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Short communication

Production of pseudorabies virus recombinant glycoprotein B and its use in an agar gel immunodiffusion (AGID) test for detection of antibodies with sensitivity and specificity equal to the virus neutralization assay.

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A B S T R A C T

Pseudorabies virus (PrV) causes Aujeszky's disease (AD), which affects mainly swine, but also cattle, sheep, and wild animals, resulting in substantial economic losses due to animal mortality and lost productivity worldwide. To combat PrV, eradication programs using PrV strains lacking the gene encoding glycoprotein E (gE) are ongoing in several countries. These eradication programs have generated a currently unmet demand for affordable, easy-to-use, and sensitive tests that can detect PrV infection in pigs infected with either wild-type virus or vaccine strain (gE-deleted) virus. To meet this demand, we used the baculovirus-insect cell system to produce recombinant glycoprotein B (gB) as antigen for an immune assay. The high GC-content (70% average) of the gB gene from the Argentinian PrV CL15 strain necessitated the use of betaine as a PCR enhancer to amplify the extracellular domain. Recombinant gB was expressed at high levels and reacted strongly with sera from PrV infected pigs. We used the recombinant gB to develop an agar gel immunodiffusion (AGID) test for detection of PrV antibodies. Compared to the gold standard virus neutralization (VN) assay, the AGID sensitivity and specificity were 95% and 96.6% respectively. Thus, recombinant gB produced in the baculovirus-insect cell system is a viable source of antigen for the detection of PrV antibodies in AGID tests. Considering its relatively lower cost, simplicity of use and result interpretation, our AGID is a valuable alternative tool to the VN assay.

Pseudorabies virus (PrV), also known as Suid Herpesvirus 1 (SHV-1), is the causative agent of Aujeszky's disease, a highly virulent disease that continues to affect the pork industry in many countries, including Argentina (Mettenleiter, 2000). In piglets, Aujeszky's disease causes central nervous system symptoms and is characterized by an acute and often fatal infection. In older pigs, it causes, among others, encephalitis, pneumonia and abortion (Nauwynck, 1997). Apart from pigs, Aujeszky's disease also affects cattle, sheep, and wild animals (Zanella et al., 2012).

Due to its associated mortality and morbidity, Aujeszky's disease has a substantial economic impact through loss of sales and reduced productivity (Bech-Nielsen et al., 1995). Cost/benefit analyses consistently indicate that eradication of the disease produces substantial economic benefits (McInerney and Kooij, 1997), and eradication programs have either been completed or are underway in several countries.

In Argentina, a PrV eradication program was started in 2010 under the auspices of the National Health Service and Food Quality (SENASA) (Resolution 474/2009). In the first stage of this program, the prevalence of PrV in Argentina was evaluated by determining the serological status of swine across the country. The results from this study showed that 19.1% of farms had infected animals, and that 9% of sows were infected (Serena et al., 2011). The presence of infection imposes restrictions on trade, and currently, facilities that provide animals for reproduction and reproductive materials (semen) must be officially certified as PrV-free. Similarly, commercial pig farms can be certified as PrV-free for export purposes.

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Eradication programs currently are based on the use of marked vaccines that lack the nonessential glycoprotein E (gE), enabling the detection of wild type PrV-infected pigs in vaccinated populations by using serological assays to detect anti-gE antibodies. When vaccination is stopped, the pig population will be fully susceptible to PrV infection. At that point, not only gE-positive PrV strains will threaten PrV-free status but also gE-negative vaccine strains that continue to circulate.

Thus, there is a demand for sensitive and specific tests that can detect exposure to PrV by detecting specific antibodies in pigs vaccinated with gE-deleted PrV strains as well as those exposed to wild-type virus. Antibodies can be detected both by non-recombinant antigens, which typically consist of lysates of virus-infected cells, and recombinant purified viral glycoproteins. Antibody detection using recombinant antigens is preferable to the use of non-recombinant antigens, as their production does not require live PrV virus. Moreover, recombinant antigens can easily be purified to high purity, and are consequently more defined than non-recombinant antigens.

