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RESEARCH ARTICLE

SURVEILLANCE OF THE ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS (EEHV) IN CHITWAN DISTRICT, NEPAL

[1]Arjun Pandit, [2]I. P. Dhakal, [3]Kamal Prasad Gairhe, [1]Hari Bahadur Rana, and [4]Dibesh Karmacharya

[1]Institute of Agriculture and Animal Science, Tribhuvan University, Nepal
[2]Faculty of Animal Science, Veterinary Science and Fisheries, Agriculture and Forestry University, Nepal
[3]Chitwan National Park, Department of National Park and Wildlife Conservation (DNPWC), Nepal
[4]Centre for Molecular Dynamics Nepal (CMDN), Kathmandu, Nepal

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ABSTRACT

Elephant End otheliotropic Herpes Virus (EEHV) has been proved as the cause for the deaths of at least 80 elephant calves worldwide till 1995, when it was first identified for the first time in North America. The study was conducted in 17 elephants (15 elephant calves and 2 adult), maintained at Chit wan Districts namely Elephant Breeding Centre and hattisar of Chit wan National Park and Gaida Wildlife Camp of Sauraha. From the selected elephants, whole blood sample, conjunctival swab and Buffy coat were collected. The samples were stored in deep freeze until taken to laboratory. DNA was extracted off conjunctiva swab, buffy coat and whole blood sample using QIAGEN Dneasy blood and tissue kit and PCR analysis was performed using 2 genes specific to EEHV, Pan EEHV Pol and EEHV1-U38. Gel electrophoresis was done using 2% agarose gel at 110 V. Statistical analysis was done using SPSS 16.0 Version and Microsoft Excel–2007. In the research site, none of the elephants (0%) were infected with EEHV infection at the research period. Though the prevalence rate of EEHV infection is found to be zero in the research site at the research period, there is need for regular monitoring of the disease.

INTRODUCTION

The onset of Elephant End otheliotropic Herpes Virus (EEHV) is sudden in elephant calves with death within 1-7 days (Garner *et al.,* 2009). Major findings included cyanosis of tongue, systemic hemorrhagic lesions, pericardial effusion and mild inflammation (Fowler and Mikota, 2006; Ehlers *et al.,* 2001; Garner *et al.,* 2009; Ehlers *et al.,* 2006; Redrobe *et al.,* 2013). Similarly, EEHV infection showed detectable DNAemia up to 28 days before the onset of clinical signs (Stanton *et al.,* 2012; Stanton *et al.,* 2013). In serum biochemistry, there was mild renal dysfunction (Richman *et al.,* 1996).

EEHV has been proved as the cause for the deaths of at least 80 elephant calves worldwide till 1995, when it was first identified for the first time in North America (Joseph, 2011). In the serological study of 109 elephants, the prevalence rate for Bovine Herpes Virus1 (BHV1) was nearly 4% while that for Bovine Herpes Virus2 (BHV2) was nearly 23% (Bhat *et al.,* 1997). However, in another study of 15 elephants, four elephants were positive for BHV2 while none were positive for BHV1 (Metzler *et al.,* 1990).

Among nine identified strains of EEHV, EEHV1 was the most common cause for death though EEHV4 was also a lethal cause of hemorrhagic disease in Asian elephants of North America (Atkins *et al.,* 2013). The EEHV2, EEHV3, EEHV6, and EEHV7 have been detected in lung nodules of asymptomatic African elephants (Garner *et al.,* 2009).
Twenty one captive born Asian elephants out of thirty EEHV cases died in the USA between 1978 and 2012 with majority of the cases in the age group of 1.5 to 6 years (Isaza, 2013). Similarly, among the total death of 42 calves in North America, 22 died of EEHV infection (Hayward, 2012).

Wellehan *et al.* (2008) identified EEHV3 and EEHV4 from conjunctival swabs in Asian elephants. Similarly, EEHV3 was found in vaginal swab from elephant with vaginitis and EEHV5 was identified from vaginal swabs of two Asian elephants with vaginal plaques. EEHV6 was also identified in conjunctival swab of African elephant.

