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Biological and environmental factors associated with the detection of elephant endotheliotropic herpesvirus in Asian elephants (*Elephas maximus*) in Thailand

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ABSTRACT. Elephant endotheliotropic herpesvirus (EEHV) infection is one of the most common diseases in young elephants, causing severe fatal hemorrhagic disease. Subclinical infection was previously described; however, information about the factors associated with virus shedding and reactivation were scarce. To identify the biological and environmental factors related with EEHV detection, blood and oral swab samples were collected from nine captive Asian elephants in Thailand for one year and tested for EEHV presence using real-time PCR. Data including hematological values, management, environmental temperature, and serum cortisol levels were also recorded and analyzed. Results showed that the viral detection frequency ranged from 0–25%. The highest detection frequency was found in the two youngest elephants, aged less than 15 years. Three types of viruses, EEHV1, EEHV4, and EEHV5, were found in this study, which also detected mixed infection in five elephants. Additionally, the study found that sample type, changes in hematological values, management and health issues, and serum cortisol levels were not associated with herpesvirus detection in the elephants. However, EEHV detection percentage was significantly increased in the summer (mid-Feb to mid-May), possibly due to body fitness reduction from food source limitation and low nutrient content. To obtain a broad aspect of EEHV management, long-term EEHV monitoring is highly recommended in every captive elephant herd.

KEY WORDS: Asian elephant (*Elephas maximus*), elephant endotheliotropic herpesvirus (EEHV), real-time PCR, Thailand

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Elephant endotheliotropic herpesvirus hemorrhagic disease (EEHV HD) has potentially severe implications for elephant conservation, owing to the severity of the disease, particularly in young individuals [14, 23]. Fatalities associated with EEHV infection have been reported worldwide, including in Thailand [27]. Similar to other herpesviruses, EEHV can be found in apparently healthy elephants and intermittent shedding has been reported previously [3, 13, 30]. Quantitative polymerase chain reaction (qPCR) assays have been developed and validated to detect the most known types of EEHV [28, 30]. These assays, which can detect the virus before the onset of clinical signs and also during shedding, are used routinely for screening both healthy and clinically ill elephants [28–30]. This technique has been applied for health and disease monitoring in a healthy population and allows us to identify the shedder in the herd [3, 13, 30], which could facilitate better management plans.

Herpesviruses are structurally fragile and only survive for short periods outside the host body [9]. Transmission typically requires close contact, particularly mucosal contact, such as during licking and nuzzling, especially between mother and offspring or between neonates [20]. It is presumed that such close contact would occur frequently in elephants, both captive and wild, and could likely account for EEHV circulation within a herd [24]. Indeed, when EEHV was monitored longitudinally, it was clearly demonstrated that EEHV was circulating within the elephant herd, and that the virus detection frequency varied among the individuals [2, 3, 13, 30]. However, the variables associated with EEHV reactivation and shedding remain unclear. To study viral shedding, a high percentage of EEHV detection using trunk wash sampling was reported [3, 30]. This is likely due to the large volume of sample obtained

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using this method, which increases the likelihood of viral detection. However, trunk wash collection requires well-trained elephants, and the personnel conducting this procedure should also be well-trained to minimize the risk of zoonotic diseases such as tuberculosis. Swab samples have been favored for use in shedding studies, which have been previously reported as potential sample sources for EEHV detection [15, 19].

Through the establishment of latency, herpesviruses can avoid elimination by the immune system and have the ability to persist in the host for life after the primary infection [12]. Since latency is controlled by the host immune system, the virus may also reactivate spontaneously and shed sporadically without the development of clinical signs [32]. A high frequency of viral shedding and reactivation is generally reported in immunocompromised patients [6, 12, 32]. Thus, it is hypothesized that stressful events and immunosuppression could induce EEHV reactivation and shedding. A previous study has reported a lack of correlation between EEHV shedding and cortisol levels [3]; however, a small sample size meant that the study's power to detect a correlation would have been limited.

