Development, evaluation, and laboratory validation of immunoassays for the diagnosis of equine infectious anemia (EIA) using recombinant protein produced from a synthetic *p26* gene of EIA virus

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Received: 14 January 2013 / Accepted: 24 July 2013 / Published online: 8 August 2013
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Abstract Equine infectious anemia (EIA)—a retroviral disease caused by equine infectious anemia virus (EIAV)—is a chronic, debilitating disease of horses, mules, and donkeys. EIAV infection has been reported worldwide and is recognized as pathogen of significant economic importance to the horse industry. This disease falls under regulatory control program in many countries including India. Control of EIA is based on identification of inapparent carriers by detection of antibodies to EIAV in serologic tests and “Stamping Out” policy. The current internationally accepted test for diagnosis of EIA is the agar gel immune-diffusion test (AGID), which detects antibodies to the major *gag* gene (*p26*) product. The objective of this study was to develop recombinant *p26* based in-house immunoassays [enzyme linked immunosorbent assays (ELISA), and AGID] for EIA diagnosis. The synthetic *p26* gene of EIAV was expressed in *Escherichia coli* and diagnostic potential of recombinant *p26* protein were evaluated in ELISA and AGID on 7,150 and 1,200 equine serum samples, respectively, and compared with commercial standard AGID kit. The relative sensitivity and specificity of the newly developed ELISA were 100 and 98.6 %, respectively. Whereas, relative sensitivity and specificity of the newly developed AGID were in complete agreement in respect to commercial AGID kit. Here, we have reported the validation of an ELISA and AGID on large number of equine serum samples using recombinant *p26* protein produced from synthetic gene which does not require handling of pathogenic EIAV. Since the indigenously developed reagents would be

economical than commercial diagnostic kit, the *rp26* based-immunoassays could be adopted for the sero-diagnosis and control of EIA in India.

Keywords Equine infectious anemia · Recombinant *p26* protein · ELISA · AGID · Sensitivity · Specificity

Introduction

Equine infectious anemia (EIA) is a retroviral disease caused by EIA virus (EIAV), which is a macrophage-tropic lentivirus. EIA is a chronic and debilitating disease of all equidae, including horses, mules, and donkeys [6]. The disease is also called as swamp fever or equine pernicious anemia. It is manifested either as an acute fatal infection or more commonly as a chronic infection; characterized by recurrent febrile episode, anemia, thrombocytopenia, weakness, and oedematous lesions in the lower parts of the body [13]. The chronic cases typically progress to life-long inapparent carriers, presumably due to the development of stable protective host immunity [9]. The asymptomatic carrier animals become a permanent source of infection to other horses at the farm [12]. EIAV infection is predominantly spread by blood-feeding insect vectors (mainly horseflies and deerflies) but can also be transmitted via contaminated syringe/needles. The hot and humid rainy season, when the vectors are very active and in abundance, favour the transmission of the virus [14].

Serological evidence of EIAV infection has been reported in many countries around the world and the disease is most prevalent in Europe and Americas. In Asia, disease is less prevalent and had not been reported for many years. In India, the first case of EIA was detected in Karnataka in 1987;

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thereafter intense sero-surveillance throughout the country diagnosed 228 positive cases in the following years [35]. However, recent emergence of EIAV infection in Asian continent like, Japan [17], Mongolia [20], China, Thailand, Uzbekistan, Philippines, and Malaysia during 2008–2012 [http://www.oie.int/wahis_2/public/wahid.php/Wahidhome] and detection of EIAV infection in 2010 and 2012 in India [16] after a long gap underlines the importance of continued surveillance to monitor the disease prevalence and identifying the genetic variation among field strains of EIAV. During the last two decades, the detection of EIAV antibodies by a number of ELISAs has been described and compared with ‘gold standard’ agar gel immuno-diffusion (AGID) test [3, 21, 22, 24, 25, 28, 29, 31, 32]. The ELISA has been reported to be more sensitive than AGID in detecting acute cases of EIA and helpful in disease eradication programme [10]. However, commercial EIA ELISA kit is expensive and is not affordable by poor equine owners in Asian countries. Therefore, availability of cost-effective EIA diagnostic ELISA is the need of the region.

