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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. 07-086.498

Solicitud de patente titulada: "MUTANTES DE LA PROTEINA N DEL VIRUS
DEL SINDROME REPRODUCTOR Y RESPIRATORIO DE LOS PORCINOS".

Solicitante: PFIZER PRODUCTS INC.

Nuestra Ref.: P2007/000131

RESPUESTA AL OFICIO No. 8754 NOTIFICADO POR FIJACIÓN EN LISTA

No. 286, DE FECHA 30 DE MAYO DE 2012.

(REQUERIMIENTO ARTÍCULO 46)

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad solicitante, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del plazo legal previsto en el artículo 46 de la Decisión 486:

1. Adjunto copia completa del siguiente documento requerido por el Examinador en el examen de búsqueda de anterioridades, según lo previsto en el Artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina:

YANG L ET AL. "Antigenic and genetic variations of the 15 kD nucleocapsid protein of porcine reproductive and respiratory syndrome virus isolates". Archives of Virology. 1999, 144(3): 525-46.

Atentamente,

CARLOS R. OLARTE
C.C. No. 79.782.747 de Bogotá
T.P. 74.295

Anexo: Artículo mencionado

Antigenic and genetic variations of the 15 kD nucleocapsid protein of porcine reproductive and respiratory syndrome virus isolates

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Summary. The antigenic variability of the 15 kD nucleocapsid protein of porcine reproductive and respiratory syndrome (PRRS) virus was characterized with a panel of 24 monoclonal antibodies (MAbs) raised against the American PRRS virus isolate ISU-P. Five continuous epitopes designated EpORF7-A through E were revealed by the reactivity pattern of these MAbs with 67 American field isolates, two modified-live vaccine viruses, and the European Lelystad virus as determined by the indirect immunofluorescence assay and Western immunoblotting and confirmed by additivity and blocking enzyme-linked immunosorbent assays. The reactivity pattern of isolates in the IFA permitted their subdivision into 4 American antigenic groups which represented 84.1, 11.6, 2.9 and 1.4% of viruses tested. The antigenic variation among isolates was correlated to single, group specific nucleotide substitutions and mediated by a combination of at least 4 of the 5 epitopes. EpORF7-A was conserved in all American isolates and the Lelystad virus which constituted a separate antigenic group. Consequently, monoclonal antibodies specific for EpORF7-A may prove useful as the antigenic basis for a universal diagnostic test for the PRRS virus. EpORF7-C, D and E were only present in the American isolates tested.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a major disease of swine throughout the world. Clinical manifestations are characterized by repro-

ductive failure in sows and gilts, pneumonia in grower/finisher pigs, and increased morbidity and mortality in preweaning pigs [11]. The syndrome was first reported as devastating outbreaks of reproductive failure in North Carolina, Minnesota, and Iowa in the United States during 1987 and 1988 [12, 14]. In the years following, PRRS spread throughout the United States and Canada [12]. In 1990, the disease was recognized in Germany and soon afterwards in other Western European countries [35]. Presently, PRRS is endemic in most swine producing countries throughout the world.

The etiologic agent responsible for the syndrome was first isolated by researchers in the Netherlands and identified as the Lelystad virus [33]. Soon after, a virus (ATCC VR-2332) was isolated in the United States using the proprietary cell line CL2621 and identified as the swine infertility and respiratory syndrome (SIRS) virus [1, 5]. Currently, the International Office of Epizootics (O.I.E.) recognizes the terms Síndrome Disgénésico y Respiratorio del Cerdo (Spanish), Syndrome Dysgénésique et Respiratoire du Porc (French), and Porcine Reproductive and Respiratory Syndrome (English).

The PRRS virus is an enveloped RNA virus that is currently classified as a member of the family *Arteriviridae* [6, 22, 28]. The genome is a positive sense, 15 kb single strand of RNA consisting of 8 open reading frames (ORFs) which are expressed as a nested set of subgenomic RNAs. To date, 6 structural proteins with molecular masses of 15, 19, 25, 30, 31, and 45 kD and their corresponding genes have been reported for the European Lelystad PRRS virus isolates [23, 24, 31]. However, only 3 proteins with molecular masses of 15, 19, and 25 kD have been consistently detected in purified virions and lysates of cells infected with North American virus isolates [26, 27].

Previous studies have shown that field isolates of PRRS virus vary antigenically. Antigenic variation among PRRS virus isolates was first demonstrated between North American and European PRRS virus isolates by the immunoperoxidase monolayer assay using PRRS virus-specific polyclonal antibodies [34]. Similar antigenic variation between the European and North American isolates was also shown by the indirect fluorescent antibody test using monoclonal antibodies (MAbs) specific for the 15 kD nucleocapsid protein [8, 16, 19, 26]. The MAbs responsible for these distinctions represented at least one epitope that was unique to American isolates, one epitope unique to European isolates and at least 3 epitopes present in both American and European isolates. Genomic diversity of ORF 7 of European and American isolates has also been demonstrated by nucleotide sequence analysis [17, 21, 25, 30]. In the following study we describe the generation of a panel of MAbs to further characterize the antigenic nature of the 15 kD nucleocapsid protein and to determine if a sufficient degree of antigenic variation exists in the 15 kD protein among field isolates that can provide the basis of a reliable virus marker system for use in epidemiological studies and for monitoring virus traffic in swine populations.

