Western Blot Assay Using Recombinant p26 Antigen for Detection of Equine Infectious Anemia Virus-Specific Antibodies

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We analyzed the performance of a single-band Western blot (WB) test using recombinant p26 (rp26) capsid protein of equine infectious anemia virus. According to the results obtained, the rp26 WB test is a reliable confirmatory diagnostic tool to be used as a complementary test after an enzyme-linked immunosorbent assay or agar gel immunodiffusion test yielding doubtful results.

Equine infectious anemia (EIA) has a severe economic impact on the equine industry and falls under a regulatory control program in many countries, including Argentina (10). To date, the only routinely approved and used methods for the diagnosis of EIA are the agar gel immunodiffusion (AGID) test and the enzyme-linked immunosorbent assay (ELISA). Even when both tests have been demonstrated to be good diagnostic tools, several factors can contribute to yield conflicting results that need to be confirmed using a reliable and highly specific diagnostic tool. Possible factors contributing to misleading results are (i) low levels of equine infectious anemia virus (EIAV)-specific antibodies as a consequence of an individual animal’s immune response or very recent infection, (ii) samples producing a nonspecific reaction line in the AGID assay because of the presence of nonspecific equine antibodies to cellular or host-derived proteins present on the test antigen, and (iii) replicate samples yielding discordant results by the same or different tests. Western blotting (WB) is used as a confirmatory diagnostic test for viral diseases, since it is a highly sensitive and specific assay.

In the present study, a novel confirmatory single-band WB test was developed and validated according to international guidelines (6, 7, 13) for EIAV antibody detection based on the use of recombinant virus protein p26 (rp26) expressed in Escherichia coli. We designed the WB assay using the p26 viral core protein since, together with gp90 and gp45, it is a main target of the host immune response (5). In fact, EIAV rp26 has proven to be a reliable reagent for AGID and ELISA (1, 2, 11) and is currently included in commercial tests (EIA AGID test kit [VMRD Inc.] and ViraCHEK/EIA [Synbiotics Corp.]). A fragment of the gag gene encoding the protein p26 of EIAV (GenBank/EMBL DNA database accession number DQ452090) was successfully amplified by reverse transcription-nested PCR from total RNA extracted from the spleen tissue of a horse experimentally infected with a native strain of EIAV. The primers were designed according to the complete EIAV Wyoming strain genomic sequence (1F, 5′-GTAATTGGCGCTAAGTCTAG-3′, and 1R, 5′-GAATTCATAGATGGGCTGGAAAC-3′, and 2F, 5′-AAGCTTATAGATGGGCTGGAAACA-3′, and 2R, 5′-AAGCTTGTATCTCTCCAAAGTCC-3′). The amplified fragment was cloned into the pRSetC expression vector (Invitrogen), and rp26 was overexpressed by the induction of bacterial recombinant clones with IPTG (isopropyl-β-D-thiogalactopyranoside) and purified using a nickel-charged resin (Probond; Invitrogen) under native conditions. We fully characterized the rp26 protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, WB with EIAV-positive control reference serum of equine origin (kindly provided by the OIE Reference Laboratory for Equine Infectious Anemia, Maisons-Alfort, France), and nucleotide sequence analysis of plasmid DNA (1). The purity and stability of rp26 were demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis since no significant evidence of bacterial protein contamination or specific protein degradation was observed.

A quantity of 0.25 μg of purified EIAV rp26 protein/cm of membrane was used as the antigen in the rp26 WB assay developed for the confirmatory diagnosis of EIA. The assay was carried out with horse serum samples diluted 1:50 in WB diluent (1% nonfat milk in Tris-buffered saline–TWEEN) and horseradish peroxidase-conjugated goat anti-horse immunoglobulin G (KPL) diluted 1:3,000 in WB diluent. The DyaSystems EIA AGID test kit from IDEXX Laboratories (Maine) was used as the standard of comparison since it is commercially available and officially approved in Argentina by the national animal health authority (Servicio Nacional de Sanidad y Calidad Agroalimentaria [SENASA]).

The rp26 WB assay detection limit was demonstrated to be higher than that of the AGID test, since eight diluted positive serum samples gave end point titers that were from 32- to 128-fold higher than the AGID titers. The analytical specificity was very good, since no cross-reactivity was observed between rp26 and 28 reference serum samples with antibodies to other pathogens (Thelidia equi, equine herpesvirus type 1, equine influenza virus, and equine viral arteritis virus), even with eight poorly preserved samples. Replicates of eight serum samples analyzed for repeatability (same day, same analyst) and reproducibility (different days, different analysts) by rp26 WB showed 100% concordance of results within and between as—
says. One hundred percent agreement with the expected results was obtained with the three proficiency test panels from Dirección de Laboratorios y Control Técnico-SENASA and one panel from the National Veterinary Services Laboratory (NVSL) checked by rp26 WB. The accuracy trial demonstrated great concordance (98.9%) between the commercially available AGID test and the developed rp26 WB test, with only six discordant results for the 657 field serum samples analyzed (Table 1). Of the 76 samples that were classified as nonspecific by the commercial AGID test, 73 were clearly negative and 3 were classified as positive by rp26 WB. The presence of nonspecific precipitation lines is very difficult to interpret and can result in false-negative results when low levels of EIAV-specific antibodies are present in the sample. This situation is solved by using rp26 as the antigen in the AGID test (1) or WB since rp26 does not carry nonspecific cellular or host-derived proteins because it is produced in a heterologous system, from which it is purified to homogeneity. Among the 340 AGID test-negative samples, 3 samples could be detected as positive by rp26 WB. New serum samples from the same animals could not be obtained since the samples were generous gifts from the archives of SENASA-qualified laboratories.