The objective of this study was to evaluate the recombinant p26 protein of EIAV derived from a synthetic p26 gene in indirect ELISA and AGID to detect anti-EIAV antibodies in equine serum samples. Using synthetic technology, the EIAV p26 was synthesized and expressed in *Escherichia coli* expression system without handling the EIAV. The results obtained in developed ELISA and AGID assay were compared with commercially available imported AGID test kits (VMRD, Pullman WA, USA & IDEXX, Westbrook, USA), officially approved by Department of Animal Husbandry, Dairying & Fishery Sciences, Ministry of Agriculture, Govt. of India. This is the first report of evaluation of diagnostic assay for EIA using recombinant protein derived from synthetic gene. The study shows the importance of gene synthesis technology for developing diagnostic for trans-boundary infectious diseases which may not be prevalent at present but have potential to re-emerge.

Materials and methods

Synthesis and expression of EIAV p26 gene in *E. coli*

A 675 bp fragment of the EIAV *gag* gene encoding the p26 protein of EIAV derived from accession no. DQ452090 was commercially synthesized in pUC57 cloning vector (GeneScript, Piscataway, NJ, USA). The gene was engineered to carry *Bam*HI and *Hind*III restriction sites at 5' and 3' end, respectively, for the purpose of directional sub-cloning in prokaryotic expression vector pQE30.

Cloned p26 gene was released from pUC57 plasmid by *Bam*HI and *Hind*III enzyme digestion and released insert was eluted from agarose gel using MinElute Gel Extraction Kit

(Qiagen, Hilden, Germany) as per manufacturer's instructions. Thereafter, the eluted p26 insert was sub-cloned into *Bam*HI and *Hind*III restricted prokaryotic expression vector pQE-30 (Qiagen, Hilden, Germany). The recombinant plasmid construct containing p26 expression cassette was then transformed into competent *E. coli* M15 cells. The positive transformants were screened by selecting single colony expressing recombinant p26 (rp26) protein by 15 % sodium dodecyl-sulphate poly-acrylamide gel electrophoresis (SDS-PAGE).

Purification of recombinant p26 (rp26) protein

Escherichia coli expressing rp26 protein was grown in 300 ml LB broth containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml) at 37 °C in a shaking incubator until the optical density reached to 0.6–0.8 at 600 nm. Induction of recombinant protein was mediated by addition of 1 M of β-D isopropyl thiogalactosidase (IPTG) and the culture was incubated for an additional 6 h. The cells were harvested by centrifugation at 6,000×g for 10 min, and rp26 protein was purified by Ni⁺-NTA column chromatography under denaturing condition as per the manufacturer's instructions (Qiagen, Hilden, Germany). Quality of purification was checked on SDS-PAGE stained with coomassie brilliant blue dye. Aliquots of purified protein were pooled, dialyzed against phosphate buffered saline (PBS, pH 7.2), and protein concentration was measured by Lowry's method using commercial protein estimation kit (Merck Bioscience, Bengaluru, India). Purified rp26 protein was stored at –70 °C as 0.5 mg/ml stocks in 0.5 ml aliquots.

Determination of p26 specific antibody by Western Blot

The specific reactivity of rp26 protein to EIAV antibody was determined by western blot analysis as previously described method [34]. The membrane having protein transferred on it was blocked with 6 % skim milk in PBS-T (PBS containing 0.05 % Tween-20) for overnight at 4 °C, washed twice with PBS-T, cut into strips and incubated with 1:200 dilution of EIAV reference positive, EIAV-infected field horse serum and reference negative serum in plastic tray on rocker for 2 h at 37 °C. The strips were washed thrice with PBS-T and incubated with 1:10,000 dilution of anti-horse IgG HRP conjugate (Sigma-Aldrich, St. Louis, USA) for 1 h on rocker at 37 °C. The strips were finally washed with PBS-T and developed with Tris-buffer (pH 7.6) supplemented with diaminobenzidine (6 mg/10 ml) in the presence of 30 µl of 30 % H₂O₂.

