlaid with the anti-fadant; and visualized with a fluorescence microscope.⁴

Radioactive labeling of virus and cells. Confluent IBRS cells grown in EMEM were infected with LPMV at a multiplicity of infection of 5–10 fluorescence forming units/cell. When the cells showed 25% cytopathic effect the growth medium was changed to methionine-free EMEM. After 40 min of methionine starvation, the LPMV-infected cells were radioactively labeled with 60 mCi/ml ³⁵S methionine and further incubated at 37 C for another 6 hr.¹⁴

Radioimmunoprecipitation analysis. Radioimmunoprecipitation (RIP) was carried out essentially as previously described.¹⁴ Mock-infected cells and LPMV-infected cells, labeled as above, were disrupted on ice in lysis buffer (0.02 M potassium phosphate, 0.1 M NaCl, 0.5% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], and 1 mM phenylmethylsulphonyl fluoride in 0.01 M Tris-HCl, pH 7.8) for 2 hr, followed by centrifugation at 5,000 X g for 10 min. Samples of 0.1 ml of the supernatant were supplemented with 0.4 ml buffer, and 15 µl of culture supernatant of MAb 2:64 was added and the tube was shaken on ice for 1 hr. The complex was adsorbed by adding 150 µl of a 1:10 slurry of protein G-sepharose CL-4B^f in lysis buffer. The suspensions were incubated on ice for 1 hr, shaken, and washed four times in lysis buffer. After the last washing, the pellets were resuspended in SDS buffer (0.4 M boric acid, 0.45 M Tris-HCl, 3% SDS, 5% sucrose, 5 mM EDTA, 1%

2-mercaptoethanol, and 0.01% bromophenolblue) and boiled for 3 min. The supernatants were then analysed by SDS polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE. SDS-PAGE was carried out essentially as previously described.⁷ An electrophoresis calibration kit^f was used as a molecular marker, and the gels were fixed and stained with Coomassie blue according to standard procedures. In experiments using radioactively labeled virus, the gels (11% acrylamide) were vacuum dried on filter paper before autoradiography.

Mab subclass determination. Subclass was determined according to the manufacturer's recommendation.^g

B-ELISA reagents and procedures

To perform the b-ELISA plates were coated with 100 μ l of partially purified whole virus antigen overnight at 4 C at a protein concentration of 3 μ g/ml in 50 mM carbonate buffer, pH 9.6. Blocking was done with 2% BSA in PBS for 1 hr at room temperature. Pig sera were diluted 1/10 in PBST and allowed to react for 1 hr at room temperature. HRP-conjugated MAb diluted in PBST was added and incubated for 1 hr at room temperature. Substrate used was 3,5,3-tetramethylbenzidine and 3.5 mM H₂O₂ in a substrate buffer (0.1 M sodium acetate, pH 6.0). The enzyme reaction was stopped after 10 min by adding 50 μ l of 5 M H₂SO₄, and the optical density (OD) was read at 450 nm with a spectrophotometer.^d

The results were interpreted by calculating the percent inhibition (PI) according to the following formula:¹⁰ PI = [(negative control OD - sample OD) \div negative control OD] \times 100. All sera with a PI >35 are considered true positive, and all with a PI <25 are considered true negatives.

Results

Selection and characterization of the MAb. MAb 2:64 was selected for its ability to block positive LPMV sera (data not shown). This MAb which was characterized by RIP, mainly precipitates a protein of 66 kD (Fig. 1a). Other weakly precipitated bands of higher molecular mass were also observed. No precipitation bands were observed from uninfected control cells (Fig. 1b). These results were verified by an IIF test on LPMV-infected and uninfected cells using the MAb 2:64 as primary antibody. The results show a clear and intensive staining of the membrane of infected cells, particularly in areas of cell-cell contact (Fig. 1c). Uninfected cells were negative for fluorescence staining (data not shown). The subclass was determined to be IgG1.

