Journal of Virological Methods 202 (2014) 15–18

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Production of equine herpesvirus 1 recombinant glycoprotein D and development of an agar gel immunodiffusion test for serological diagnosis

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Article history:
Received 31 October 2013
Received in revised form 24 February 2014
Accepted 27 February 2014
Available online 11 March 2014

Keywords:
Agar gel immunodiffusion test
Equine herpesvirus 1
Recombinant glycoprotein D
Virus neutralization

ABSTRACT

Equine herpesvirus 1 and 4 (EHV-1 and 4) infect most of the world's horses, causing serious clinical illness. Viral glycoproteins have been identified as the immunodominant antigens that generate the antiviral serological responses to EHV-1 and EHV-4 in infected horses. Here, glycoprotein D of EHV-1 was expressed by a recombinant baculovirus, purified and evaluated by a simple agar gel immunodiffusion test (AGID). Compared with virus neutralization, serological analysis by AGID showed good specificity (100%) and sensitivity (99.5%). The estimated Kappa values for repeatability and reproducibility were satisfactory. Thus, this rapid, inexpensive, simple and highly specific AGID test seems to be a valuable alternative tool for serological detection of antibodies against both EHV-1 and EHV-4.

Equine herpesvirus 1 (EHV-1) and Equine herpesvirus 4 (EHV-4) are ubiquitous herpesviruses that infect most of the world's horses at some time during their lives, causing respiratory disease, abortion and neurological disorders (Allen and Bryans, 1986). After the initial lytic phase of infection in the equine respiratory tract, EHV-1 and EHV-4 enter a latent state in both circulating lymphocytes and lymphocytes in draining lymph nodes, as well as in sensory nerve-cell bodies within the trigeminal ganglia. Recurrent shedding of the virus from asymptomatic carriers may spread the disease in the equine population (Baxi et al., 1996). Several studies have identified glycoproteins B, C, and D (gB, gC and gD, respectively) as the immunodominant antigens that generate antiviral serological responses to EHV-1 and EHV-4 in infected horses (Allen et al., 1992). EHV-1 and EHV-4 are closely related both genetically and antigenically. Diagnoses of both viruses are based on virus isolation, histopathology, immunohistochemical staining, or antibody detection, but they can show cross-reactivity in many serological tests such as virus neutralization and some ELISAs (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.05.09_EQUINE_RHINO.pdf, access September 24, 2013). Serological tests such as virus neutralization and ELISA require special facilities or commercial equipment. In contrast, the agar gel immunodiffusion test (AGID), which was designed for other equine viruses as an effective tool for the detection of specific antibodies (Coggins and Norcross, 1970), is a fast technique that does not require special conditions and can be performed at any veterinary diagnostic laboratory.

In this work, a purified recombinant gD of EHV-1 was produced and evaluated by a simple AGID test performed to examine a serum

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http://dx.doi.org/10.1016/j.jviromet.2014.02.025
0166-0934/

samples with the same specificity and sensitivity as virus neutralization in a short time.