Table 1. Details of each elephant and the elephant endotheliotropic herpesvirus (EEHV) detection frequency in blood and oral swab samples collected during the study period (57 weeks)

Name code	Sex	Age (years)	No. of sampling week	No. of positive week
SD	F	10	57	11 (19%)
JM	F	26	57	9 (16%)
JP	F	44	57	6 (10%)
KT	F	54	44	5 (11%)
NT	F	77	12	1 (8%)
KK	M	15	12	3 (25%)
BL	M	29	10	1 (10%)
MK	M	31	12	0 (0%)
PK	M	44	9	1 (11%)

F, female; M, male.

To determine the variations in EEHV detection, viral reactivation and shedding pattern, and biological and environmental factors associated with the frequency of EEHV detection, longitudinal EEHV monitoring was conducted in a captive elephant herd in Thailand. Samples including blood and oral swabs were collected from nine Asian elephants for one year and tested for the presence of EEHV using real-time PCR. Data including hematological values, management and medical records, environmental temperature, and serum cortisol levels (SCLs) were recorded and their association with EEHV detection was determined in this study. Results from this study could provide a better understanding of viral reactivation patterns, and could improve the EEHV monitoring and management plan in the future.

MATERIALS AND METHODS

Animals and sample collection

This study was approved by the Kasetsart University Institutional Animal Care and Use for Scientific Research Committee (No. ACKU61-VET-069). A herd of captive Asian elephants (*Elephas maximus*) at Khow Keaw Open Zoo Thailand, which had previously reported clinical EEHV infection in elephant, was monitored for EEHV infection for 57 weeks. This study started at the beginning of January (referring to the 1st week) and continued until the end of January in the next year (the 57th week). In addition, the seasons were divided according to the Thailand climatological classification: summer, rainy, and winter. Summer was from 8th to 20th weeks, rainy from 21st to 42nd weeks, and winter from 1st to 7th and 43rd to 57th weeks.

A herd consisting of nine Asian elephants trained with positive reinforcement-based standard training techniques, the details of which are listed in Table 1, was selected for the study. The elephants shared the exhibit area together during the daytime, while they were held separately during nighttime in the holding area. The male elephant was separated from the group during the musth period and stayed at the restriction area. The elephants were mainly fed pineapple leaves and grass. Various types of fruit were occasionally provided according to availability in the market. Water was provided via a hose pipe three times per day and a small pond of clean water was available *ad libitum* in the exhibit area. Samples were collected weekly from four elephants (SD, JM, JP, and KT) and once a month from the others. Samples were collected in the morning from the holding areas of each elephant to avoid cross-contamination between elephants. All elephants were physically examined and considered healthy during the sampling period. The animals included in this study were well-trained; however, if an elephant demonstrated reluctance to participate or cooperate in sample collection at a particular time, that elephant was excluded from the sampling regime due to safety reasons and to avoid the stress affected by sampling.

Blood and oral swab samples were collected from each elephant to identify the reactivated and shedding stages, respectively. A minimum of 5 ml blood was collected from the auricular ear vein and then divided into EDTA-coated and plain tubes. Oral swabs were collected following a previously published protocol [13]. Briefly, a sterile cotton swab soaked in phosphate buffer saline (PBS) or sterile saline was used to swab the oral cavity of the elephant. The swab was then kept in a 1.5 ml tube containing 250 µl PBS solution, which was centrifuged at 6,000 × *g* for 3 min at room temperature. The swab was then discarded and the supernatant was used for DNA extraction. All samples were stored at 4°C during transportation. If DNA could not be extracted within 48 hr, the samples were stored at −20°C until further processing.

Real-time PCR for EEHV detection

DNA was extracted from 200 µl whole blood or swab solution using FavorPrep™ Viral Nucleic Acid Extraction Kit (Favorgen Biotech Corp.; Ping-Tung, Taiwan), following the manufacturer instructions. To evaluate the quality of extracted DNA, elephant tumor necrosis factor (TNF) real-time PCR assay was used to determine the presence of elephant genomic DNA, as previously described [29]. DNA extraction was repeated once if the sample was TNF-negative. Only the TNF-positive samples were tested for

EEHV.