Several ELISA tests have been developed using recombinant PrV glycoproteins as antigen. However, these require specialized laboratory facilities or expensive commercial equipment. In contrast, Agar Gel Immunodiffusion (AGID) tests are low cost, very easy to perform, do not require expensive equipment and can be read with the naked eye. Although a PrV AGID test using non-recombinant antigens has previously been reported (Smith and Stewart, 1978), no AGID test based on recombinant antigens has been reported for PrV diagnosis, and there are no commercial kits available for this purpose. Thus, we set out to develop a simple AGID test for PrV based on recombinant proteins.

PrV encodes three major glycoproteins (gB, gC and gD) that induce protective immune responses (van Rooij et al., 1998; Yoon et al., 2006). Several B-cell epitopes on gB and gC, as well as T-cell epitopes on gC can induce both humoral and cytotoxic responses (van Rooij et al., 1998, 2000). Glycoprotein B (gB) is a major constituent of the viral envelope that occurs as a complex consisting of three forms: gBa, gBb and gBc. The primary translation product consists of 913 amino acids, and the mature form of the glycoprotein after removal of the signal sequence (gBa) comprises 855 amino acids (Fig. 1A). The mature protein is a 120 kDa polypeptide that can carry up to 6 *N*-linked glycans (Fig. 1A), and can be cleaved between Arginine 444 and Serine 445 by a cellular protease to yield gBb (68 kDa) and gBc (55 kDa) (Hampl et al., 1984; Whealy et al., 1990; Wolfer et al., 1990). Glycoprotein B is indispensable for viral replication due to it essential role in membrane fusion events during virus infection and cell-to-cell spread of virus. Furthermore, gB is highly conserved, present in all PrV strains, and is highly immunogenic, leading to high anti-gB antibody titers in infected and vaccinated animals (Robbins et al., 1987). Thus, we selected gB as the antigen for our study.

To produce recombinant gB, we chose the baculovirus insect cell system (BICS), as this system has the ability to produce large quantities of properly folded, correctly processed and antigenically active glycoproteins. Furthermore, these can be secreted into the extracellular medium to facilitate purification. Finally, our recent experiences with the BICS demonstrated our ability to use this system to produce large amounts of recombinant viral glycoproteins (Larsen et al., 2013; Serena et al., 2013). Thus, we designed a soluble, secreted PrV gB that lacks the signal anchor (gBa-sol), with tags added to facilitate purification and detection of the recombinant protein (Fig. 1C)

PrV viral DNA was obtained by infecting Continuous Porcine Kidney (CPK) cells with the CL-15 PrV strain as described previously (Echeverria et al., 1994). Infected cells were collected, treated with proteinase K and total DNA was extracted using a commercial kit (Promega). The gBa-sol ORF was PCR amplified using primers gBa SP (CAC CGC CAC CCC CAA CGA CGT CTC C) and gBa ASP (CTA GTT GTG GTC CAC CTT GAC CAC GC) (IDTDNA, Coralville, IO). The underlined CACC sequence in gBa SP was introduced to facilitate directional cloning into the pENTR™/D-TOPO® vector (Thermo Fisher Scientific, Carlsbad, CA), and a stop codon was introduced in the gBa ASP primer. As the gBa-sol ORF has an unusually high GC content (70% average, 96% maximum, 50% minimum, 50-bps sliding window analysis, Fig. 1B), we used Phusion DNA polymerase (New England Biolabs, Beverly, MA) in a reaction mixture supplemented with 1 M betaine, an effective PCR enhancer that allows for the amplification of extremely GC-rich sequences (Henke et al., 1997). The PCR included 0.2 μL of Phusion DNA polymerase, 10 μL of Phusion GC Buffer, all four dNTPs at final concentrations of 0.2 μM each, primers at a final concentration of 1 μM each, and 10 μL of 5 M betaine free-base (Sigma-Aldrich, St. Louis, MO) in a total volume of 50 μL. The cycling conditions were as follows: an initial denaturation step at 98° for 30 s, followed by 5 cycles of 1) 20 s denaturation at 98 °C, 2) 20 s annealing at 66, and 3) 120 s extension at 72°, followed by 25 cycles of 1) 20 s denaturation at 98 °C, 20 s annealing at 69 °C, and 120 s extension at 72 °C, followed by a final extension for 10 min at 72 °C. The PCR was performed in a Biometra TProfessional thermal cycler (Biometra, Göttingen, Germany) and the amplification products were purified on a 1% (w/v) agarose gel in TAE buffer, stained with ethidium bromide, and extracted using a commercial kit (QIAquick Gel Extraction kit, Qiagen), according to the manufacturer's instructions. The PCR product was obtained in a good yield, and matched the expected size (2038 bps, Fig. 2A).

