Sero-surveillance of equine infectious anemia virus in equines in India during more than a decade (1999–2012)

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Received: 14 January 2013 / Accepted: 20 June 2013 / Published online: 16 July 2013
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Abstract Equine infectious anemia (EIA) is a retroviral infection of horses. Horses infected by EIA virus (EIAV) become inapparent carriers that remain asymptomatic for the remainder of their life span and serve as infection source to other horses. In this study, agar gel immunodiffusion test and ELISA were used to investigate the presence of antibodies to EIAV in equines. A total of 67,042 equine serum samples from 19 states and two union territories were tested during April 1999 to September 2012. The results revealed that none of the animals were positive for antibodies to EIAV from 1999 to December 2009. However, two EIAV sero-positive cases one each from indigenous and thoroughbred equines were detected in 2010 and 2012, respectively. Occurrence of EIA after a long gap of 11 years is indicative of reemergence of EIA in India which warrants concerted efforts in nationwide surveillance and monitoring for detection and elimination of EIAV carrier animals to prevent EIA outbreak.

Keywords Equine infectious anaemia · Sero-prevalence · AGID · ELISA · India

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Equine infectious anemia (EIA) is a retroviral disease caused by EIA virus (EIAV), a macrophage tropic lentivirus belonging Retroviridae family [3]. EIA is a chronic, debilitating disease of all equidae, including horses, mules, and donkeys. The disease is manifested either as an acute fatal infection or more commonly as a chronic infection; in which equines commonly experience a recurrent febrile episode, anaemia, thrombocytopenia, weakness, and oedema in the lower parts of the body [3]. EIAV infection is predominantly spread by blood-feeding insect vectors (mainly horse- and deerflies) but can also be transmitted via contaminated syringe needles and blood transfusion from carrier animals. Horses infected by EIAV, either die in the acute attack or typically progress to lifelong (long-term) inapparent carriers, presumably due to the development of enduring protective host immunity. These inapparent carriers remain infected for life and become a permanent source of infection to other horses at the farm [5]. There is neither any specific cure nor vaccine available for this disease. Thereby to eradicate the disease many country adopted a stringent policy by imposing statutory testing, elimination of infected animals, and import restrictions. The diagnosis of EIA is currently exclusively dependent on indirect serological detection techniques such as agar gel immunodiffusion test (AGID) and ELISA. The AGID [2] prescribed by the Office International des Épizooties (OIE) for international trade (OIE terrestrial manual, 2008; http://www.oie.int/fileadmin/home/eng/health_standards/tahm/2.05.06_eia.pdf), and EIAV antibody test ELISA (VMRD, USA) is also being used in India for EIA control program.

In India, EIAV infection was first diagnosed in 1987 in a thoroughbred horse from Karnataka state, thereafter intense sero-surveillance throughout the country had led to the diagnosis of 228 EIA-positive cases in following years

[14]. Continuous sero-surveillance and control program of EIA on national basis revealed decreasing trend of EIA occurrence from 3.20 % in 1987 to 0.03 % in 1997. The last positive cases of EIA were reported in October 1998 (two cases from Uttar Pradesh) [15] and February 1999 (one case from Delhi) [7]. Although, no positive case of EIA was found in the consecutive years and the occurrence of disease seemed to be very rare; continuous sero-monitoring of equine population by active and passive surveillance from April 1999 to September 2012 was put in order to investigate its possible countrywide distribution. This paper presents outcome of this surveillance and discusses the possibility of re-emergence of EIA in India, with a potential risk to neighbouring countries.

In the present study, EIAV sero-surveillance was conducted in equine inhabited areas which encompass the 19 states and two union territories of India. The indigenous equines were randomly selected in each region and included stables that housed at least four animals, in order to have an efficient sampling process. Most of the residing thoroughbred horses were sampled in each stable. The sampled horses were from different breeds and genders and were at least 6 months of old. Blood samples were collected aseptically from the jugular vein of the animals and transported to the laboratory at 4 °C. The animals were either sampled by a scientist of National Research Centre on Equines (NRCE), Hisar or by state veterinary officers and the samples were sent to the NRCE by courier. Serum was separated by centrifugation at $3,000\times g$ for 10 min, stored in aliquots at -20°C until testing. The clinical status of the equine was not considered for this study. The data were sorted out according to the year, origin of the samples, and type of equines.