For recombinant gD production, rabbit kidney RK13 cells (Argentine Cell Bank Association, Argentina) infected with the EHV-1 AR8 strain were used. Infected cells were collected, treated with proteinase K (Promega, Madison, Wisconsin, USA) and total DNA was extracted with a commercial kit (Promega). DNA was quantified by measuring absorbance at an OD_{260}/OD_{280} ratio in a SmartSpec™ 3000 spectrophotometer (BIO-RAD, California, USA). A polymerase chain reaction (PCR) was used to generate a 1373-bp fragment encompassing the EHV-1 gD open reading frame (ORF) flanked by 5′SnaBI and 3′XmaJI sites, using a primer pair (5′-AGTTACGTATTTATGCCTGCTGTGCTG-3′ and 5′-GTTTACCTAGGCTGGGTATATTTAACATCC-3′). The PCR was carried out in a Mastercycler Gradient (Eppendorf, Hamburg, Germany), in a final volume of 25 μl, using 2.5 μl of 10X Taq buffer with KCl, 2.5 U of Taq DNA polymerase (Fermentas, Tecnolab, Buenos Aires, Argentina), 1.5 μM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Promega), 0.5 μM of each primer and 2 μl of DNA sample (concentration 5.6 μg/ml). The DNA was amplified with an initial denaturation step of 94 °C for 4 min, followed by 30 cycles consisting of 94 °C denaturation for 30 s, 60 °C annealing for 20 s, and 72 °C extension for 1 min. The PCR products were run on 1.5% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM ethylenediamine tetra-acetic acid–EDTA–, pH 8), stained with ethidium bromide at a final concentration of 0.5 μg/ml and purified using a gel extraction kit Wizard SV Gel & PCR Clean Up (Promega). The PCR product coding for EHV-1 gD was cloned into the vector pCR2.1-TOPO using the TOPO TA Cloning Kit (Life Technologies Corporation, Carlsbad, California, USA) and then digested with restriction endonucleases corresponding to restriction sites added to the primers. The fragment obtained corresponding to the gD gene was inserted into the multiple cloning site of the transfer plasmid pFastBacHTB between the StuI and XbaI sites (isoschizomers of SnaBI and XmaJI, respectively) and transformed into DH10Bac cells (Invitrogen, Carlsbad, California, USA). These restriction sites allowed fusion of EHV-1 gD in frame with the N-terminal 6X His tag and controlled by *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin promoter. The pFastBac-gD construction was sequenced using the primers pFastBacF: 5′-TATTCCGGATTATTCATACC-3′ (3994–4014) and pFastBacR: 5′-TTCAGGTTCAGGGGGAGGTG-3′ (4298–4318) and the sequence of the resulting ORF was examined using the Vector NTI Advance ™ software version 11.0 (Invitrogen). Recombinant baculovirus was obtained in *Spodoptera frugiperda* (Sf21) cells (Gibco, Grand Island, NY, USA) transfected with recombinant bacmid DNA. For glycoprotein expression High Five cells (Invitrogen) were infected with 1PFU of recombinant baculovirus/cell. The infected cell extracts were harvested at 12, 24 and 48 h post-infection and examined by 10% SDS-PAGE and Western Blot according to standard procedures. The location of gD EHV-1 in infected insect cells was determined by immunofluorescence using horse anti-EHV-1 polyclonal antibody, provided by Dr. T. Kumanomido (Equine Research Institute, Japan Racing Association, Tochigi, Japan) and anti-horse fluorescein isothiocyanate conjugated (Sigma–Aldrich, Saint Louis, Missouri, USA). Visualization was performed on an epifluorescence microscope Olympus System – Model BHS (Olympus Corporation, New York, USA).

After determining the time of greatest expression of gD, infected cells were harvested, lysed by freezing and thawing, clarified by centrifugation and purified by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA agarose (Qiagen, Maryland, USA). The elution was performed with 50 mM sodium phosphate buffer and 500 mM sodium chloride at decreasing pHs (6, 5 and 4). SDS-PAGE and Western blot were used to detect the purified protein. Purified gD was concentrated in a cellulose membrane (M.W. 14,000, Sigma–Aldrich) with polyethylene glycol 6000 (Biopack, Buenos Aires, Argentina) and its concentration was determined by the standard method of Bradford, 1976.

The AGID test was performed according to the previously described method for Equine infectious anemia (EIA) virus (Coggins and Norcross, 1970), but using two different agar solutions: one consisting of 1% Difco™ agar granulated (Becton Dickson, Sparks, Maryland, USA) in 0.145 M borate buffer (9 g H3BO3, plus 2 g NaOH per liter) pH 8.6 (±0.2) and the other consisting of 1% Difco agar in Tris buffer 0.2 M and Na Cl 0.85%, pH 7.2. The gD antigen was assayed at decreasing concentrations of 300, 150, 75, 50, 37.5, 18.7 and 9.4 μg/ml using one EHV-1-positive reference horse serum (1:64 and 1:8 neutralizing antibody titers for EHV-1 and -4, respectively) and one negative horse serum (Equine Research Institute, Japan Racing Association, Tochigi, Japan). The AGID was carried out in a Petri dish using a punch of six wells of 4 mm (~40 μl) in diameter and 3 mm apart and incubated at room temperature in a humid chamber. The results were read at 48 h. After determining the optimal concentration of gD, reference sera for EIA virus, Equine Influenza virus (EIV), Equine arteritis virus (EAV) and Equine herpesvirus 2 (EHV-2) were tested. Then, two-fold serial dilutions in phosphate buffer saline (PBS) of an EHV-1-positive horse serum (neutralizing antibody titers 1:128) were examined by AGID. A total panel of 396 serum samples previously tested by virus neutralization and some of which ($n = 38$) showed severe hemolysis were also examined by AGID. In addition, the 10 sera of the panel with 1:256 neutralizing antibody titers were examined in two-fold serial dilutions. Repeatability and reproducibility of AGID were evaluated four times with 32 sera (16 positive and 16 negative) and by four different operators. The Kappa index of concordance was determined at both intra- and inter-operator level.