Table 1. The geographic origins of PRRS virus isolates and modified-live vaccine viruses tested and their antigenic groups as determined by seroreactivity with a panel of 24 monoclonal antibodies in the indirect immunofluorescence antibody test

Geographic origin	Antigenic group				
	I	II	III	IV	V
Southeastern U.S.	29	2		1	
Midwestern U.S.	27 ^a	5	2		
Southwestern U.S.	1				
Canada		1			
Guatemala	1				
The Netherlands					1 ^b
Total	58	8	2	1	1

^a Includes PRRS viruses ISU-P, VR-2332 and the 2 modified-live vaccine viruses, PRIME PAC PRRS (Schering-Plough Animal Health Corporation, Omaha, NE, USA) and RespPRRS/Repro (NOBL Laboratories, Sioux Center, IA, USA)

^bThe Lelystad virus

Materials and methods

Viruses, cells, and media

A total of 70 PRRS viruses were used in the present study. These viruses included isolate ISU-P, ATCC VR-2332, 2 modified-live vaccine viruses, PRIME PAC PRRS (Schering-Plough Animal Health Corporation, Omaha, NE, USA) and RespPRRS/Repro (NOBL Laboratories, Sioux Center, IA, USA), the Lelystad virus [33] and 65 field isolates that were isolated between 1989 and 1995 from Canada, Guatemala, and 13 states of the United States of America (Table 1). All PRRS virus isolates were propagated and passaged at least one time after initial isolation in MARC-145 cells, a clone of an African green monkey kidney cell line [15]. The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS).

Antigen preparation for MAb production

Antigen for mouse immunization

Semi-purified whole virus preparations sufficient to immunize 16 mice were prepared from eleven 75 cm² cell culture flasks of MARC-145 cell monolayers which were inoculated with PRRS virus isolate ISU-P at a multiplicity of infection of approximately 5. Inoculated cells were incubated at 37 °C in a humid atmosphere containing approximately 5% CO₂. Four days later virus was harvested by 3 cycles of freezing/thawing at -80 °C/+37 °C followed by clarification at 2,000 × g for 30 min at 4 °C. The supernatant was subsequently centrifuged at 90,000 × g in a Beckman Ti 60 rotor for 2 h at 4 °C. The resulting pellet was resuspended in approximately 1 ml of 0.01 M phosphate buffered saline (PBS), pH 7.4, overlaid onto a

discontinuous sucrose gradient (30% and 60%), and centrifuged at $120,000 \times g$ in a Beckman SW 28 rotor for 70 min at 4°C . The band at the interface of the 2 sucrose layers was collected. Two thirds of this material, which contained virus particles, was diluted in PBS to a total volume of 4 ml and emulsified in an equal volume of Freund's incomplete adjuvant (GIBCO Laboratories, Grand Island, NY, USA). This preparation was stored at 4°C until used. The remaining material was diluted to 4 ml in PBS and stored at -70°C until used.

Antigen for Western immunoblotting and the enzyme-linked immunosorbent assay (ELISA)

MARC-145 cells were infected with PRRS virus ISU-P as described. Cells were harvested 4 days later, washed 2 times in PBS and pelleted by centrifugation at $500 \times g$ for 15 min at 4°C . Ten volumes of 0.25 M Tris/0.11 M tricine buffer, pH 8.6, containing 0.14 mM calcium lactate and 1% (V/V) Triton X-100 were added to one volume of packed cells, gently agitated overnight at 4°C and subsequently centrifuged at $2,000 \times g$ for 20 min at 4°C . The supernatant containing solubilized antigen was harvested and stored at -20°C until used.

MAb production

Monoclonal antibody secreting hybridomas were generated from 6 to 8 week-old specific pathogen free BALB/c mice. Each mouse was given two 0.25 ml injections of antigen with adjuvant subcutaneously 2 weeks apart. Two to 3 weeks later, mice were given a third 0.25 ml injection of antigen without adjuvant. Three days later, mouse spleens were removed. Fusions of mouse spleen cells with mouse SP2/O myeloma cells were carried out using the protocol described by Zola [38]. Hybridomas were screened for PRRS virus specific antibody secretion by the indirect ELISA. An indirect immunofluorescence assay (IFA) using normal and PRRS virus ISU-P infected MARC-145 cell monolayers as antigen [10] was used to confirm the identity of MAb secreting hybridomas. Antibody positive hybridoma populations were cloned 3 times with the EPIC 752 cell sorter (Coulter Corporation, Hialeah, FL, USA). Positive clones were identified by the IFA. The subclass and light chain type of individual MAbs were determined with a commercial mouse MAb isotyping kit (Boehringer Mannheim Corporation, Indianapolis, IN, USA).

Ascitic fluid containing specific MAbs was produced in 10 week-old BALB/c mice. All mice were primed with 0.5 ml pristane (2, 6, 10, 14-tetramethyl-pentadecane) (Sigma Chemical Company, St. Louis, MO, USA) given intraperitoneally 10 days before inoculation with specific hybridomas. Each mouse was injected with approximately 10^7 hybridoma cell in 0.5 ml PBS by the intraperitoneal route. Ascitic fluid was harvested from anesthetized mice between 7 and 10 days post inoculation. Monoclonal antibodies were extracted from ascitic fluid by protein A affinity chromatography using the protocol described by the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA) and stored at -20°C .

Indirect enzyme-linked immunosorbent assay (ELISA)

Viral antigen and MARC-145 cell control antigen preparations were diluted to optimum concentrations in antigen coating buffer consisting 0.02 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 9.6. One hundred μl of each preparation was added to individual wells in alternate rows of 96-well polystyrene microtiter plates (Immunlon I, Dynatech Laboratories, Inc., Chantilly, VA, USA) and incubated first at room temperature for 1 hr and subsequently overnight at 4°C . Excess viral and control antigen were removed and nonspecific binding sites in each well were blocked by adding PBS containing 10% FBS and incubating overnight at 4°C . The blocking solution was removed and the plates were stored in airtight plastic bags at -20°C until used.

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Supernatants from hybridoma cultures were tested for antibody without dilution. Ascitic fluid was tested for antibody by first diluting it 1:100 in PBS containing 10% FBS. One hundred μ l samples were added in duplicate to viral and cell antigen coated wells, incubated for 1 h at 37 °C and washed 4 times with washing buffer (PBS with 0.05% Tween 20). Subsequently 100 μ l of goat anti-mouse IgA+IgG+IgM(H+L) labeled with horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) was added to each well and incubated for 1 hr at 37 °C. The plates were washed 4 times and 100 μ l ABTS peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) was added to each well. Reactions were allowed to develop for 20 min at room temperature. The optical density (OD) of each well was determined at 405 nm using a computerized V max microtiter plate reader (Molecular Devices Corporation, Palo Alto, CA, USA). A signal to noise ratio of 1.5 was considered positive in the screening test. Serological reactivity in the additivity and blocking ELISA was expressed as a corrected OD which was calculated by subtracting the mean OD generated from cell control antigen from the mean OD generated from viral antigen.