Among the 46 evaluated healthy elephants in South India using real time PCR of trunk washes, prevalence rate for EEHV1, EEHV3 or EEHV4 and EEHV5 were 6.5%, 8.7% and 19.6% respectively. However, EEHV2 and EEHV6 were not identified in that study (Ling *et al.,* 2013). Ling's study found that major EEHV1 gene subtype distribution patterns from India have shown the same diversity as in case of Asian elephants of North America and Europe.

Five Kenyan juvenile elephants with trunk nodules and one killed elephant with lung nodules had shown more than one herpes viruses at a time. In those elephants, EEHV1B, EEHV2, EEHV3A, EEHV3B, EEHV6, EEHV7A, and EEHV7B were identified (Pearson, 2013). Among these five elephants, four elephants were positive for EEHV3 and

* Corresponding author: Arjun Pandit
Department of Institute of Agriculture and Animal Science, Tribhuvan University, Nepal

EEHV4, four elephants were positive for EEHV6 and one elephant was positive for EEHV1. (Nofs *et al*., 2013).
Till now, EEHV1A, EEHV1B and EEHV5 have been detected only in Asian elephants while EEHV2, EEHV3, EEHV6, and EEHV7 have been detected only in African elephants (Cracknell *et al.,* 2006; Hardman *et al.,* 2012 and Hayward, 2012). Four types of EEHV (EEHV1A, EEHV1B, EEHV3 and EEHV4) have caused fatality in young Asian elephants, whereas EEHV5 has been found in the blood of apparently healthy adult Asian elephants. EEHV3 and EEHV4 are phylogenetically similar and were first identified in 2009 (Sripiboon *et al.,* 2013).

In the study of six asymptomatic Asian elephants in United Kingdom, prevalence rate of EEHV1 strain was 100% using TaqMan Real-time PCR. Though the virus was detected in trunk washes, conjunctival swabs, swabs of palate and swabs of vulva, highest prevalence was detected in conjunctival swabs (Hardman *et al.,* 2012).

Fifteen cases tested in South India with sudden hemorrhagic deaths between the period of 2007-2011 using the preserved samples in collaboration of Laboratory at the National Zoological Park in Washington DC, Johns Hopkins University, Baltimore and Kerala Forest Department, resulted nine positive cases for EEHV1 after first or second round amplification. By DNA sequencing results, eight of these cases were confirmed as EEHV1A and one was confirmed as EEHV1B strains (Zachariah *et al.,* 2013).

However, in the EEHV workshop held in January 2013 in Houston, Texas, identification of 11 types namely, EEHV1A, EEHV1B, EEHV2, EEHV3A, EEHV3B, EEHV4, EEHV5A, EEHV5B, EEHV6, EEHV7A and EEHV7B was proposed. Among them, EEHV1, EEHV4 and EEHV5 were found to be distributed in Asian elephants while EEHV2, EEHV3, EEHV6 and EEHV7 were distributed in African elephants. EEHV1, EEHV3 or EEHV4 and EEHV5 were found in trunk wash samples in Indian asymptomatic elephants. EEHV2, EEHV3A and EEHV3B, EEHV6, and EEHV7A and EEHV7B were found in lung and trunk wash samples in the elephants of Kenya (Pearson, 2013).

The survey report on the EEHV in Thai camp elephants has reported the absence of EEHV in the elephants of Thailand (Hildebrandt *et al.,* 2001). Similarly, lymph node biopsies and PCR of whole blood of 39 Asian elephants revealed the absence of EEHV (Hildebrandt *et al.,* 2005; Hildebrandt *et al.,* 2006). However, the death of 3 year old female elephant named "Seima" in Cambodia in May 6, 2004 was tested with liver tissue sample. DNA extraction and DNA sequencing showed 99% similarity with U. S. A. strain of EEHV1 strain and 97% similarity with the European strain of EEHV1 (Reid *et al.,* 2006). In Thailand, a 2-year old male captive elephant calf died due to EEHV1B while another 3-year old male captive elephant calf died due to EEHV3 in 2009 (Sripiboon *et al.,* 2011).