The virus neutralization test was performed in microplates using two-fold serial dilutions of sera (two replicate wells) mixed with an equal volume (25 μl) of 100 TCID50 of the AR8 EHV-1 strain and after 60 min of incubation in a 5% CO_2 atmosphere, RK-13 cells (100 μl of 5×10^5 cells/ml) were added. The results were read 5 days later and neutralizing antibody titers were expressed as the highest serum dilution that protects 100% of the cell monolayer from virus destruction in both wells. The effectiveness of the present AGID test was compared with the virus neutralization test, and the sensitivity and specificity were evaluated with ROC Curve analysis (Dohoo et al., 2009).

Western blot results showed that EHV-1 gD was expressed in readily detectable antigenic forms of gD after 12 h of infection with a maximum concentration at 48 h. The High Five cells infected with the recombinant baculovirus showed a similar banding pattern in all samples at different times post-infection, with one main band of 65 kDa and another band at a lower concentration (Fig. 1). After purification using Ni-NTA, SDS-PAGE also revealed two specific bands of 58 and 65 kDa in cell samples at 48 h post-infection. Fluorescence specificity was clearly seen on the cell surface and in the cytoplasm but not in cell nuclei (data not shown). After purification, recombinant gD was obtained at 300 μg/ml with a high purity level, as evaluated by the Western blot.

The AGID reaction was observed when the borate buffer was used. Clear precipitation lines were obtained using antigen concentrations of 50 μg/ml and EHV-1-positive reference horse serum (Fig. 2A) and up to a 1/8 dilution of the EHV-1-positive horse serum (Fig. 2B). No positive reactions were observed with reference EIA, EIV, EAV and EHV-2 sera, since these viruses are antigenically, genetically, and pathogenetically distinct from EHV-1 and EHV-4 (Allen et al., 2004).

The AGID test detected 182 negative and 213 positive sera with different neutralizing antibody titers (1:256 $n = 10$; 1:128 $n = 21$; 1:64 $n = 22$; 1:32 $n = 46$; 1:16 $n = 82$ and 1:8 $n = 32$). One serum with doubtful neutralizing antibody titer at a 1:4 dilution was considered

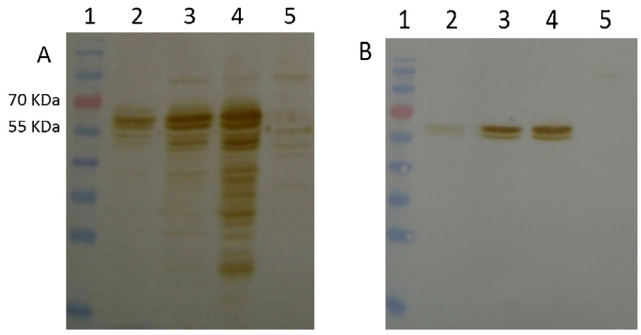

Fig. 1. Western blot analysis of recombinant baculovirus-infected High Five cell lysates. Lane 1: molecular weight marker (PageRuler Prestained Protein Ladder, Thermo Scientific, USA); lanes 2–4: 12, 24 and 48 h post-infection; lane 5: High Five control cells. (A) Serum from an EHV-1-infected horse; (B) gD specific monoclonal antibodies.

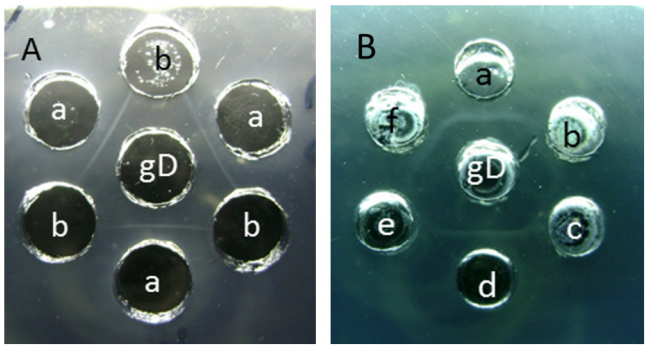

Fig. 2. Agar gel immunodiffusion test with recombinant EHV-1 gD. (A) EHV-1-positive reference horse serum (a) (1:64 and 1:8 virus neutralizing titers for EHV-1 and EHV-4, respectively) and negative horse serum (b); (B) EHV-1-positive horse serum with 1:128 virus neutralizing titer (a and d) and 1:64 (f), 1:32 (b), 1:16 (c) and 1:8 (e) dilutions.