Additivity ELISA

The additivity ELISA was conducted using a modification of the methods originally described by Friguet et al. [9] and Levieux et al. [18]. In brief, MAbs representing each MAb group (Table 2) were diluted to concentrations that would consistently react with all available epitopes in antigen coated wells. Fifty μ l of such an optimally diluted MAb was added to viral and cell antigen coated wells and incubated for 30 min at 37 °C. Subsequently 50 μ l of the homologous MAb or heterologous MAb were added to the same wells and reacted for an additional hour at 37 °C. Each reaction was conducted in duplicate. Antigen/antibody reactivity was visualized with horseradish peroxidase conjugate. Corrected ODs were determined at 405 nm for at least 3 replicates. Mean additivity values were calculated using

Table 2. Characterization of PRRS virus isolates and monoclonal antibodies specific for the 15 kD nucleocapsid protein as determined by their seroreactivity in the indirect immunofluorescence antibody test

PRRS virus		Monoclonal antibody group				
Group	No. of isolates	A (n=7)	B (n=3)	C (n=3)	D (n=1)	E (n=10)
I	58 ^a	+	+	+	+	+
II	8	+	–	+	+	+
III	2	+	+	–	+	+
IV	1	+	+	+	–	+
V	1 ^b	+	+ ^c	–	–	–

^aIncludes PRRS viruses ISU-P, VR-2332 and the 2 modified-live vaccine viruses, PRIME PAC PRRS (Schering-Plough Animal Health Corporation, Omaha, NE, USA) and RespPRRS/Repro (NOBL Laboratories, Sioux Center, IA, USA)
^bThe Lelystad virus
^cWeak positive reaction

the formula:

$$AV = \frac{\text{Corrected OD MAb } a + b}{(\text{Corrected OD MAb } a) + (\text{Corrected OD MAb } b)} \times 100\%$$

where MAbs “a” and “b” are any 2 MAbs tested for additivity.

The additivity value (AV) of a homologous MAb combination should not be significantly greater than 50% whereas the AV resulting from a combination of 2 MAbs representing different and widely separated epitopes should approach 100%. Differences between homologous and heterologous MAb combinations were analyzed by Student’s *t*-test.

Blocking ELISA

The blocking ELISA was performed essentially as described by Cecilia et al. [30]. In brief, affinity purified IgG preparations representing each of the 5 MAb groups summarized in Table 2 were labeled with biotin hydrazide according to the manufacturer’s instructions (Pierce, Rockford, IL, USA) and diluted to a concentration that would consistently react with all available epitopes in antigen coated wells. Purified unlabeled MAbs representing each MAb group were serially diluted 10-fold in PBS containing 10% FBS and used for blocking. Viral and cell antigen preparations were treated in duplicate with either blocking MAb or diluted and subsequently reacted with homologous or heterologous biotinylated MAbs. Antigen/antibody reactivity was visualized with peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). Percent binding was calculated for each MAb interaction by comparing the corrected ODs generated in viral and cell antigen preparations treated with blocking MAb to the corrected ODs generated in antigen preparations that were treated with only diluent. The mean percent binding representing at least 3 replicates was calculated and plotted against dilution using the linear regression model. The one-way analysis of variance was used to test for differences at each dilution between homologous and heterologous interactions and among heterologous interactions mediated by individual blocking MAbs. Significant inhibition of binding by blocking antibodies was considered to be an indication of identity or interference by adjacent or overlapping epitopes.

Western immunoblotting

Western immunoblotting was done as previously described by Yoon et al. [37] with the exception that antigen preparations were mixed 1:1 with sample buffer containing 5% 2-mercaptoethanol.

Virus neutralization test

The microtiter serum virus neutralization test was used to assess the susceptibility of PRRS virus isolate ISU-P, the Lelystad virus and 4 individual American isolates representing virus antigenic groups I through IV to neutralization by MAbs representing groups A through E. Viruses were diluted in DMEM containing 10% FBS to yield approximate 200 TCID₅₀ in 50 µl. Ascitic fluid representing each MAb group was serially diluted two-fold in DMEM containing 10% FBS. Fifty µl aliquots of virus and MAb were added together in duplicated wells and incubated at 37°C for 1 h. Subsequently 100 µl containing approximately 10⁵ MARC-145 cells were added to virus/MAb mixtures and incubated for 7 days and observed for cytopathic effect.

RT-PCR

Viral RNA was extracted from 16 group I, 8 group II, 2 group III and 1 group IV viruses using the protocol described by Boom et al. [2]. Individual viral RNA preparations were

retrotranscribed using the protocol of the GeneAmp RNA PCR kit (Perkin Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA). Amplification of cDNA representing ORF 7 was mediated with the 2 oligonucleotide primers 5'-TAAATATGCCAAATAACAAC-3' and 5'-TAGGTGACTTAGAGGCACA-3'. These primers were originally designed by Mardassi et al. [20] and synthesized for use in this study by the Iowa State University Nucleic Acid Facility. The amplification was performed for 40 cycles by denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extending at 72 °C for 1 min. The time of the final extension step was 8 min. The PCR products were purified using a PCR DNA purification kit (Promega Corporation, Madison, WI, USA) by using the instructions of the manufacturer.

Sequencing and sequence analysis

DNA sequencing was performed in triplicate on both strands with the Applied Biosystems 377 automated sequencer (Applied Biosystems, Foster City, CA, USA) using *Taq* polymerase, fluorescein labeled dNTPs and the 2 primers previously described. Sequence analysis was conducted using programs of the GCG package developed by Genetics Computer Group, Inc. of Madison, Wisconsin. Nucleotide sequences were compared using the BESTFIT program and translated to amino acid sequences by the TRANSLATE program. Multiple alignment of the sequences was done with the PILEUP program.