In order to establish their real status, the discordant samples were sent to the NVSL, an EIA reference laboratory in the United States, where they were fully checked by AGID, two commercial ELISAs, and WB with the cell-adapted strain of EIAV as the antigen. Considering all the results together, the positive results obtained for these six discordant samples by using rp26 WB were concluded to be true positives since they matched those obtained with the NVSL set of serologic tests. Only one of the six sera gave a uniform positive reaction in the different tests at the NVSL (Table 2). The other five discordant samples were clearly negative by AGID but positive by competitive ELISA (CELISA) and by WB with p26 and at least one of the following proteins: gp90 or gp45 (Table 2). The absence of an AGID reaction in both laboratories can be explained based on the greater analytical sensitivity of the WB assay than of the AGID. The discrepancy between the results of the AGID test and the SA II ELISA is of concern, but considering that the assays detect antibodies against p26 and gp45, the absence of a reaction may be the consequence of low levels of antibodies in the samples, as was previously reported for animals after a recent infection or during an acute episode of the disease and/or animals in which the antibody levels never rose high enough to be detectable by AGID or ELISA (4, 8, 12). It is very unlikely that p26 reactivity is due to a nonspecific reaction, as was observed by other authors when cell-adapted virus was used as the antigen (3, 9), since the rp26

TABLE 1. Comparison of rp26 WB and commercial AGID test kit for detection of antibodies to EIAV in serum

| rp26 WB result | Total no. of samples | Nonspecific<sup>a</sup> | Negative | Positive |
|---------------|----------------------|-------------------------|----------|----------|
| Negative      | 404                  | 76                      | 337      | 0        |
| Positive      | 163                  | 3                       | 3        | 148      |
| Total         | 567                  | 79                      | 340      | 148      |

<sup>a</sup> The commercial AGID test (DyaSystems EIA AGID test kit [IDEXX Laboratories]) was considered to be the standard of comparison, according to the World Organization for Animal Health (OIE) validation guidelines. The results obtained with the rp26 WB were classified as true positive, true negative, false positive, or false negative.

<sup>b</sup> A nonspecific result was recorded for serum samples found to have nonspecific precipitation lines by the commercial AGID test.

TABLE 2. Analysis of serum samples with discordant results

| Serum sample | INTA result by: | AGID<sup>a</sup> | rp26 WB | NVSL result by: | AGID<sup>a</sup> | WB<sup>b</sup> | CELISA<sup>c</sup> | SA II ELISA<sup>d</sup> |
|--------------|-----------------|------------------|---------|-----------------|------------------|--------------|-------------------|-------------------------|
| 243          | Negative        | Positive         | Negative| gp90: positive  | Positive         | Negative<sup>e</sup> |                   |                         |
| 279          | Negative        | Positive         | Negative| gp90: positive  | Positive         | Negative<sup>e</sup> |                   |                         |
| 347          | Negative<sup>f</sup> | Positive       | Negative| gp90: positive  | Positive         |             |                   |                         |
| 280          | Negative<sup>f</sup> | Positive       | Negative| gp90: positive  | Positive         |             |                   |                         |
| 382          | Negative<sup>f</sup> | Positive       | Negative| gp90: positive  | Positive         |             |                   |                         |
| 500          | Inconclusive    | Positive         | Positive|                 | Positive         |             |                   |                         |

<sup>a</sup> DyaSystems EIA AGID test kit (IDEXX Laboratories).
<br><sup>b</sup> WB with cell-derived viral lysate.
<br><sup>c</sup> DyaSystems EIA CELISA test kit (IDEXX Laboratories).
<br><sup>d</sup> SA II ELISA (Centaur Laboratories Inc.)
<br><sup>e</sup> Sample showed some color development visually but was negative by optical density readings.
<br><sup>f</sup> Sample with nonspecific reaction.
<br><sup>g</sup> ND, not done.
antigen is produced in a heterologous system, from which it is purified to homogeneity.

In conclusion, we have defined the most important quality parameters and have found that the assay’s performance is adequate to make it a reliable diagnostic tool to be used as a follow-up test after a doubtful ELISA or AGID test result. This tool is particularly relevant since there is no laboratory in Argentina that can confirm discordant or inconclusive test results. We do not suggest the use of rp26 WB as a substitute for conventional serological assays but only as a complementary test, available at the national level for the confirmation of conflicting results, that will contribute to resolving legal and/or sanitary situations that can be propitious to the dissemination of EIA in Argentina.

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