In the PCR examination of conjunctival swabs of eight Asian elephants with epiphora, blepharitis, and conjunctivitis, two novel her pesvir uses distinct from EEHV were identified which might be the members of the family Gamma her pesvirinae. This fact showed the need of further understanding and characterization of those viruses to understand their role in elephant health (Wellehan *et al.,* 2006).

A survey on the prevalence of EEHV carried out in 57 Asian elephants and 17 African elephants of 12 zoos and 3 circuses in Europe and 1 zoo in Israel, reported six positive cases for EEHV out of 57 Asian elephants. However, none of the 17 elephants were positive for the disease (Fickel *et al.,* 2001).

In Thailand, a death of a two–year old male elephant in 2005 and three–year old male elephant in April 2011 were proved to be due to EEHV4 subtype, which was the first confirmed case of EEHV4 in Asian elephants (Sripiboon *et al.,* 2013).

In February 2011, a 42 year old wild born female Asian elephant in Methai presented with generalized depression, cyanosis of tongue and bilateral swelling of temporal glands was tested for EEHV with trunk wash sample and found to be infected by EEHV5 (Schmitt *et al.,* 2012).

In the Elephant Breeding Centre of Chitwan National Park, Nepal, out of 8 elephant calves, only one female elephant calf of about 2 years old died due to suspected EEHV with symptoms of classical EEHV, in spite of treatment with Acyclovir in November 2012. DNA sequencing and conventional PCR confirmed the death of the calf to be due to EEHV1A (Gairhe *et al.,* 2013). Similarly, in 2011, an elephant calf died with similar symptoms of EEHV. Two other suspected cases of EEHV were reported there and one similar case was reported from Bardia National park of Nepal (Picciotto, 2012). Only one EEHV unconfirmed case which was seen in Sukhlaphanta Wildlife Reserve was recovered by administration of Famcyclovir (Gairhe, personal communication). EEHV detection in captive born elephants of Nepal may be a challenge for raising captive born elephant calves (Nolen, 2011; Gairhe, 2011, Gairhe, 2012).

A study conducted in 14 elephants by three different sampling techniques (conjunctival swabs, respiratory secretions and whole blood) reported no positive EEHV infection in Nepal (Picciotto, 2012).

MATERIALS AND METHODS

Study area

The study was conducted in the elephants of three locations of Chitwan National Park, Elephant Breeding Centre at Khorsor, Chitwan National Park Hattisar and Gaida Wildlife Camp Hattisar at Sauraha. Among these three locations, Gaida Wildlife Camp Hattisar at Sauraha is under private ownership while two other locations are under the government ownership.

Chit wan National Park (CNP)

The park is situated in south central Nepal, covering 932 km^2 in the subtropical lowlands of the inner Terai. The area comprising the Tikauli forest from Rapti River to the foothills of the Mahabharat extending over an area of 175 km^2 was declared Mahendra Mriga Kunj (Mahendra Deer Park) by the late King Mahendra in 1959. In 1963, the area south of Rapti River was demarcated as a rhinoceros sanctuary. The area was gazetted as the country's first national park in 1973, recognizing its unique ecosystems of international significance. UNESCO declared CNP a World Heritage Site in 1984. In 1996, an area of 750 km^2 surrounding the park was declared a buffer zone, which consists of forests and private lands including cultivated lands (DNPWC, 2010).

The park is home to more than 50 mammal species, over 525 birds, and 55 amphibians and reptiles. The endangered fauna found in the park are: one-horned rhinoceros, gaur, royal bengal tiger, wild elephant, four horned antelope, pangolin, golden monitor lizard, python, bengal florican, lesser florican, giant hornbill, black stork, white stork, etc. (DNPWC, 2010).

Figure 1 Location of the study site

Elephant Breeding Centre (EBC)

Elephant Breeding Centre is located near Bodreni village across the Khageri river at Bacchauli village development Committee-1, Chitwan. It is 12 kilometers south west of Tandi Bazar.