Prediction of the nucleocapsid protein structure

Unique structural features and characteristics of the nucleocapsid protein of all PRRS viruses representing antigenic groups I through V that were sequenced were predicted from the translated amino acid sequences using the PEPTIDESTRUCTURE program of the GCG package.

Parameters that were used to compare amino acid sequences included hydrophilicity, surface probability, flexibility, Jameson-Wolf (JW) antigenic indices [13], Chou-Fasman (CF) turns [5], CF alpha helices, CF beta sheets, Garnier-Osguthorpe-Robson (GOR) turns, GOR alpha helices, and GOR beta sheets.

Results

Generation and characterization of MAbs

Twenty-four MAbs were generated. All were of the IgG1 subclass and possessed kappa light chains. These MAbs were specific for the 15 kD nucleocapsid protein of PRRS virus as determined by Western immunoblotting. All MAbs reacted with PRRS virus isolate ISU-P in the IFA and produced bright cytoplasmic fluorescence. None of the MAbs representing each group neutralized PRRS virus isolate ISU-P, the Lelystad virus or the 4 other American isolates representing antigenic groups I through IV.

Reactivity of MAbs with PRRS virus isolates

Five distinct groups of MAbs (A–E) and four distinct groups of American isolates (I–IV) and the Lelystad virus (group V) were revealed by seroreactivity as determined by Western immunoblotting and the IFA (Table 2). Monoclonal antibodies in group A were denominated as A1 through A7, group B as B1 through B3, group C as C1 through C3, group D as D1, and group E as E1 through E10. Virus

group I represented 84.1% (58/69) of all American isolates tested including the 2 vaccine viruses and isolate ISU-P. Each isolate in this group reacted with all 5 groups of MAbs. Virus groups II, III, and IV represented 11.6% (8/69), 2.9% (2/69) and 1.4% (1/69) of the American isolates respectively. Each of these virus groups was characterized by their reactivity with at least 4 of the 5 groups of MAbs. The Lelystad virus (group V) was only recognized by MAb groups A and B. Group A MAbs reacted strongly with Lelystad virus in the IFA as indicated by bright fluorescence. In contrast, group B MAbs reacted weakly with this virus in the IFA and not at all when evaluated by Western immunoblotting, possibly due to partial identity. The geographic origins of the PRRS viruses and their antigenic groups are summarized in Table 1.

Epitopic analysis of the 15 kD nucleocapsid protein

Results of additivity assays are summarized in Table 3. Additivity values for any 2 MAbs tested that equal or approach 50% indicate identity as revealed by the calculated mean additivity value for all homologous reactions of 51.5±0.4%. Conversely, additivity values for any 2 MAbs tested that differ significantly from 50% and approach 100% are indications that the 2 MAbs tested represent differ-

Table 3. Epitopic analysis of the 15 kD nucleocapsid protein of PRRS virus isolate ISU-P by the additivity test using group representative MAbs A1, B1, C1, D1 and E1

Added MAb	Test MAb				
	A	B	C	D	E
None	0.97 ^a	0.65	1.34	0.89	0.71
A	50.5% (0.98/1.94) ^b				
B	78.4% (1.27/1.62)	51.3% (0.67/1.30)			
C	82.7% (1.91/2.31)	85.4% (1.70/1.99)	53.0% (1.42/2.68)		
D	84.4% (1.57/1.86)	82.5% (1.27/1.54)	84.3% (1.88/2.23)	51.1% (0.91/1.78)	
E	75.6% (1.27/1.68)	71.3% (0.97/1.36)	86.3% (1.77/2.05)	82.5% (1.32/1.60)	51.4% (0.73/1.42)

^aOptical density was measured at 405 nm
^bAdditivity values expressed as binding percent were calculated using the formula:

$$AV = \frac{\text{Corrected OD MAb } a + b}{(\text{Corrected OD MAb } a) + (\text{Corrected OD MAb } b)} \times 100\%$$

where MAbs “a” and “b” are any 2 MAbs tested for additivity. The measured OD values and theoretical sum of OD values of any 2 MAbs are indicated in parenthesis. The mean additivity values for any 2 heterologous MAbs are significantly increased (P < 0.001), as determined by Student’s *t*-test

Table 4. Epitopic analysis of the 15 kD nucleocapsid protein of PRRS virus isolate ISU-P by the blocking ELISA using group representative MAb A1, B1, C1, D1 and E1

Biotinylated MAb	Blocking MAb				
	A	B	C	D	E
A	+ ^a	–	–	–	–
B	– ^b	+	–	–	± ^c
C	–	–	+	–	±
D	–	–	–	+	±
E	–	±	±	–	+

^a Significant (P<0.001) inhibition

^b No inhibition

^c Partial inhibition as indicated by significant blocking (P<0.05) at a magnitude of 1.8–2.0 less than that which was observed in homologous interactions

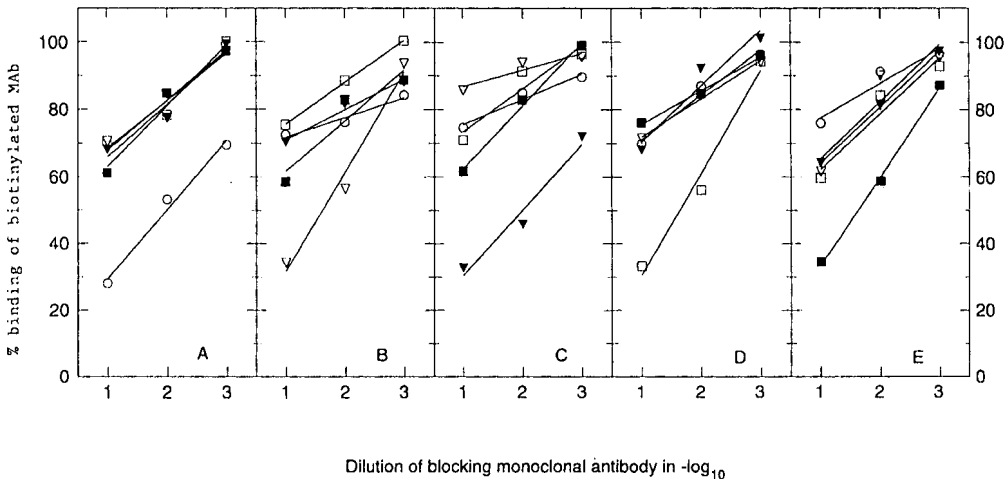


Fig. 1. Competitive binding curves of 5 representative biotinylated MAbs A (○), B (▽), C (▼), D (□), and E (■) in the presence of blocking MAbs A through E

ent epitopes. The mean additivity values for all heterologous comparisons was $81.3 \pm 4.8\%$ and ranged from 71.3% to 86.3%. These values were significantly different ($p < 0.001$) from homologous additivity values indicating that MAbs representing groups A through E represent 5 different continuous epitopes hereafter referred to as EpORF7-A through E.