Following gel purification, the PCR product was cloned into the pENTR™/D-TOPO® vector using the Directional TOPO® Cloning Kit according to the manufacturer's instructions. Clones containing the gBa-sol insert were amplified in LB broth and plasmids were purified with a commercial kit (Qiagen). To confirm the sequence of the insert, plasmids were sequenced and an error-free clone was used in an LR recombination reaction with Ac6.9GT baculovirus DNA, as described previously (Toth et al., 2011). Upon recombination with this viral vector, the gBa-sol ORF is fused with the Honeybee mellitin signal sequence, as well as StrepTagII and 8xHis purification tags. Baculovirus clones were plaque-purified once and amplified in Sf9 cells, as described previously (Summers and Smith, 1987).

Baculovirus clones were initially screened by immunoblotting using a monoclonal anti-5xHis antibody (Thermo Fisher Scientific), and a clone expressing PrV gBa-sol was identified, further amplified in Sf9 cells (Fig. 2B) and sequenced (GenBank accession number KT223434). Further SDS-PAGE and immunoblotting analysis were performed using infected High Five™ cell supernatants collected at three days post-infection with a mouse anti-HisTag monoclonal antibody (GE Healthcare) and a swine polyclonal PrV antibody as the primary antibodies, and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody or rabbit anti-swine antibody (Sigma) as the secondary antibody (Fig. 2C). The supernatant culture containing recombinant gBa-sol protein was concentrated four-fold using a 14 kDa cutoff cellulose membrane (Sigma–Aldrich) with polyethylene glycol 6000 (Biopack, Buenos Aires, Argentina). Protein concentrations of the concentrated supernatant were determined by Bradford assay.

Virus neutralization assays were performed with serial twofold dilutions of porcine sera heat-inactivated at 56 °C for 30 min, prepared using serum-free medium in a 96-well flat-bottomed tissue culture plates (Nunc, Rochester, NY). Virus suspension with a titer of 100 $TCID_{50}$ in 25 μL was added to each serum dilution well and the mixture was incubated for 1 h at 37 °C and 5% CO_2, followed by addition of 100 μL of CPK cell suspension (3×10^5 cells/mL) to each well and incubation for 72 h, as described previously (Komaniwa et al., 1981). Although recent reports indicate superior sensitivity using 24 h incubations at 4 °C (OIE, 2012), we used a 1 h incubation at 37 °C for ease and speed of detection. Appropriate serum, virus