Control and field serum

To evaluate the test performance, including the sensitivity and specificity, of the rp26-based ELISA and AGID, sets of

reference serum and field serum maintained in the serum repository of NRCE were used. Serum samples ($n = 14$) positive for EIAV antibodies were used as positive control which included seven reference positive procured from (NVSL Ames, IA, USA; VMRD, Pullman WA, USA, and IDEXX, Westbrook, USA) and seven EIAV-infected equine serum collected in routine diagnosis process under institute sero-surveillance programme. Description of different positive serum samples is given in Table 1.

Negative control serum samples were collected from a pack of EIAV-negative horses housed in a race course where all the animals are tested for EIAV antibodies during every movement or at least once a year by AGID. A panel of 30 known negative serum samples was prepared and serum from two horses was pooled. Thus, total 15 negative serum pools were made from 30 serum samples. Reference negative control ($n = 2$) serum (VMRD, Pullman WA, USA) was also included in the assay.

Equine serum samples received from different race courses and private farms for regular testing or collected from field for sero-surveillance of equine disease were evaluated by ELISA and AGID using rp26 protein.

Standardization of rp26ELISA

The optimal rp26-ELISA reagent concentrations were evaluated by block titration using different rp26 protein concentrations (50–300 ng/well), four dilutions of the positive and negative control serum (from 1/100 to 1/800), and four dilutions (1/10,000–1/20,000) of rabbit anti-horse

IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, USA). The optimal p26-ELISA reagent concentrations were assumed to be those showing the highest discrimination between positive and negative standard sera.

In brief, 96-well microtitre plates (Greiner Bio One, Neuburg, Germany) were coated with rp26 protein (at the referred concentration) by incubating overnight at 4 °C followed by washing the plates with PBS-T and blocking with 5 % of skimmed milk in PBS-T for 1 h at 37 °C. After blocking, serum samples were diluted in dilution buffer [2 % (w/v) skimmed milk in PBS-T] and added in a volume of 100 µl/well, and the plates were incubated for 1 h at 37 °C. Plates were washed four times with washing buffer (PBS-T) and the anti-horse HRP conjugate (100 µl/well) in dilution buffer were added. Plates were incubated for 1 h at 37 °C and then washed four times with washing buffer. Following washing steps, in each well 100 µl of phosphate-citrate substrate buffer containing 200 µmol of orthophenylene-diamine (Sigma-Aldrich, St. Louis, USA) was added and incubated at 37 °C for 10 min. Finally, the reaction was stopped by addition of 50 µl of 1 M H₂SO₄ and the absorbance was read at 492 nm in a microtitre plate reader (BioTek Powerwave XS2, Highland Park, Winoski, USA).

Standardization of agar gel immunodiffusion (AGID) Test using rp26

The rp26-AGID test was performed according to Coggins test [7]. Different concentration of rp26 protein (ranging

Table 1 Description of EIAV positive and negative control serum used in the study

Positive control serum	Serum ID	Strength	Source
Reference standard	P1	Weak positive	NVSL, Ames, IA, USA
	P2	Weak positive	VMRD, Pullman, USA
	P3	Medium positive	VMRD, Pullman, USA
	P4	Strong positive	NVSL, Ames, IA, USA
	P5	Strong positive	VMRD, Pullman, USA
	P6	Known positive control serum of AGID kit	Idexx laboratories, Westbrook USA
	P7	Known positive control serum of AGID kit	VMRD, Pullman, USA
	N1–2	Known negative	NVSL, Ames, USA
EIAV-infected equine serum	From cases detected at		Year of collection
	P8	Tohana, Haryana	1996
	P9	Faizabad, Uttar Pradesh	1998
	P10	Pune, Maharashtra	1996
	P11	Delhi	1991
	P12	Delhi	1994
	P13	Jaipur, Rajasthan	1996
	P14	Haldwani, Uttarakhand	2010

P positive, N negative control serum

from 5 to 40 µg/ml) in different buffer composition (denatured and renatured) were evaluated in AGID test for determining the optimum effective concentration of rp26 protein. The test was simultaneously compared with the commercially procured OIE-approved AGID kits (VMRD, Pullman WA, USA, and IDEXX, Westbrook, USA) to confirm the complete identity of precipitations lines. Positive control serum were diluted twofold from 1/2 to 1/32 in borate buffer (pH 8.5) and tested against the rp26 at its best concentration. All of the AGID tests were visually interpreted independently by at least two experts, both after 24 and 48 h of incubation.