Results of blocking assays that are summarized in Table 4 and Fig. 1 also support the existence of at least 5 epitopes on the 15 kD protein. The mean percent binding for all homologous interactions at the lowest MAb dilution was

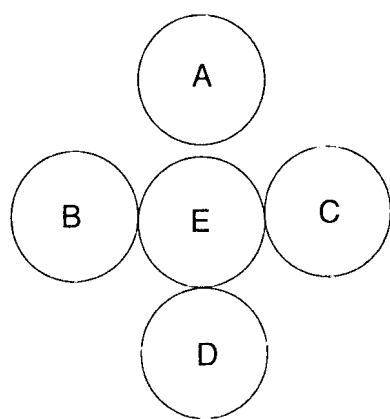


Fig. 2. The spatial relationship of 5 continuous epitopes (EpORF7-A through E) of the 15 kD nucleocapsid protein of PRRS virus isolate ISU-P. Group representative MAb A1, B1, C1, D1, and E1 were used. Circles that touch indicate that the epitopes are adjacent or overlap

32.6±2.7 and ranged from 28.0±2.3 to 34.3±1.1. All homologous interactions were significantly different from heterologous interactions ($p<0.001$). No significant differences were detected among heterologous interactions when MAb representing EpORF7-A, B, C and D were used for blocking with the exception that the MAbs representing EpORF7-B and C interfered ($p<0.05$) with subsequent reactivity by the MAb representing EpORF7-E. Similar interference of reactivity of MAbs representing EpORF7-B, C and D was observed when MAb representing EpORF7-E was used for blocking ($p<0.05$). In both instances the magnitude of interference as demonstrated by percent binding among heterologous interactions was less than that observed among homologous interactions and ranged from 58.5±2.4 to 64.2±1.8. These observations suggest that EpORF7-A, B, C, and D are spatially separate from each other while EpORF7-E overlaps with or is adjacent to EpORF7-B, C and D. The postulated spatial relationship of these epitopes is presented in Fig. 2.

Comparison of ORF 7 of representative viruses of the 5 antigenic groups

The nucleotide sequences of ORF 7 of 15, 8, 2, and 1 representative viruses of antigenic groups I,II, III, and IV respectively have been deposited in the GenBank with the accession numbers AF043949- AF043974. The nucleotide sequence of ORF 7 of RespPRRS/Repro vaccine virus (NOBL Laboratories) has been deposited in the GenBank with the accession number AF054970. These nucleotide sequences are presented in Fig. 3. The deduced amino acid sequences of these viruses are summarized in Fig. 4. The nucleotide and deduced amino acid sequences of ORF 7 of Lelystad virus and ATCC VR-2332 [22, 25] are presented for the purpose of comparison. The ORF 7 of all American PRRS virus isolates analyzed consisted of 372 nucleotides which encode 123 amino acids. In contrast, ORF 7 of Lelystad virus was 387 nucleotides long and hence 128 amino acids. Furthermore, 2 nucleotide segments corresponding to positions 38-51 and 372-387 in ORF 7 of the Lelystad virus were not present in American viruses. Similarly,

	CONSENSUS	ATGCCAAATAACAACGGCAAGCAGCAGAGAAAAAGAA-----GGGGATGGCCAGCCAGTCAATCAGCTGTGCCAGATCCTGGGTAAGATCA	100
Group I	VR-2332	G.....	
	NOBL ^a	G.....	
	S-P ^b	A.....	
	ISU-P	G.....	
	89-46448	G.....	
	91-46907	G.....	
	92-01205	G.....	C.....A.....
	92-11824	G.....	
	93-6351	A.....	A.....
	93-14620	A.....	A.....C.....
	93-22330	G.....	A.....
	93-27687	G.....	
	93-44927	G.....	
	94-18310	A.....	A.....C.....
	95-13536	G.....	
	95-15299	G.....	
	95-33010	A.....	A.....G.....A.....
Group II	89-46489	A.....	
	89-47361	A.....	
	89-47363	A.....	
	92-19698	A.....	A.....C.....
	92-22332	G.....	
	93-22326	A.....	A.....
	93-47324 ^c	G.....GA.....	
	94-36893	A.....G.....	A.....T.....
Group III	92-6725	G.....	
	IA-D21	A.....	A.....
Group IV	28523	A.....G.....	A.....A.....T.....T.....
Group V	Lelystad	...G.CGG...A...CAG.GC...A...A..G..A.GTACAGCTCCGAT.....	

Fig. 3 (continued)

✓

	CONSENSUS	TCCTCCAGCAAAACCACTCCAGAGCCCAAGCGACCGGGAAGAAAAATAAGAAGAAAAACCGGAGAGCCCCATTTTCCTCTAGCGACTGAAGATGATCT	200
Group I	VR-2332	T.....	
	NOBL ^a	T.....	
	S-pb	T.....C.....G.....T.....C.....	
	ISU P	C.....	
	89-46448	T.....	
	91-46907	G.G.....T.....C.....	
	92-01205	G.....T.....C.....	
	92-11824	T.....C.....	
	93-6351	C.....C.....	
	93-14620	T.....C.C.....T.....G.C.C.....	
	93-22330	C.....	
	93-27687	C.....	
	93-44927	G.....T.....C.....	
	94-18310	G.....C.....	
	95-13536	T.....	
	95-15299	T.....	
	95-33010	T.....G.....C.....C.....	
Group II	89-46489	T.....G.....	
	89-47361	T.....G.....	
	89-47363	T.....G.....	
	92-19698	G.....C.....C.....	
	92-22332	T.....C.G.....C.....T.....C.....C.....	
	93-22326	T.....G.....C.....C.....	
	93-47324 ^C	G.....G.....G.....	
Group III	92-6725	T.....C.....	
	1A-021	T.....G.....C.....C.....C.....	
Group IV	28523	G.....C.....	
Group V	Lelystad	.AAAGTCC..GCG....-----C.ACCTAGGGG.GGAC.GGGG..G.....G..T.....A.....C..G..TG.....CA.	