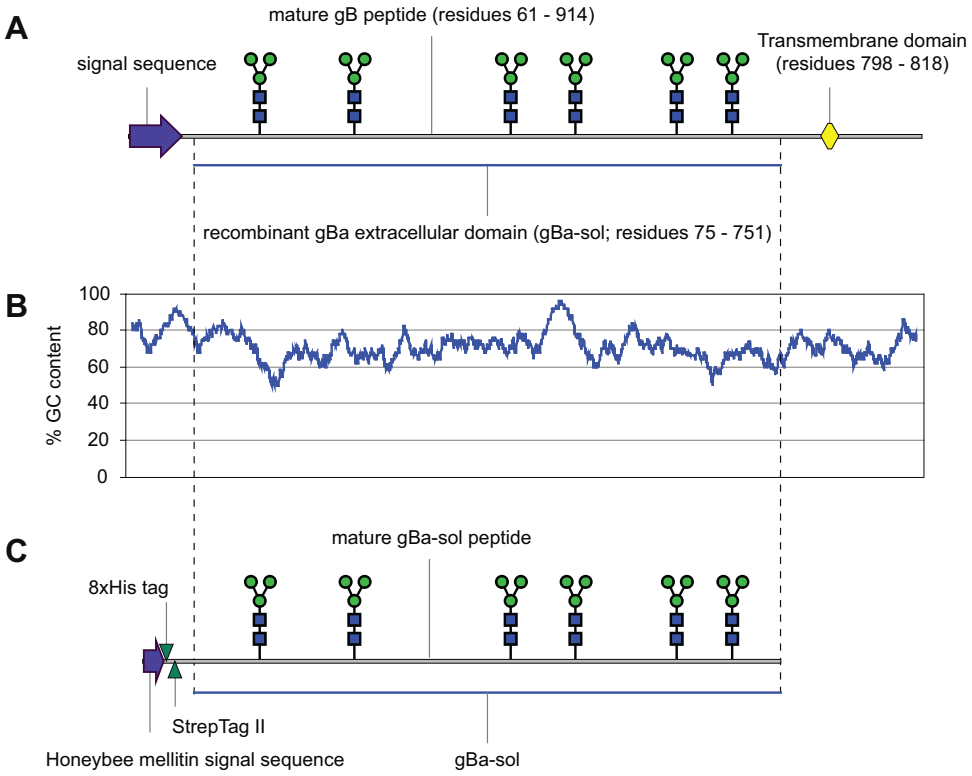

Fig. 1. A schematic overview of native (A) and recombinant (C) PrV glycoprotein B (gB) with potential *N*-glycosylation sites indicated by cartoons. The portion used in the recombinant protein, and other relevant protein features are indicated. (B) Guanine-cytosine content of the gB protein ORF in a 50 bps sliding window analysis.

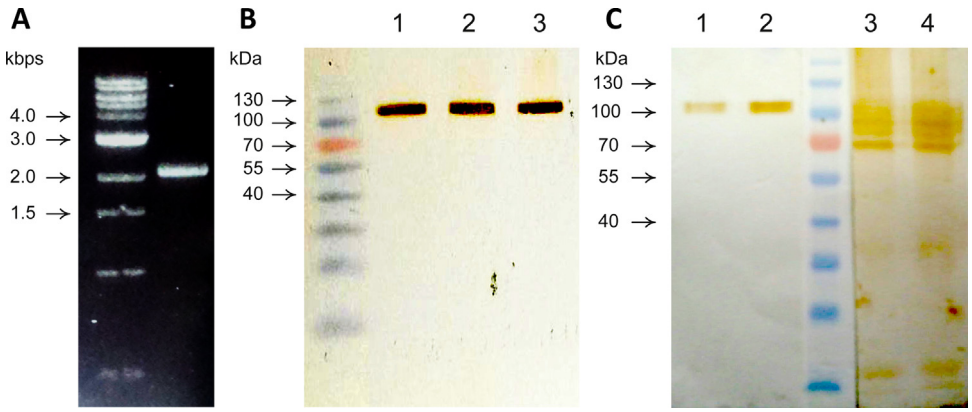

Fig. 2. (A) PCR amplification of the gBa-sol ORF. 1000 bps marker (NEB, left lane), PCR amplification reaction containing the 2038 bps gBa-sol amplimer (right lane) (B) Western blot screen showing three positive clones (lanes 1, 2, 3) (C) Western blot of concentrated gB showing a positive reaction with serum from PrV-infected pigs (lanes 1, 2) and anti-His tag (lanes 3, 4). The MW marker in B and C is PageRuler™ Prestained Protein Ladder (Fermentas).

and cell control were included, and plates were observed under a microscope to assay cytopathic effects.

AGID tests were performed using 1% agar in different buffers: 140 mM boric acid with 50 mM NaOH (pH 8.6, Agar A), 200 mM Tris buffer with 0.85% NaCl (pH 7.2, Agar B), or 50 mM Tris buffer with 0.85% NaCl (pH 8.0, Agar C). Tests were carried out in Petri dishes with six 4 mm wells (~40 μL volume), and incubated at room temperature in a humid chamber for 48 h. Different protein concentrations (1000, 500, 250 and 125 μg/ml) and dilutions of PrV positive serum samples (undiluted, 1:2, 1:4 and 1:8) were tested. A panel of 297 swine serum samples previously analyzed by virus neutralization (VN) was obtained from the serum bank of the Virology Laboratory of the Veterinary Faculty of La Plata City (Buenos Aires, Argentina) and used for AGID tests with the gBa-sol protein as the antigen.