The analytical sensitivity or detection limit of rp26-AGID was determined by testing serial dilutions of known positive samples. The relative sensitivity and specificity of rp26-AGID assay in compare to the commercial AGID test kits were estimated on 1,200 horse serum samples. In case of disagreement in the interpretation between analysts or between tests, the sample was retested.

Data analysis

After the best concentration for each of the ELISA reagents was established, serum from panels of positive controls, negative controls and 7,150 field serum samples were tested in duplicate by rp26 ELISA as described above in duplicates. The absorbance data of ELISA (O.D. values) were analyzed using Windows 7 Microsoft Excel programme (Microsoft Corp, Redmond, WA) and were normalized using the following formula:

$$\text{Percent positivity (PP \%)} = \left[\frac{(\text{OD}_{492} \text{ sample serum} - \text{OD}_{492} \text{ negative control})}{(\text{OD}_{492} \text{ positive control} - \text{OD}_{492} \text{ negative control})} \right] \times 100\%.$$

The optimum cut-off value of the ELISA was determined by receiver operative curves analysis using normalized OD₄₉₂ value (PP %) [30]. Relative sensitivity and specificity of the assay were determined by following calculation: Sensitivity = True positive / (True positive + False negative), and specificity = True negative / (True negative + False positive). The results of calculations were expressed as percentages.

Results

Expression and purification of rp26

PCR and restriction enzyme digestion of the recombinant plasmid yielded expected band of 675 bp of EIAV *gag* gene encoding the p26 protein (Fig. 1, lane 1, 3). Further, DNA sequence analysis (BLASTN) of the clone confirmed

the identity of the EIAV p26 gene. The sequencing indicated 100 % homology with the GenBank sequence DQ452090 (data not shown).

The recombinant *E. coli* carrying pQE30:p26 cassette expressed a protein with a molecular weight around 26 kDa that was not observed in the control cultures (Fig. 2, lane 1, 2). SDS-PAGE analysis of eluted fractions revealed that rp26 protein was purified to homogeneity. The resulting protein was highly pure as no contaminating protein band could be seen from the flow through and wash fraction (Fig. 2, lane 3–5). Intensity of protein band varies in different eluates and highest intensity of protein band was observed in fraction 4 and 5 (Fig. 2, lane 9, 10). The final yield of purified recombinant protein was about 10 mg/l of induced bacterial culture.

Validation of rp26 in western blot assay

In western blot rp26 protein showed specific reactivity with the EIAV reference and EIAV antibody positive field serum samples as demonstrated by positive reaction band at 26 kDa position (Fig. 3). Reference negative control serum showed no reactivity against rp26.

Diagnostic efficacy of indirect ELISA test using rp26 protein

After block titration, optimal concentrations of reagents for rp26 ELISA were found to be 200 ng of rp26/well for antigen coating, 1/400 serum dilution and 1/15,000 conjugate dilution. The percent positivity percentage (PP %) value of 22.2 in the assay was determined as cut-off value to obtain the maximum benefit of the assay (Fig. 4). Serum