Traditionally, captive elephants were not raised for breeding in Nepal though calves were born in camps occasionally. However, breeding of elephants started functionally after the establishment of EBC in 1986. During the period of 1987 to June 2013, thirty nine elephant calves were born in this centre. Among them, twin calves were born once while other births were single. During this period, two calves died of suspected EEHV, one died of training injuries and another died of enteric disease (EBC, 2013).

Gaida Wildlife Camp Hattisar

Gaida Wildlife Camp, a private resort and including its Hattisar is located inside the Chitwan National Park.

Selection of Elephants

Altogether 15 elephant calves and 2 adult elephants with calves were selected. Elephant calves belonged to the age group from 4.5 years to 15 years of age while adult elephants were of 43 to 47 years old. The two adult elephants were having grown up calves of age about 10 years. Elephants selected from different locations are given below:

Elephant Breeding Centre (EBC), Khorsor: 10 (8 elephant calves and 2 adult elephants). Chitwan Hattisar of Chitwan National Park (CNP): 5 elephant calves. Gaida Wildlife Camp Hattisar, Sauraha: 2 elephant calves.

Sample collection and processing for EEHV study

From the elephants selected, blood sample and conjunctival swabs were collected aseptically.

Blood collection

Blood (~20 ml) was collected from auricular veins into EDTA tubes (whole blood) and Serum separator tubes (for blood biochemistry) using 19 gauze winged blood collection sets. Serum was separated within an hour and transferred to cryovials and preserved in deep freeze at -20˚C.

Type of samples

- **Whole blood sample:** Blood sample was collected in EDTA tubes, stored in deep freeze (NTNC laboratory) and taken to the laboratory (CMDN) for EEHV analysis.
- **Buffy coat:** After separation of serum, the remaining cellular sample was collected as the buffy coat.
- **Serum samples:** After collecting blood in gel tube, it was centrifuged for about 20 minutes at 5,000 rpm to separate serum from blood cells. Then, the serum was collected in cryovials and remaining cellular portion left in the original tube.
- **Conjunctival swab:** The conjunctival swabs were collected by inserting a sterile cotton–tipped swab into the conjunctiva and rubbing it along the inside of the eyelid and the cotton tip was removed by breaking and stored in a 2 ml cryotube filled with 0.3 ml saline. The sample was collected three times every week.

Storage of the sample

The entire samples i.e. whole blood, conjunctival swab and buffy coat sample were stored at -20˚C until taken to Centre for Molecular Dynamics, Nepal (CMDN), Kathmandu.

DNA extraction

The DNA was extracted off conjunctival swab, whole blood and buffy coat sample using QIAGEN DNeasy Blood and Tissue Kit (Qiagen Group, Texas, United States of America). The final extracted DNA was preserved in AE buffer. There were a total of 17 elephant samples.

To the original sample tube (~300 µL sample), 25 µL of proteinase K and 300 µL buffer ATL was added and thoroughly vortexed. After overnight incubation at 55˚C, Proteinase K was inactivated at 95˚C, for 10 minutes and centrifuged at 4000 rpm for 5 minutes. The supernatant was then transferred to a new 1.5 ml vial, with 200 µL buffer AL added and incubated at 55˚C for a further 10 minutes. Then, 200 µL ethanol was added and centrifuged for 1 minute (8000 rpm). The reaction mixture (~1000 µL) was then transferred to the kit provided spin column, centrifuged for 1 minute (8000 rpm); with the flow through (in collection tube) discarded and the spin column transferred to a new collection tube. The 500 µL buffer AW1 was added to the spin column, centrifuged for 1 minute (8000 rpm) and as earlier, the spin column was transferred to a new collection with the flow-through discarded. The 500 µL buffer AW2 was added to the spin column, centrifuged this time at 14,000 rpm for 3 minutes and transferred to a new collection tube. The final elution step was carried out by adding 60 µL buffer AE to the spin column, incubated at room temperature for 1 minute, then centrifuged at 1 min (8000 rpm) with the flow-through collected and stored at -20˚C.