Clean precipitation lines were obtained with 500 μg/mL protein, and undiluted PrV positive serum samples in Agar A. Under these conditions, we did not observe positive reactions with PrV-negative control sera (Fig. 3). When we compared the results obtained using our newly developed AGID test to those obtained with VN assays, 170 out of 297 pig serum samples were positive in both assays, nine serum samples that were positive by neutralization scored negative with AGID, and four samples that were negative by neutralization scored positive with AGID. The Kappa index (0.90, 95% confidence interval 0.86–0.95), sensitivity (SS, 94.97% (170/179)) and specificity (SP, 96.61% (114/118)) values were satisfactory, and indicate substantial agreement between the two methods. Thus, the reliability of our AGID test was comparable to that of the gold standard virus neutralization test commonly used in diagnostic laboratories.

Fig. 3. Sample AGID test with recombinant PrV-1 gBa-sol. C: PrV-positive reference serum. 1: negative VN serum, 2: 1:8 VN positive serum, 3: 1:32 VN positive serum. gB: concentrated cell culture supernatant at 500 μg/mL. Serum samples were used undiluted.

In summary, we expressed recombinant PrV gB using the baculovirus-insect cell system, and used the protein as antigen to successfully develop an AGID test. The advantages of using recombinant viral antigens as compared to non-recombinant antigens for immuno-detection include their relatively straightforward and safe production. As recombinant antigens lack viral nucleic acids or other viral proteins, they are less toxic and can be produced even if the virus cannot be cultivated. We engineered the recombinant gB protein to allow easy detection, as well as recovery and purification of the protein from the supernatant cell culture. Our AGID employing recombinant PrV gB is a simple and reliable serological assay based on a homogeneous and continuously available source of antigen that guarantees its use and availability for veterinary PrV diagnosis.

PrV infection can be diagnosed based on virus isolation, histopathology, immunohistochemical staining and antibody detection by virus neutralization (VN) and ELISA assays. VN has been recognized as the reference method for serology and is considered the gold standard test for PrV detection. Nonetheless, VN has been widely replaced by ELISA assays because of their suitability for large-scale testing. Both VN and ELISA assays are expensive, time consuming, and require specialized equipment and trained technicians. We demonstrate that our new AGID test is a valuable alternative tool that identifies positive animals with a specificity and sensitivity similar to that of VN, with the benefit that it is a fast technique that can be performed at any veterinary diagnostic laboratory.

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References

Bech-Nielsen, S., Bowman, G.L., Miller, G.Y., Orloski-Snider, K.A., Burkholder, R.H., Dodaro, S.J., 1995. Pseudorabies (Aujeszky's disease) eradication progress and program costs in Ohio, USA. Prev. Vet. Med. 22, 41–53.

Echeverria, M.G., Norimine, J., Galosi, C.M., Oliva, G.A., Etcheverrigaray, M.E., Nosetto, E.O., Tohya, Y., Mikami, T., 1994. The genotype of Aujeszky's disease viruses isolated in Argentina. J. Vet. Med. Sci. 56, 985–987.

Hampl, H., Ben-Porat, T., Ehrlicher, L., Habermehl, K.O., Kaplan, A.S., 1984. Characterization of the envelope proteins of pseudorabies virus. J. Virol. 52, 583–590.

Henke, W., Herdel, K., Jung, K., Schnorr, D., Loening, S.A., 1997. Betaine improves the PCR amplification of GC-rich DNA sequences. Nucl. Acids Res. 25, 3957–3958.

Komaniwa, H., Fukusho, A., Shimizu, Y., 1981. Micro method for performing titration and neutralization test of hog cholera virus using established porcine kidney cell strain. Natl. Inst. Anim. Health Q. (Tokyo) 21, 153–158.

Larsen, A., Gonzalez, E.T., Serena, M.S., Echeverria, M.G., Mortola, E., 2013. Expression of p24 gag protein of bovine leukemia virus in insect cells and its use in immunodetection of the disease. Mol. Biotechnol. 54, 475–483.