Fig. 1 Confirmation of EIAV p26 clone by PCR and restriction enzyme digestion. Lane M 100 bp DNA ladder (Fermentas), Lane 1 PCR amplified p26 gene (675 bp), Lane 2 negative control PCR, Lane 3 *Bam*HI and *Hind*III cut p26 gene insert

samples showing PP % greater than or equal to 22.2 and less than that were regarded as positive and negative, respectively. A total of 7,150 equine serum samples were tested in indirect ELISA which comprised of 5,550 serum from horses, 1,087 serum samples from donkeys, and 513 serum samples from mules. All of the test samples were also simultaneously compared with commercial AGID test. Of the 7,150 serum samples, 20 samples were found false positive in ELISA (Fig. 5) but all of them were negative in reference AGID and newly developed rp26-AGID test. False positive results in ELISA have been linked to poor quality of serum (rotten and lysed) as repeat samples was found negative in subsequent testing. Thus, the specificity of the rp26-ELISA was therefore 98.6 % (at 95 % confidence interval: 95.8–99.4 %). Interestingly, there were

none that were negative in ELISA but positive in the AGID assay. Relative sensitivity, positive predictive value, and negative predictive value of the ELISA were 100, 41.17 and 100 %, respectively.

Agar Gel Immunodiffusion (AGID) Test using rp26 protein

AGID test was optimized using the rp26 protein in different buffer composition. Sharp precipitin line half way between p26 antigen and the positive control serum wells were observed at rp26 concentration of 17 µg/ml. Among the different buffer composition glycine (pH 9.0) = 0.9 mM, EDTA = 0.09 mM, glycerol = 0.0004 %, urea = 0.02 M, Tris Cl = 0.03 mM, NaH₂PO₄ = 0.38 mM, tween-

Fig. 2 SDS-PAGE analysis of nickel affinity purification of p26 protein. Lane 1 Uninduced control, 2 IPTG induced cell lysate, Lane 3 flow throw, Lane 4 Wash 1, Lane 5 Wash 2 Lane M Protein molecular weight marker (Invitrogen), Lanes 6–10 Eluate fraction 1–5

Fig. 4 Indirect enzyme-linked immunosorbent assay: distribution of PP % value of positive and negative serum samples. The horizontal line represents the cut-off value [percent positivity (PP % = 22.2)]

Fig. 3 Specific immune-reactivity of rp26 protein in western blot for detection of antibodies to EIAV in serum. Lane p26, and M Amido black stained recombinant p26 protein, and protein molecular weight marker respectively, upon transfer on PVDF membrane. Lanes 1–7

Reference positive serum of varying strength as described in materials and methods (P1–P7). Lanes 8–14 EIAV infected equine serum as per materials and methods (P8–P14), Lanes 15, 16 Reference EIAV negative control serum

Fig. 5 Determination of cut-off value of indirect enzyme-linked immunosorbent assay, according to sensitivity and specificity values

20 = 0.005 %, 1:50 PMSF (25 mg/ml) were found optimal for increasing solubility as well as to prevent degradation of the rp26 protein in AGID. A clear precipitation line between positive serum well and rp26 protein (17 µg/ml) well was observed from neat serum to ¼ dilutions, and then gradually precipitin line became thin and weak at higher serum dilution. The detection limit of rp26-AGID was shown to be similar to that of the commercial AGID kits (data not shown). For evaluating the rp26-AGID assay, neat serum samples ($n = 1200$) was used during the study and compared with commercial AGID kit. The rp26 AGID test results showed total correlation with cent percent agreement with commercial AGID kits (Table 2). Considering the commercial AGID test as the ‘standard of comparison’ according to the OIE validation guidelines, none of the serum samples could be classified as false positive or false negative by the rp26-AGID. The relative sensitivity of the rp26-AGID compared with that of the commercial AGID test was 100 % and the relative specificity was 100 %.

Discussion

Detection of sero-positive equines and subsequent elimination of the EIA positive animals is the only option to control the disease. In an effort to develop a more rapid and sensitive diagnostic capability, several attempts have been made on development of enzyme-linked immunosorbent assays (ELISAs) based on p26 protein purified from EIAV strain [28, 29, 32], recombinant proteins [1, 2, 15, 22, 25, 33] and peptides [27, 31]. The EIAV p26 protein encoded

Table 2 Comparison of commercial AGID test kit and rp26-AGID test kit for detection of serum antibodies to EIA

	VMRD/Idexx EIA AGID test kit		Total
	Negative	Positive	
rp26-AGID			
Negative	1,200 (tn)	0 (fn)	1,200
Positive	0 (fp)	14 (tp)	14
Total	1,200	14	1,214

The commercial AGID test was considered as the ‘standard of comparison’ according to the OIE validation guidelines. The results obtained with the rp26-AGID were classified as *tp* true positive, *tn* true negative, *fp* false positive and *fn* false negative

by *gag* gene is one of the highly conserved structural proteins among the viral isolates even from very different geographical locations [4, 5, 26]. Thus, the present diagnosis of EIAV infection relies on detection of antibodies to p26 protein. During the last few years, the detection of EIAV antibodies directed against p26 protein by ELISA and AGID has been described and these tests are commercialized under various formats [21].