Polymerase chain reaction (PCR) analysis

Nested PCRs were performed using 2 genes specific to EEHV, Pan EEHV Pol and EEHV1-U38. The primers are attached as follows:

A PCR reaction of total volume 25 µl was prepared containing 22 µl of 1X Invitrogen Bluemix PCR Master mix, 1 µl each forward and reverse primer (0.4 µM) to which 1 µl of extracted

undiluted DNA was added. Negative control was maintained to check for contamination.

Table 1 List of primers used in polymerase chain reaction (PCR) analysis

Gene	Primer name	Sequence 5'--3'
Pan EEHV POL	R1-LGH6711	GTATTTGATTTYGCNAGYYTGTAYCC
	R2-LGH6712	TGYAAYGCCGTNTAYGGATTYCGGG
	L1-LGH6710	ACAAACACGCTGTCRGTRTCYCCRTA
EEHV1-Specific U38	R1-LGH7445	GATTTTGCGAGYCTGTAYCC
	L2-LGH7448	CTGTCTACAGGGCARTCAAC
	L1-LGH7446	CACGCTGTCAGTATCTCCGTA

N PCR#	Genes	PRIMERS (fwd/rvs)	Product
1	Pan EEHV POL--1st Round	6711/6710	530 bp
	Pan EEHV POL--2nd Round	6712/6710	250 bp
5	EEHV1-U38--1st Round	7445/ 7446	530 bp
	EEHV1-U38--2nd Round (1A)	7448/7445	490 bp

The PCR thermo-cycling protocol was:

Table 2 Thermo-cycling protocol of PCR

Thermo cycling	Temperature	Time period
Initial denaturation	95°C	2 mins
Denaturation	95°C	40 sec
Annealing	50°C	45sec
Elongation	73°C	60 sec
Number of cycles		45
Final elongation	72°C	7 min
Cool down	4°C	Hold

Gel Electrophoresis

Post PCR, amplified samples were run on a 2% agarose gel at 110 V. Images are attached on the results section.

RESULTS

General management status of EEHV based on questionnaire survey

In the research site, none of the elephants were monitored for the presence of EEHV at any time. In the survey with mahouts of 17 elephants, all of them (100%) have some knowledge about EEHV. Among the total population of elephants, all the elephants (100%) have access to veterinarian at any time.

Among the different locations, 29% of the elephants were located at the site where there was no any death due to EEHV infection. However, 71% of the elephants were located at the site where death of calves due to EEHV infection occurred at earlier years. In other words, majority of elephants (71%) were in close contact with the EEHV infected premises in their life time (Figure 2).

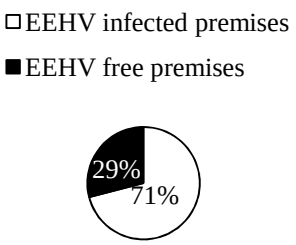

Figure 2. Distribution of elephant according to contact with dead calves due to EEHV

Prevalence of EEHV in the research site

In the research site, all the mahouts of elephant with calves (100%) used to monitor the clinical signs regularly for edema and cyanosis of tongue. None of the elephants had shown such clinical signs during the research period.

Similarly, none of the elephants were infected with EEHV infection during the time of research as confirmed by Polymerase Chain Reaction (PCR) analysis of various samples (Figure 3).

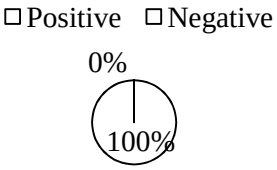

Figure 3 Prevalence of EEHV in the research site

Gel electrophoresis for nested PCR EEHV Pan-Pol 1st and 2nd round

All the samples (A to Q, Q7) were not amplified in round 1 of PCR. However, in round 2, positive control (Pos) was showing up in expected region of 250 base pair (bp) as shown by ladder (far left) between 200 and 300 bp. Most samples were appearing to bind higher (>250 bp) or lower than the product size and sample H (Round 2) had multiple bands indicating that the primers might have bound to multiple binding sites on the gene. NTC (No Template Control) was used as a PCR contamination control. The ladder 100 bp starts at 100 bp goes on till 1000 bp; however our region of interest was between 200-300 bp.