Fig. 3 (continued)

	Group I	CONSENSUS	CAGACATCACTTCACCCCTAGTGAGCGGCAATTGTGCTCTGTCGTCAATCCAGACTGCCTTTAATCAAGCGCGCTCGAACTTGTACCCCTGTCAGATTCAGGG	300
		VR-2332	T.....C.....G.....C.....	
		NOBL ^a	T.....C.....G.....C.....	
		S-P ^b	C.....T.....A.....T.....C.....C.....	
		ISU-P	T.....G.....C.....G.....	
		89-46448	T.....G.....C.....	
		91-46907	C.....	
		92-01205		
		92-11824	T.....G.....C.....	
		93-6351	G.....C.....	
		93-14620	T..G.....C.....	
		93-22330	A..G.....T.....C.....	
		93-27687	T.....G.....C.....	
		93-44927		
		94-18310	T.....A.....G.....G.....	
		95-13536	T.....C.....GG.....C.....	
		95-15299	T.....C.....G.....C.....	
		95-33010	G.....C..G.....	
	Group II	89-46489		
		89-47361		
		89-47363		
		92-19698		
		92-22332	T.....T.....	
		93-22326	..G.....C.....T..A.....	
		93-47324 ^C	T.....G.....C.....T.....	
		94-36893	A.....C.....	
	Group III	92-6725	T.....C.....G.....	
		IA-D21	..G.....G.....	
	Group IV	28523	..G.....A.....T.....	
	Group V	Lelystad	.C.G..C...C.....AG.C...A..CTCCC.C..CT..CAA..G.....G..T..C.....A.....GCGT.G..T...TCCAGC...	

Fig. 3 (continued)



	CONSENSUS	AGGATAAGTTACACTGTTGGAGTTTAGTTTTCCTGACCCATCATACTGTCCGCTGATCCGGCTCAGCAGCATCACCCCTCAGCATCA-----	387
Group I	VR-2332	T.....	
	NOBL ^a	T.....	
	S p ^b	C.....T.....	
	ISU-P	T.....T.....	
	89-46448	T.....	
	91-46937	..A.....A.....C.....T.....	
	92-C1235	..A.....A.....C.....T.....	
	92-11824	T.....T.....	
	93-6351	T.....T.....	
	93-14620	T.....G.....	
	93-22330	T.....	
	93-27687	T.....	
	93-44927	..A.....A.....C.....T.....	
	94-18310	T.....	
	95-13536	T.....	
	95-15299	T.....	
	95-33010	T.....C.....	
Group II	89-46489	C.....	
	89-47361	C.....	
	89-47363	C.....	
	92-19698	C.....T.....	
	92-22332	T.....C.....	
	93-22326	C.....C.....	
	93-47324 ^c	..C.....T.....T.....T.....C.....	
Group III	92-6725	T.....C.....	
	IA-D21	..A.....C.....	
Group IV	28523	T.....	
Group V	Lelystad	..A.G.C.....TTCAGG.T.....TGC.....ATTGC.....A.....T.....G.....T.....TA.A..C..CA..TCAGGGTGCAAGTTAA	

Fig. 3. Comparison of the nucleotide sequences of ORF 7 of representative PRRS viruses of antigenic groups I-V. The 15 nucleotides at the 5' end of ORF 7 are primer-derived. Dots represent bases identical to the consensus sequence, dashes indicate gaps introduced into the sequences, and the bold nucleotides are group-specific substitutions. The Lelystad virus and VR-2332 sequences used in this alignment were obtained from the GenBank [22, 25]. *a* The modified-live vaccine virus RespPRRS/Repro, manufactured by NOBL Laboratories, Sioux Center, IA, USA. *b* The modified-live vaccine virus PRIME PAC PRRS, manufactured by Schering-Plough Animal Health Corporation, Omaha, NE, USA. *c* A Canadian PRRS virus isolate

	CONSENSUS	MPNANGKOOKKKK---GDCQFVNOICOMLGKIIAONQSRGKGPGKKKKQNPKEHFLATEDDVRRHFTPSEROCLSSIoTAEQAGTCTLSDSG	100
Group I	VR-2332	R-----	
	NOBL ^a	R-----	
	S-P ^b	Y-----	
	ISU-P	R-----	
	89-46448	R-----	
	91-46907	R.I-----	
	92-01205	R.I-----	
	92-11824	R-----	
	93-6351	N-----	
	93-14620	N-----	
	93-22330	R-----	
	93-27687	R-----	
	93-44927	R.I-----	
	94-18310	N-----	
	95-13536	R-----	
	95-15299	R-----	
	95-33010	N.S-----	
Group II	89-46489	-----	
	89-47361	-----	
	89-47363	-----	
	92-19698	N-----	
	92-22332	R-----	
	93-22326	N-----	
	93-47324 ^c	R-----	
Group III	92-6765	R-----	
	IA-D21	N-----	
Group IV	28523	N-----	
Group V	Lelystad	.AGK.QS.K...STAPM.N.....L..AM.KS.R---QPR.CQA...K.....A...I...L.QT..S...Q.....AS.....	