Sera from experimentally infected piglets. Blood samples from 3 piglets experimentally infected with LPMV were collected on days 0, 5, 8, 11, and 14, and selected sera from the animals were tested by indirect ELISA and HI, IIF, and VN tests.⁸ In the present study, the b-ELISA was used to retest the sera from the 3 piglets. The results were in agreement with those of the previous study⁸ (see Table 1). The results presented

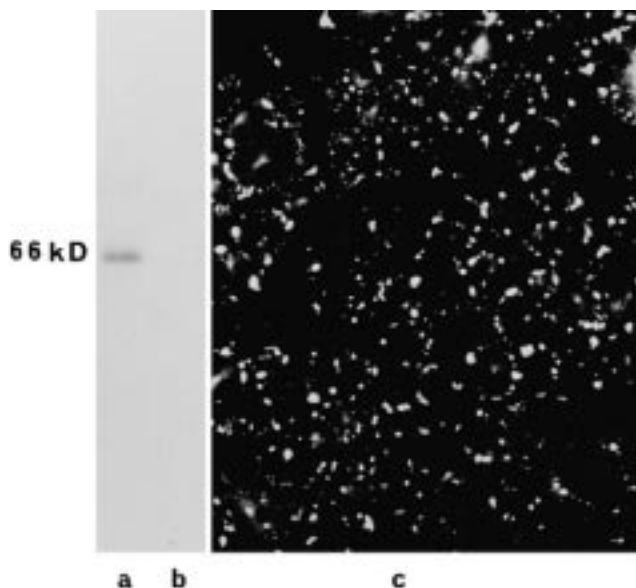


Figure 1. a. RIP of radioactively labeled cells infected with LPMV strain 85. b. RIP of radioactively labeled uninfected control cells. c. IIF study of LPMV-infected cells stained with the MAb.

in Table 2 concerning the experimentally infected boars in Mexico show that all 3 boars were positive on the b-ELISA on day 9, i.e., in agreement with the results of the previous experiment.⁸

Field sera from Mexico. All 597 field sera from Mexico were tested by b-ELISA: 431 were positive, and 166 were negative. Sixty of the 597 sera, 10 strong positive, 10 clear negative, and all 40 in the "gray-zone" (PI-25-35), were selected and further analyzed by VN and IIF tests. The results from the different tests agreed with those of the b-ELISA except for 5 sera that scored false negative with a PI of 21-34 and 5 false-positive sera, 4 of which had PIs of 37-42 and 1 of which had a PI of 84 (data not shown).

Sensitivity and specificity of the b-ELISA. Based on these results, the specificity and sensitivity of the blocking test were calculated: sensitivity = [true positive \div (true positive + false negative)] \times 100 or [426 \div (426 + 5)] \times 100 = 99%; specificity = [true negative \div (true negative + false positive)] \times 100 or [161 \div (161 + 5)] \times 100 = 97%.

Discussion

Recently, 4 antibody detection techniques (indirect ELISA, HI, IIF, and VN) for screening antibodies to LPMV were compared.⁸ The results showed that all 4 tests gave satisfactory results. However, these tests have the following disadvantages. The HI test is quick but gives false positives at low dilutions because of the nonspecific inhibitors, which can only be completely removed by pretreatment of the sera with heparin/MgCl₂.⁸ The IIF and the VN tests are time con-

Table 1. Comparison of serologic tests for the detection of LPMV antibody in the serum of 3 experimentally infected pigs.

Pig no.*	Serologic test†					
	b-ELISA	HI	IIF	VN		i-ELISA
				cpe	ha	
0 days						
1	2	<4	<32	<12	<12	0.316
2	-4	<4	<32	<12	<12	0.285
3	2	<4	<32	<12	<12	0.237
5 days						
1	6	<4	<32	<12	<12	0.317
2	-5	<4	<32	<12	<12	0.218
3	7	<4	<32	<12	<12	0.261
8 days						
1	23	4	1,024	18	12	0.792
2	62	4	2,048	12	18	1.773
3	28	8	2,048	36	24	1.266
11 days						
1	74	32	16,384	4,608	1,536	3.373
2	62	8	8,192	576	768	3.003
3	28	8	8,192	288	288	3.280
14 days						
1	70	32	32,768	4,608	2,304	3.404
2	72	16	16,384	2,304	1,152	3.209
3	50	32	16,384	2,304	2,304	3.234

* pi = postinfection.