negative by virus neutralization and was not detected by AGID. In addition, 38 hemolyzed samples showed results similar to those with virus neutralization (14 negative and 24 positive). The 10 sera (1:256 neutralizing antibody titers) of the panel examined were also found positive by AGID up to 1:8 dilutions. The comparative results of AGID and virus neutralization are shown in Table 1. The Kappa values for repeatability and reproducibility estimated were satisfactory with an interval range from 0.75 (IC95% 0.71–0.82) to 0.93 (IC95% 0.87–0.98).

In summary, the expression of EHV-1 recombinant gD and its use as antigen to develop an AGID test were described. The EHV-1 gD was expressed by using a recombinant baculovirus and two bands were observed probably due to degradation products or different stages of glycosylation or translation of gD. These findings are in line with those found by other authors, who suggested that the presence of more than one band may be due to incomplete translation or proteolytic degradation (Flowers et al., 1995; Love et al., 1993). After purification, two bands were also detected. However, this result did not interfere with the AGID test. The specific fluorescence observed suggests that the gD was processed correctly and transported in the cells, as it has been shown in previous reports (Love et al., 1993; Whalley et al., 1995).

Serological testing also showed good specificity and sensitivity of AGID, as well as an excellent analytical specificity with poorly preserved samples.

Serological testing can be a useful adjunct procedure for assisting for the diagnosis of horses (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.05.09_EQUINE_RHINO.pdf, access February 8 2014).

EHV-1 and EHV-4 are endemic viral agents with different seroprevalence among horses in most parts of the world. However, in Argentina and other countries, some farms still remain negative for the detection of antibodies, which could indicate no viral activity. In addition, since in Argentina vaccination against EHV-1 and EHV-4 is not mandatory, some farms or breeding facilities are more susceptible to infection. Taking into account the abortigenic potential of EHV-1 (Crabb and Studdert, 1995), the immunological status of animals in these farms should be known and monitored. Moreover, when clinical signs consistent with EHV-1 or EHV-4 infections are present, a rapid and sensitive serological test may be helpful for an early diagnosis and prophylaxis previous to the confirmatory results by other techniques, such as virus isolation or PCR. The serodiagnosis that can differentiate infected from vaccinated animals is based on the significant increases of a four-fold or greater increase in antibody titers in paired sera taken during the acute and convalescent stages of the disease (Allen et al., 2004). However, sometimes, serological results are not reliable, especially when the first sample is not taken at the beginning of the disease (Walter et al., 2013). The virus neutralization test is widely used to detect EHV-1 and EHV-4 antibodies, but requires sterile conditions and adequate facilities which are not available in all laboratories, and the result takes 4–5 days. In contrast, ELISA is a highly sensitive and automatized procedure, mainly useful for a large number of samples. However, in many diagnostic laboratories, the equipment for virus neutralization is not available, veterinarians cannot access to the commercial ELISA test or the number of samples to be examined is scarce.

In this study, we showed that AGID is a simple and reliable serological assay based on a homogeneous and permanent source of antigen that guarantees its use and availability for veterinary diagnosis. It can be used for antibody detection of two-fold dilutions from pure paired samples, and seroconversion can be also shown from second samples which were diluted more than four times with respect to the first sample.

In conclusion, although AGID does not differentiate antibodies against EHV-1 or EHV-4, it seems to be a valuable alternative tool because it identifies positive animals and seroconversion in infected animals, with a specificity and sensitivity similar to those of virus neutralization.

Table 1
Comparison of results obtained from 396 horse sera analyzed by virus neutralization and agar gel immunodiffusion test (AGID).[a]

Test	Virus neutralization		
	+	–	Total
AGID+	213	0	213
AGID–	1	182	183
Total	214	182	396
Sensitivity		99.5%	
Specificity		100%	
Predictive + value		100%	
Predictive – value		99.5%	

[a] + = positive; – = negative.

Acknowledgments

This study was supported by grants from CIC-PBA, the Department for Science and Technology of the National University of La Plata (SeCyT-UNLP), and by ANPCyT (PICT 2011-1123), Argentina. We also thank the technician Claudio A. Leguizamón for his support.

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