For the detection of EIAV-specific antibodies in equine serum, OIE-approved commercial AGID kits obtained from VMRD (USA) was used according to the manufactures instructions. Confirmation of positive reaction was determined as per OIE guidelines. Besides AGID, a 'modified sandwich' ELISA kit (VMRD, USA)—for the detection of antibodies against EIAV p26 antigen—was also included in testing protocol from 2005 onwards for resolving the suspected results obtained in AGID or vice versa. The kit was used as per the instructions given in the manual. In addition to these commercially available assays, the immunoblot test (western blot) using recombinant EIAV p26 protein was also used to further confirm the results. The recombinant EIAV p26 protein was produced in *E. coli*. Homogeneously purified recombinant EIAV p26 protein was used for developing specific in-house diagnostic assay (unpublished data). Approximately, 800 ng of recombinant EIAV p26 protein was transferred onto PVDF membrane and cut into strips and incubated with 1:200 dilution of EIAV reference positive (VMRD, USA),

reference negative (NSVL, USA) and EIAV-suspected field serum in plastic bags on rocker for 2 h at 37 °C. The strips were washed with PBS-T (0.05 % Tween 20 in PBS) and incubated with 1:10,000 dilution of HRP-conjugated rabbit anti-horse IgG antibody (Sigma-Aldrich, St. Louis, USA) for 1 h on rocker at 37 °C. The strips were finally washed with PBS-T and developed with diaminobenzidine (6 mg/10 ml Tris buffer pH 7.6) in presence of 30 μl of 30 % H_2O_2 . Finally, the reaction was stopped by emerging the strips in distilled water.

Between April 1999 and September 2012, a total of 67,042 equine blood samples which includes 51,867 from thoroughbred and 15,175 from indigenous equines from the study area were submitted to or collected by NRCE for testing for EIAV-specific antibodies. The number of samples that were tested for EIA diagnosis is presented in Table 1. On an average, 5,000 serum samples were obtained during each year. As number of equine population was less in Manipur, Meghalaya, and Assam in compared to other densely equine inhabited states; the numbers of equine population sampled from these areas were minimal. For thoroughbred horses, highest number of horses were tested from Karnataka ($n = 16,144$), followed by Delhi ($n = 10,096$), Tamil Nadu ($n = 7,026$), and Andhra Pradesh ($n = 5,124$). While highest number of indigenous equines were from Rajasthan ($n = 3,381$), followed by Uttarakhand ($n = 1,681$), Uttar Pradesh ($n = 1,158$), Jammu and Kashmir ($n = 1,137$), and Madhya Pradesh ($n = 1,126$).

No EIAV-sero-positive animal was detected during the period from April 1999 to December 2009. The first serologically EIAV-positive mule was detected in Haldwani district of Uttarakhand in January 2010. The second case of EIAV-positive thoroughbred horse was detected in Manesar, Haryana in September 2012. Besides ELISA and AGID, specific recognition of EIAV antibody in two suspected equine serum samples were also demonstrated by western blot which gave positive reaction with recombinant EIAV p26 protein (Fig. 1). Viewing the uniform results in this three-pronged assay, the sero-reactor animal was declared as EIAV carrier. This strategy may be useful in resolving the diagnostic issue of suspected samples. EIAV-positive mule was quarantined for up to 10 months before euthanization for observation of any clinical signs. During the observation time, two febrile episodes were noticed. However, no clinical signs were observed in EIAV infected horse detected in 2012.

EIA infection is widely reported in several countries in European and American continent [(1, 4, 10, 13); World Animal Health Information Database, WAHID Interface Version 1.2. http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home, last accessed on 14 January 2013]. Although EIA has not been reported in the South-East Asia

Table 1 State wise number of equines serologically surveyed for equine infectious anemia virus infection during 1999–2012

Sr. nos.	States	1999–2004		2004–2009		2009–2012 (up to Sep)		Total		Grand total
		T	I	T	I	T	I	T	I	
1	Andhra Pradesh	2391	603	1773	0	960	0	5124	603	5727
2	Chandigarh	16	30	99	99	59	0	174	129	303
3	Delhi	2171	6	4612	92	3313	0	10096	98	10194
4	Gujarat	239	24	132	395	141	438	512	857	1369
5	Haryana	314	241	487	254	763 ^a	411	1564	906	2470
6	Himachal Pradesh	0	101	127	129	0	133	127	363	490
7	Jammu and Kashmir	0	422	174	375	0	340	174	1137	1311
8	Karnataka	7392	571	4859	0	3893	0	16144	571	16715
9	Madhya Pradesh	125	164	185	853	168	109	478	1126	1604
10	Maharashtra	421	300	616	510	1689	0	2726	810	3536
11	Punjab	309	207	412	434	314	100	1035	741	1776
12	Rajasthan	207	621	989	708	1223	2052	2419	3381	5800
13	Tamil Nadu	3438	12	2229	509	1359	0	7026	521	7547
14	Uttar Pradesh	191	313	250	397	338	448	779	1158	1937
15	Uttarakhand	62	195	79	358	250	1128 ^a	391	1681	2072
16	West Bengal	1162	135	851	195	798	0	2811	330	3141
17	Chhatisgarh	0	0	15	234	31	320	46	554	600
18	Manipur	0	0	45	98	38	28	83	126	209
19	Meghalaya	0	21	0	0	0	0	0	21	21
20	Assam	12	0	0	0	120	0	132	0	132
21	Bihar	26	62	0	0	0	0	26	62	88
	Total	18476	4028	17934	5640	15457	5507	51867	15175	67042

T thoroughbred horse, *I* indigenous equines (horse, donkey, mule, and pony)

^a Indicates detection of single case of EIA in 2010 (mule from Uttarakhand) and in 2012 (thoroughbred horse from Haryana). All the samples were tested using Coggins test; modified sandwich ELISA (VMRD) and western blot using in-house developed recombinant EIAV p26 were also used to confirm the positive reactors

for a long time, the presence of hematophagous vectors and hot-humid climate remains the most important risk factors in this region. In recent past, the disease incidence was detected in South-Eastern Asia e.g., China, Japan, Mongolia, Thailand, Uzbekistan, Philippines, Malaysia [(8, 11); WAHID].