† b-ELISA values are expressed as percent inhibition of sera diluted 1/10; HI = hemagglutination inhibition titer following heparin/MgCl₂ treatment of sera, expressed as the reciprocal of the last serum dilution that completely inhibited virus hemagglutination; IIF = indirect immunofluorescence titer, expressed as the reciprocal of the last serum dilution at which cytoplasmic fluorescence was observed; VN = virus neutralization titers, expressed as the reciprocal of the last serum dilution that completely inhibited the production of cytopathic effect (cpe) or the production of hemagglutinin (ha). Indirect (i) ELISA values are expressed as mean optical density of 4 replicates determined at a wavelength of 450 nm, with a cutoff value of 0.378.

suming and expensive, and the indirect ELISA suffers from poor specificity, mainly because of the nonspecific reaction of the anti-swine conjugate in the test.

As an attempt to develop a more specific, sensitive, less time consuming, and easy to perform test for screening large numbers of sera, a panel of MAbs were prepared and a b-ELISA based on an MAb directed against the HN protein of the LPMV was standardized. Stable hybridoma clones were selected by an indirect ELISA. Some clones were further characterized by RIP analysis. The results indicated that the majority of MAbs were directed either to the HN or to the nucleoprotein of the LPMV. The MAb 2:64 was selected for its ability to block LPMV-specific sera in a b-ELISA. The RIP procedure using the MAb 2:64 on radio-labeled LPMV-infected cells precipitated a protein of 66 kD (Fig. 1a). The size and the “fuzzy”

Table 2. Detection of LPMV antibodies by blocking ELISA* in the serum of experimentally infected boars in Mexico.

Days pi†	Pig 1	Pig 2	Pig 3
0	20	16	17
9	68	68	59
12	73	66	72
15	75	69	65
19	74	76	63
32	81	75	74
38	86	84	81

* Values are expressed as percent inhibition of sera diluted 1/10.

† pi = postinfection.

appearance of the band suggests that the precipitated band corresponds to the HN, because this protein has an appearance of a glycosylated protein.¹⁷ The bands of higher molecular mass were considered coprecipitated proteins and were not further analyzed. To verify these results, MAb 2:64 was used as a primary antibody in an IIF test. LPMV-infected cells showed an intensive staining of the cell membrane, further corroborating that this MAb recognizes the HN protein of LPMV. No fluorescence was observed in the cytoplasm of the cells.

The b-ELISA cut-off values, expressed as PI, have been determined on a selected number of negative, positive, and weakly positive serum samples. The results of the b-ELISA were verified by comparing them to those obtained⁸ in an indirect ELISA and HI and VN tests. The b-ELISA was standardized on a large number of field pig sera from Mexico and on sera from experimentally infected animals. The results of the b-ELISA show a sensitivity of 99% and a specificity of 97%. This assay can detect infection at an early stage in pigs.

Evidence exists that LPMV infection is “widespread” in Mexico. However, the exact extent of the infection and the distribution throughout pig herds in Mexico is still vague. To date, field testing of pig sera for antibodies to this virus has been conducted using the HI test, which is grossly inaccurate.⁸ Because the results of different serologic tests are similar and only differ in terms of performance, time, and availability of laboratory facilities, the selection of a test is dependant on the purposes of the study.

The b-ELISA based on a MAb against the HN protein is robust, highly specific and sensitive, and suitable for screening large numbers of sera. This b-ELISA has been standardized for performance and reproducibility. Because this ELISA should be prepared as a ready-to-use kit, it should be easy to use for epidemiologic studies in Mexico and for outbreaks of undiagnosed disease in pigs in other Central/South American countries and the United States. This test will also be practical for monitoring the results of vac-

ination programs, which will soon be implemented in intensive pig-production units in Mexico.

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Sources and manufacturers

- a. Nunc, Roskilde, Denmark.
- b. Sorvall, Du Pont, Wilmington, DE.
- c. Sigma, St. Louis, MO.
- d. Flow Laboratories, Irvine, UK.
- e. Dakopatts, Roskilde, Denmark.
- f. Pharmacia, Uppsala, Sweden.
- g. Innogenetics, Antwerp, Belgium.

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