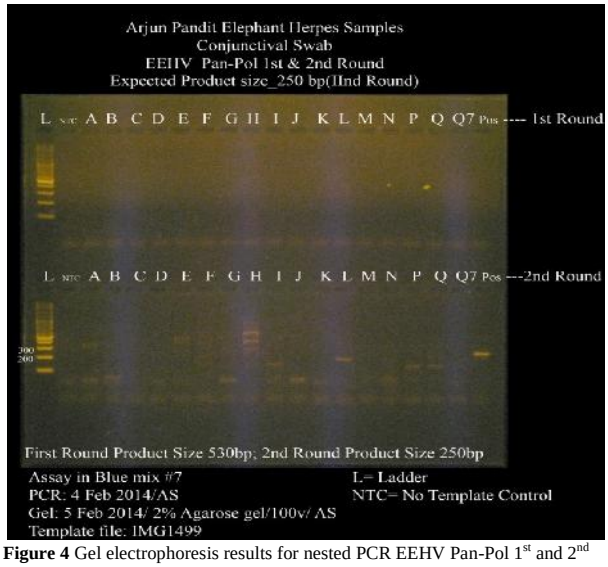

Figure 4 Gel electrophoresis results for nested PCR EEHV Pan-Pol 1st and 2nd round

None of the samples detected the presence of EEHV with our EEHV Pan POL 6710/6712 primers during second round PCR within all the samples (Figure 4, Lane A-Q, Q7).

Gel electrophoresis for nested PCR EEHV U38 1st and 2nd round

The samples were not amplified in round 1 of PCR. However, in round 2, positive control (Pos) was showing up in expected region of 490 base pair as shown by ladder (far left). Most samples were appearing to bind higher (>490 bp) or lower than the product size and sample H (Round 2) had multiple bands indicating that the primers might have bound to multiple binding sites on the gene. NTC (No Template Control) was used as a PCR contamination control.

None of the samples detected the presence of EEHV with our EEHV1 U38 7448/7445 primers during second round PCR within all the samples (Figure 5, Lane H, I, L, P, Q)

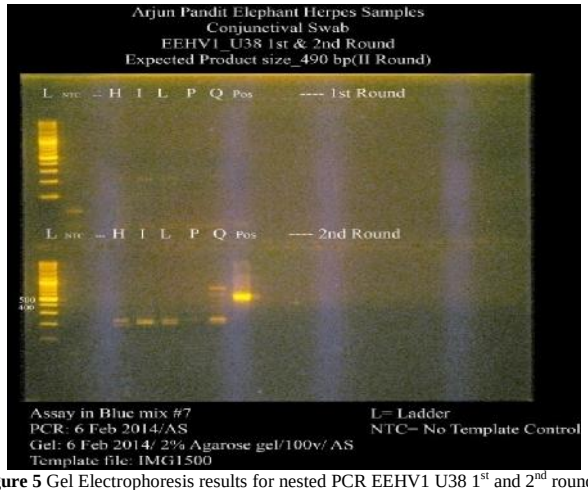

Figure 5 Gel Electrophoresis results for nested PCR EEHV1 U38 1st and 2nd round

Serum Creatinine Level (Blood biochemistry)

Serum creatinine value in sampled elephants ranged from 0.9 to 1.2 mg/dl. Two elephants had the value below normal range (1.0 to 2.0 mg/dl). However, the subnormal value was nearly normal (0.9 mg/dl) (Figure 6).

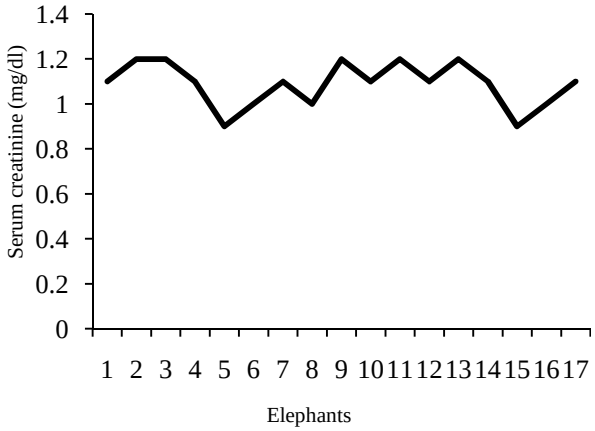

Figure 6 Serum creatinine values in tested elephants

DISCUSSION

Regular monitoring program for EEHV infection do not occur in the research site and majority of the elephants were in close contact with one another. Few elephant calves that died of EEHV infection create suspicion of high prevalence of the disease. However, the death of elephant calves by EEHV infection taught mahouts well about the clinical signs and mortality pattern of the disease.