EIA is a notifiable disease and control measures are presently being followed under the guidelines of ‘The prevention and Control of Infectious and Contagious Diseases in animals Act-2009’, ‘National Policy on EIA’ and regulations administered by the Ministry of Agriculture of India. Previous studies suggested that EIAV infections in thoroughbred equines had been endemic in certain states of India during 1987–1999 [7, 14, 15]. Our results indicate the absence of the disease in all of the equines (thoroughbred and indigenous) tested during the period of 1999–2009. Surprisingly, a single case of EIA-positive mule was detected in January 2010 from Uttarakhand state in North India. Following detection of EIA-positive reactor, surveillance was heightened in the area and 1,048 equines

were tested from the affected area but ended with negative results. Again, one thoroughbred horse showing presence of EIAV antibody was detected in September 2012 from Haryana state. The two animals infected with EIAV were quarantined, euthanized, and subjected to postmortem examination. However, no gross lesions and histological findings were observed in postmortem examination. Attempts to isolate the EIAV and PCR amplify the EIAV-specific gene sequences from these two animals using published primer [1, 9, 12] failed, probably due to a very low level of viremia at the time of sample collection or extensive genetic variation within target gene sequences among different EIAV field isolates.

This is the most comprehensive serological study ever conducted in India giving valuable insights into the EIAV disease incidence in equines. Further, this is to clarify that the disease was restricted only to thoroughbred horse in India before the first incidence in indigenous equine in 2010. Hence, more emphasis was given on surveillance of thoroughbred equines, with repeated sampling and testing

Fig. 1 Western blot of EIAV affected mule and horse serum using recombinant EIAV p26 protein. *Lanes 1–2* reference EIAV strong and weak positive serum, respectively (VMRD, USA), *Lane 3* serum from EIAV infected mule detected in 2010, *Lane 4* serum from EIAV infected horse detected in 2012, *Lanes 5–6* reference EIAV negative control serum (NSVL, USA)

resulting in testing of more samples from some states like Delhi and Karnataka causes skewed sample distribution in view of number of equine population of particular states. The states of Jammu and Kashmir, Uttar Pradesh, Uttarakhand, and Bihar have got limited number of thoroughbred equines resulting in relatively less number of samples. Therefore, sample was not equally distributed across the states. Intense surveillance of indigenous equines is equally important and need special attention in future surveillance programme. The study reveals overall countrywide sero-prevalence of EIA in the thoroughbred equines was brought down to zero; although two cases of EIA incidence in indigenous and thoroughbred equines were observed during the time period of the survey. From the available data, it was impossible to trace the original source for the current EIAV infection in the study area, despite our best efforts to test all in-contact equine populations including all possible movements of these equines within or among different equine population. Similarly, emergence of EIA infection after a long gap of 17 years has been reported in Japan in 2011 [8]. Movement of wild ponies and non-race horse are considered to be the major cause of dissemination of EIAV infection to native Japanese equines [6, 8]. In the present scenario, presence of inapparent carrier animal may be the source of infection found in the study.

India possesses 1.19 million equines (18th Livestock Census, 2007, DAHDF, Ministry of Agriculture, Government of India). Major population of equines comprising about 1.15 million indigenous animals which includes donkeys (0.44 m), mules (0.14 m) and ponies (0.57 m). The major population is distributed in northern part of the country with maximum population in Uttar Pradesh and Jammu and Kashmir followed by Rajasthan, Bihar and Gujarat and provides livelihood to the poor owner living in arid, semi-arid and hilly regions through transport and draught. Moreover, utilization of equines in army, police, racing industry, agricultural and allied operations, tourism, sports and ceremonial events has added valued strength to the equine sector. India has a paradoxical situation to deal with the health problems of equines. On one hand, the elite thoroughbred horses pose the danger of the ingress of exotic diseases to indigenous equids. On the other hand, the donkeys and mules act as potential source of infection to thoroughbred and other elite horses. Therefore, repeated sampling and more concerted state wise sero-surveillance is recommended to know the actual EIA status among equine populations in India.

Acknowledgments The authors thank the previous and current staff members of the National Research Centre on Equines for skilful technical assistance with serological investigations during the past decades. The help of Sri Sitaram and Sri Gurudutt is also acknowledged. We thank all those who performed fieldwork during these 10 years. We also thank the State Animal Husbandry Departments, vets, and equine owners for sending serum samples and the Indian Council of Agricultural Research for funding the monitoring and epidemiological research.

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