Antigenic and genic variations in the 15 kD protein of PRRS virus

[Handwritten signature]

	CONSENSUS	R LSYTV E ESLPTHTTIVRLIRVTASPSA-----128
Group I	VR-2332	
	NOBL ^a	
	S-p ^b	
	ISU P	
	89-46448	
	91-46907	
	92-01205	
	92-11824	
	93-6351	
	93-14620	
	93-22330	
	93-27687	
	93-44927	
	94-18310	
	95-13536	
	95-15299	
	95-33010	
Group II	89-46489	 P
	89-47361	 P
	89-47363	 P
	92-19698	 PS
	92-22332	 P
	93-22326	 P
	93-47324 ^c	 SP
Group III	94-36893	 PH
Group III	92-6725	 P
	1A D21	 P
Group IV	28523	 H
Group V	Lelystad	KV.FQ...M..VA.....T..SQGAS

Fig. 4. Alignment of deduced amino acid sequences encoded by ORF 7 representative PRRS viruses of antigenic groups I–V. The 5 amino acids at the amino terminal of the 15 kD nucleocapsid protein are primer-derived. Dots represent residues identical to the consensus sequence, dashes indicate gaps introduced into the sequences, and the bold amino acids are group specific substitutions. *a* The modified-live vaccine virus RespPRRS/Repro, manufactured by NOBL Laboratories, Sioux Center, IA, USA. *b* The modified-live vaccine virus PRIME PAC PRRS, manufactured by Schering-Plough Animal Health Corporation, Omaha, NE, USA. *c* A Canadian PRRS virus isolate

2 nucleotide segments corresponding to positions 117–127 and 357–361 in ORF 7 of American viruses were not present in the Lelystad virus.

Analysis of ORF 7 of the American isolates by the GCG package program revealed single, group specific nucleotide substitutions relative to virus group I that could account for unique antigenic differences. These nucleotide substitutions occurred at positions 358, 364 and 209 in virus groups II, III, and IV, respectively, and are indicated in Fig. 4. In groups II and III, thymidine at positions 120 and 122 respectively was replaced by cytidine which changed the encoded amino acid from serine to proline. In group IV, guanine at position 70 was by adenine which changed the encoded amino acid from serine to asparagine.

The locations of these amino acid substitutions at positions 120, 122 and 70 in antigenic groups II, III and IV respectively are correlated to changes in the secondary structure of the protein that are predicted by Chou-Fasman (CF) turns

10

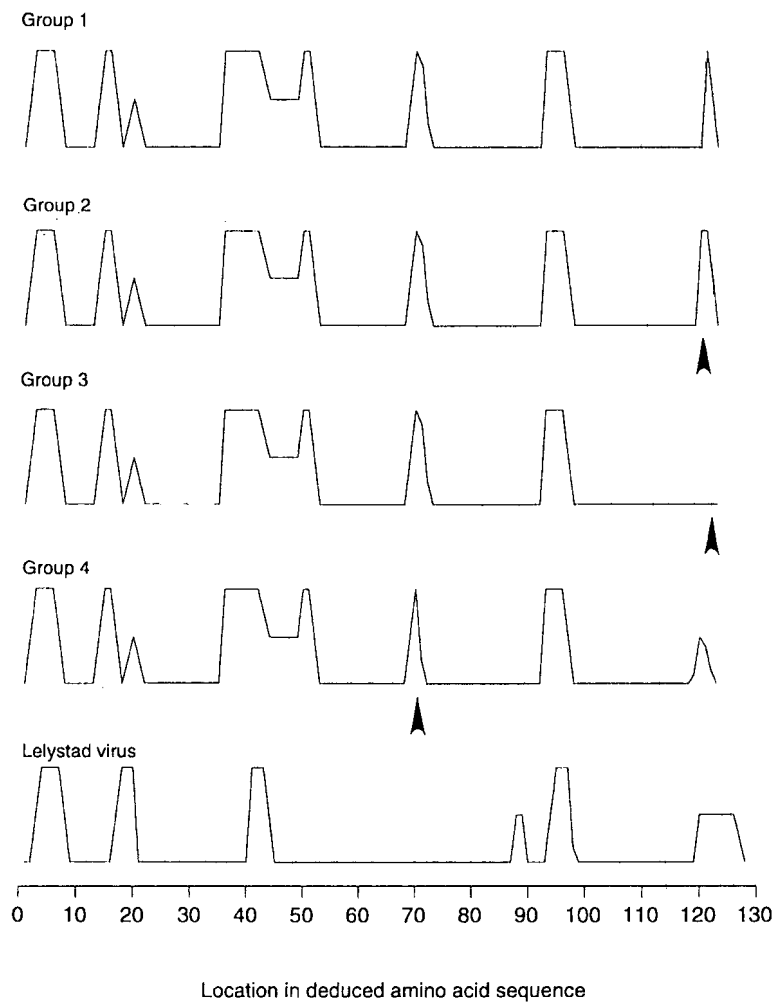


Fig. 5. Chou-Fasman turns shown for each antigenic group are representative of all PRRS virus isolates that were sequenced. Note that group specific changes marked with arrows are located in the carboxy terminal CF turns for antigenic groups II and III, and in the 6th CF turn for antigenic group IV

(Fig. 5). Specifically, the carboxy terminal CF trun representing group II viruses is broader at the base and its apex is flatter relative to the homologous CF turn that represents group I viruses. No turn is present in this location for group III viruses and the height of the CF turn representing the group IV virus in this location is markedly shorter than its counterparts in groups I and II. The shoulder in the peak representing the CF turn at position 70 in the group IV virus is noticeably narrower than the shoulder of the peaks representing the homologous CF turn in groups I, II, and III. No distinct group specific differences were observed between the 4 American antigenic groups when the deduced amino acid sequences were analyzed by the other parameters of the GCG package as specified in Materials and methods.