During the research period, none of the elephants showed typical signs like facial edema, cyanosis and edema of the tongue as described by Fowler and Mikota (2006), Ehlers *et al.* (2001), Garner *et al.* (2009), Ehlers *et al.* (2006), Redrobe *et al.* (2013), Stanton *et al.* (2012), and Stanton *et al.* (2013), indicating sporadic and seasonal occurrence of the disease. The creatinine level was normal in all the elephants indicating the absence of any renal dysfunction. Absence of renal dysfunction might suggest the absence of EEHV infection in those elephants during the research period which is similar to the view of Richman *et al.* (1996).

Though majority of the elephants were in close contact with the EEHV infected elephant calves in their life time, none of the elephants during research period were infected with EEHV. This may be due to the fact that the disease cannot be transmitted to any other elephants as suggested by Hayward *et al.* (2009).

As reported by Joseph (2011), the disease is of sporadic nature. In our country also, though there were few suspected and one confirmed case of EEHV, the disease was absent in the research period. The prevalence rate of the disease was found to be similar to that reported by Hildebrandt *et al.* (2001) in Thailand. The prevalence rate was also similar to that reported by Picciotto (2012) conducted in elephants of Chitwan National Park, Nepal. The findings of this study were also similar to the findings of Hildebrandt *et al.* (2005). Similarly, the prevalence rate was also similar to that reported by Fickel *et al.* (2001) in the study of 17 African elephants though the findings was contrasting to that of 57 Asian elephants in the same study. The similarity in the findings with those of Picciotto (2012), Hildebrandt *et al.* (2001), Fickel *et al.* (2001) and Hildebrandt *et al.* (2005) might be due to similar methodologies and similar type of elephants. All these studies included the asymptomatic elephants in particular research site within particular research period.

However, the prevalence rate was lower compared to that reported by Metzler *et al.* (1990), Bhat *et al.* (1997), Cracknell *et al.* (2006), Schmitt *et al.* (2012), Reid *et al.* (2006), Wellehan *et al.* (2008), Garner *et al.* (2009), Hardman *et al.* (2012), Isaza (2013), Hayward (2012), Atkins *et al.* (2013), Ling *et al.* (2013), Nofs *et al.* (2013), Sripiboon *et al.* (2013), Pearson (2013), and Zachariah *et al.* (2013). The higher prevalence rate in these studies might be due to different methodology in selection of elephants where samples were collected mostly from dead and symptomatic elephants. Virus shedding was higher in the sample elephants compared those we selected. Thus, these facts suggests for the higher prevalence rate of EEHV infection in elephants compared to our study.

The findings were contrasting to the information of Gairhe (Personal communication). One EEHV infection confirmed by PCR analysis in 2012 and three unconfirmed cases from Chitwan and one unconfirmed case from Sukhlaphanta Wildlife Reserve have shown typical symptoms and postmortem lesions as described by Fowler and Mikota (2006), Ehlers *et al.* (2001), Garner *et al.* (2009), Ehlers *et al.* (2006), Redrobe *et al.* (2013), Stanton *et al.* (2012), and Stanton *et al.*

(2013). However, none of the elephants have shown such symptoms during the research period. This might be the reason for the lower prevalence rate of EEHV infection.

CONCLUSIONS

The findings of the PCR analysis and serum biochemistry revealed the absence of the highly fatal disease Elephant Endo the liotropic Herpes Virus (EEHV) infection in the elephant calves of Chit wan district. However, there is still a great threat of this infection in the near future because there was one confirmed death due to EEHV and other few suspected cases few years ago in the research site. So, regular monitoring of EEHV is suggested.

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