McInerney, J., Kooij, D., 1997. Economic analysis of alternative AD control programmes. Vet. Microbiol. 55, 113–121.

Mettenleiter, T.C., 2000. Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis—state of the art. Vet. Res. 31, 99–115.

Nauwynck, H.J., 1997. Functional aspects of Aujeszky's disease (pseudorabies) viral proteins with relation to invasion, virulence and immunogenicity. Vet. Microbiol. 55, 3–11.

OIE, 2012. Manual of standards for diagnostic test and vaccines for terrestrial animals, Aujeszky's Disease (Chapter 2.1.2) http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.01.02_AUJESZKYS_DIS.pdf.

Robbins, A.K., Dorney, D.J., Wathen, M.W., Whealy, M.E., Gold, C., Watson, R.J., Holland, L.E., Weed, S.D., Levine, M., Glorioso, J.C., et al., 1987. The pseudorabies virus gII gene is closely related to the gB glycoprotein gene of herpes simplex virus. J. Virol. 61, 2691–2701.

Serena, M.S., Geisler, C., Metz, G.E., Corva, S.G., Mortola, E.C., Larsen, A., Jarvis, D.L., Echeverria, M.G., 2013. Expression and purification of Suid Herpesvirus-1 glycoprotein E in the baculovirus system and its use to diagnose Aujeszky's disease in infected pigs. Protein Expr. Purif. 90, 1–8.

Serena, M.S., Metz, G.E., Corva, S.G., Mortola, E.C., Echeverria, M.G., 2011. A differential ELISA based on recombinant immunodominant epitopes of the gE gene of SHV-1 in a baculovirus-insect cell system to discriminate between pigs infected naturally with pseudorabies and vaccinated pigs. J. Virol. Methods 171, 388–393.

Smith, P.C., Stewart, W.C., 1978. Agar-gel immunodiffusion assay for pseudorabies virus antibody. J. Clin. Microbiol. 7, 423–425.

Summers, M.D., Smith, G.E., 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin, Texas Agricultural Experiment Station, College Station, TX.

Toth, A.M., Geisler, C., Aumiller, J.J., Jarvis, D.L., 2011. Factors affecting recombinant Western equine encephalitis virus glycoprotein production in the baculovirus system. Protein Expr. Purif. 80, 274–282.

van Rooij, E.M., Haagmans, B.L., de Visser, Y.E., de Bruin, M.G., Boersma, W., Bianchi, A.T., 1998. Effect of vaccination route and composition of DNA vaccine on the induction of protective immunity against pseudorabies infection in pigs. Vet. Immunol. Immunopathol. 66, 113–126.

van Rooij, E.M., Haagmans, B.L., Glansbeek, H.L., de Visser, Y.E., de Bruin, M.G., Boersma, W., Bianchi, A.T., 2000. A DNA vaccine coding for glycoprotein B of pseudorabies virus induces cell-mediated immunity in pigs and reduces virus excretion early after infection. Vet. Immunol. Immunopathol. 74, 121–136.

Whealy, M.E., Robbins, A.K., Enquist, L.W., 1990. The export pathway of the pseudorabies virus gB homolog gII involves oligomer formation in the endoplasmic reticulum and protease processing in the Golgi apparatus. J. Virol. 64, 1946–1955.

Wolfer, U., Kruft, V., Sawitzky, D., Hampl, H., Wittmann-Liebold, B., Habermehl, K.O., 1990. Processing of pseudorabies virus glycoprotein gII. J. Virol. 64, 3122–3125.

Yoon, H.A., Aleyas, A.G., George, J.A., Park, S.O., Han, Y.W., Kang, S.H., Cho, J.G., Eo, S.K., 2006. Differential segregation of protective immunity by encoded antigen in DNA vaccine against pseudorabies virus. Immunol. Cell Biol. 84, 502–511.

Zanella, E.L., Miller, L.C., Lager, K.M., Bigelow, T.T., 2012. Evaluation of a real-time polymerase chain reaction assay for Pseudorabies virus. J. Vet. Diagn. Invest. 24, 739–745, LID–10.1177/1040638712447279.