Molecular biological methods such as the nested PCR, multiplex PCR, and real time PCR have been developed for diagnosis of EIAV [5, 8, 11, 18, 19, 23]. While PCR-based methods represent a potentially highly sensitive alternative for the detection of viral nucleic acids, these techniques require extensive nucleotide sequence information for approval of universal diagnostic method. Most conventional and real-time PCR methods reported are mainly suited to detect EIAV strains of limited diversity and of

nearly identical sequences and are not useful for detecting EIAV field strains among different countries [5, 19]. Once appropriate consensus primers are identified, PCR-based methods can serve as a valuable adjunct to conventional serological testing for the routine diagnosis of EIA. However, these methods need regular updating of sequence information and are inconvenient for large-scale epidemiological investigation; owing to the need for laboratory facilities, trained personnel, and more financial resources.

The serological method is a relatively satisfactory diagnostic method due to its sensitivity, inexpensiveness, and easy repeatability. Among the serological diagnostics, the AGID test is still internationally recognized as the gold standard test for the diagnosis of EIA [36] and continues to be the most commonly used diagnostic tool because of its high specificity, simplicity and cost-effective nature. However, the test has certain drawback in terms of sensitivity, and subjective interpretation of the reading of precipitation line curvature. The ELISA formats have the advantages of allowing efficient and accurate testing of large numbers of serum samples within a relatively short time. ELISA also provides valuable tool for large-scale screening tests, monitoring the prevalence as well as assessing risk factors of EIA. Therefore, in many countries, ELISA has been adopted officially for sero-monitoring of EIAV, with mandatory provision that positive reactions in any EIA ELISA be confirmed by AGID testing before regulatory actions are taken [5, 11].

Recombinant protein production in heterologous expression system provides large-scale antigen production without handling the infectious agent. In the present study, the rp26 protein was expressed in *E. coli* from a synthetic gene and evaluated an AGID and indirect ELISA for the diagnosis of EIA in equines. This is the first report of optimizing a diagnostic ELISA and AGID in large number of field serum samples ($n = 7,150$ in ELISA, and $n = 1,200$ in AGID) for diagnosis of EIA employing gene synthesis technology. Diagnostic sensitivity and specificity rp26-ELISA and rp26-AGID obtained in the present study are in complete agreements with previous findings [21, 22, 28, 29]. Our results suggest that the assays can be used for a large-scale screening test to monitor the prevalence of EIA and assess the risk factors. The only limitation of the present investigation is that the assay could not be evaluated in more number of positive serum samples from the field because the prevalence of EIA in India has been very rare since 1999; thus, positive predictive value of the assay was low. The newly developed assay will be economical being indigenous and equally specific and sensitive. The rp26-ELISA could be adopted in India as an official test method for the diagnosis of EIA. This is particularly relevant for nationwide sero-surveillance of EIA by regional laboratories from all round the country. We propose

sero-positive results of ELISA must be resolved by AGID for accurate diagnosis of carrier equines.

Here, we demonstrated comparable performance characteristics of the rp26-ELISA and rp26 AGID with the commercial AGID test. Validation of the rp26-ELISA in endemic region is recommended in future.

Acknowledgments The authors thank all staff members of the National Research Centre on Equines for skillful technical assistance with serological investigations during the past decades. The help of Sh. Sitaram, Technical Officer and Sh. Gurudutt, skilled supporting staff is highly acknowledged. Thanks are also due to the State Animal Husbandry Department, Vets and equine owners for their cooperation and the Indian Council of Agricultural Research for funding.

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