Discussion

The principal objectives of the current study were to further characterize the antigenic nature of the highly conserved 15 kD nucleocapsid protein of PRRS virus and to determine if a sufficient degree of antigenic diversity exists in this protein that could provide the basis of a marker system for PRRS viruses. Published data prior to the present study suggest that at least 5 epitopes in various combinations are associated with the 15 kD nucleocapsid protein of American and European PRRS virus isolates. Three of these epitopes are most likely discontinuous because of their inability to be recognized by MAbs in Western immunoblotting. These epitopes are represented by MAbs SDOW 17, VO 17 [7, 29], and ICH5 [29]. The MAbs SDOW 17 and VO 17 represent 2 distinctly different epitopes. The original characterization of MAbs SDOW 17 and VO 17 by Nelson et al. [26] demonstrated that MAb SDOW 17 reacted with all 63 American and 57 European isolates tested. In contrast, MAb VO 17 only recognized the American isolates. The MAb ICH5 appears to be different from MAb SDOW 17 because it failed to react in the IFA with 3 American isolates [16]. The 2 continuous epitopes are represented by MAbs WBE 1 and WBE 4 respectively [18]. Their distinctness was demonstrated by their reactivity pattern with a combined total of 44 European and 11 American PRRS virus isolates [8, 16]. Both MAbs reacted with all European isolates tested but while MAb WBE 4 did not react with any of the 11 American isolates, MAb WBE 1 reacted with 3.

In addition to the 5 epitopes described above, a sixth epitope specific for the 15 kD protein may also exist. It is continuous and is represented by MAb P3/27 which was originally shown to react with all 14 European and 12 American isolates tested [36]. In a different study [16], MAb P3/27 was shown to have the same IFA reactivity pattern as MAb WBE 1. In that study, both MAbs reacted with all 21 European and 3 American isolates tested. There are no published accounts that clearly indicate whether or not MAb P3/27 reacts with the 8 American isolates that were not recognized by WBE 1 [8] or that WBE 1 reacts with the 12 American isolates recognized by P3/27 [36]. Consequently, it remains to be clarified whether or not MAbs P3/27 and WBE 1 represent the same or different epitopes. Nevertheless, the two published accounts which indicate that MAb P3/27 reacted with 15 of 15 American isolates tested [16, 36] and that MAb WBE 1 did not react with 8 different American isolates [8] suggest that 2 MAbs represent different epitopes.

In the present study, 5 distinct continuous epitopes designated EpORF7-A through E were revealed among American isolates further contributing to our knowledge of the antigenic nature of the 15 kD protein of PRRS virus. The uniqueness of the individual epitopes was confirmed by the results of blocking and additivity ELISA assays. In the blocking assay (Table 4), heterologous MAbs failed to inhibit binding of MAbs representing each of the 5 different epitopes ($p < 0.001$). While in the additivity assay (Table 3), the optical density measured following treatment of antigen preparations with MAb representing each epitope was significantly enhanced ($p < 0.001$) after additional treatment with heterologous MAbs but not with homologous MAbs. At least 4 of the 5 continuous

epitopes were present in all viruses tested with the exception of the Lelystad virus which contained only EpORF7-A as determined by both IFA and Western immunoblotting (Table 2). Consequently, it is possible that EpORF7-A may represent the same epitope as P3/27. Analysis of a broad panel of European PRRS virus isolates will be necessary to determine which if any of the remaining 4 continuous epitopes are present in European isolates.

In an effort to determine the approximate locations of EpORF7-A through E in the 15 kD protein the deduced amino acid sequences were computer analyzed using the GCG package. None of the parameters as described in the Materials and Methods section could be correlated to antigenic sites with the possible exception of Chou-Fasman (CF) profiles, as indicated by number, location, shape and size of CF turns. Individual CF turns are a reflection of secondary structure which can influence antigenicity. Unique differences in the CF profiles of antigenic groups II, III and IV relative to the CF profile of group I were observed at locations encompassing amino acids 120, 122 and 70 respectively (Fig. 5). These amino acids represent unique group specific substitutions which appear to be correlated to the absence of EpORF7-B, C and D in antigenic groups II, II and IV respectively. This observation and the demonstration of inhibition mediated by MAbs representing EpORF7-E with MAbs representing EpORF7-B, C and D (Fig. 2) suggest that the amino acid sequences representing EpORF7-B, C and D are relatively close to one another at the carboxy terminal end of the 15 kD protein. The absence of reciprocal or one-way inhibition between MAbs representing EpORF7-A and EpORF7-B, C, D and E in the blocking assay (Table 4) suggests that EpORF7-A is spatially distant from these epitopes.

The available data is not sufficient to predict the probable location of EpORF7-A or E. However, a recent study by Rodriguez et al. [29] demonstrated the presence of a continuous epitope in the central region of the 15 kD protein in both a European (Olot/91) and an American (Quebec 807/94) PRRS virus isolate by reacting a panel of MAbs with fragments of recombinant 15 kD protein by Western immunoblotting. They further demonstrated that a highly conserved amino acid sequence extended from amino acid 50–90 in 3 American and 2 European isolates which included the Lelystad virus. Amino acids 50–66 were associated with the common epitope. These observations suggest that the common epitope described by Rodriguez et al. could be identical to EpORF7-A. Competitive assays between MAbs representing EpORF7-A and the common epitope described by Rodriguez et al. may answer this question.

The degree of antigenic diversity demonstrated among American isolates in the present study was less than expected. The distribution of the 5 epitopes associated with American PRRS isolates permitted their subdivision into 4 antigenic groups that were distinctly different from the European Lelystad virus. However, antigenic groups I and II represented 84.1% and 11.6% of all isolates tested while groups III and IV represented the remaining 4.3% of the isolates. This limited antigenic diversity among American PRRS viruses as revealed by the panel of MAbs specific for the 15 kD protein indicates that a useful marker system will require a broader panel of MAbs representing epitopes of other PRRS virus pro-

teins. Alternatively, more sensitive MAb based methodologies could be used such as the signature analysis that was originally described by Wands et al. [32]. This technique uses a panel of MAbs to generate individual antigen binding curves that reflect both the affinity by which individual MAbs bind to an epitope and the density of epitopes on the surface of the virus.

The reliable use of MAbs to identify PRRS virus isolates will also depend on the stability of the epitopes that they represent. Previous work by Le Gall et al. [16, 17] failed to demonstrate any antigenic change in the 15 kD protein following one or two passages of 6 virus isolates through pigs. However, they did observe antigenic change in an epitope of glycoprotein 3 in 2 of the 6 isolates. Consequently, the relative stability of individual epitopes that form the basis of a virus identification system must be thoroughly evaluated.

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