To test for the presence of EEHV, DNA samples were initially screened for EEHV using TaqMan probe real-time PCR with a universal EEHV probe and primers [7]. Sample, that gave threshold cycle (Ct) less than 40, was then confirmed and typed by using EEHV type-specific TaqMan probe real-time PCR for EEHV1, 3/4, and 5 [28, 30]. All qPCR reactions were conducted using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc.; Hercules, CA, USA) with Luna® Universal qPCR Master Mix (New England Biolabs Inc.; Ipswich, MA, USA). The qPCR condition for universal probe-primer was 95°C for enzyme reactivation, then 40 cycles of 95°C 10 sec, 55°C for 10 sec, and 72°C for 10 sec, while the two-step qPCR condition used for type-specific probe-primer was enzyme reactivation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Samples were considered positive when Ct value was less than 40, and negative control (sterile water) gave a zero Ct value. Only samples positive for both universal and type-specific qPCR were considered positive for this study. To confirm the results, samples were randomly selected and submitted for direct sequencing. Sequences were analyzed using BioEdit® (Ibis Biosciences; Carlsbad, CA, USA) and compared to sequences in the database (GenBank®) using Blast [1] to verify the anticipated EEHV identity.

Hematological values

Whole blood collected on the same day of EEHV monitoring was used to evaluate the hematological values manually by a skilled technician at the hematological laboratory, Khao Kheow Open Zoo. The recorded values included hematocrit value, hemoglobin level, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, segmented neutrophil count, lymphocyte count, monocyte count, eosinophil count, and total protein levels.

Serum cortisol levels

Serum collected on the same day of EEHV monitoring was used to determine the level of serum cortisol by enzyme immunoassay (EIA) at Conservation Research and Animal Health, Khao Kheow Open Zoo, Thailand. The EIA analysis was conducted following a standardized protocol that has been validated for Asian elephants [5]. Briefly, polyclonal cortisol antiserum at dilution 1:8,500 was coated on the plate and incubated overnight (at least 12 hr) at 4°C. After washing, 50 µl sample, standard, and control at the desired concentration were added, then 50 µl cortisol-horseradish peroxidase (HRP) working solution (1:20,000) was added immediately, and incubated for 1 hr at room temperature. The plate was then washed, 100 µl ABTS substrate added to each well, and incubated at room temperature for 30–60 min in dark. The absorbance of the plate was measured at 540 nm, with the value for the negative control being 1 or less. The SCLs were quantified by comparing with standard cortisol levels.

Data records

Records on management including food, activities, sleeping period, staff changing, and infrastructure reconstruction were obtained by interviewing the elephant keepers on a monthly basis. Medical records and behavior changes were recorded by the zoo veterinarians. Moreover, environmental data including temperature, humidity, and rainfall accumulated level were collected weekly from SriRacha Meteorological Station, where the zoo was located.

Statistical analysis

Descriptive analysis was used to evaluate the EEHV herd status. Correlations between EEHV detection and hematological values, SCLs, management changes, environmental data, and season were analyzed using logistic regression by Stata® (Stata Corp LLC.; College Station, TX, USA). Significant differences were set at $P<0.05$.

RESULTS

Variations in EEHV detection

Eight of the nine elephants tested positive for EEHV on qPCR at least once during the study period. The variation in viral detection frequency in each elephant ranged from 0–25% (Table 1). The elephants with the two highest EEHV detection percentages were SD (19%) and KK (25%), the two youngest elephants in this study. Moreover, a variation in the EEHV detection frequency was also observed in each week and month. Out of the 57 weeks, 26 weeks were EEHV-positive (Fig. 1). The samples collected from 18th–20th weeks (end of April to the mid of May) had the highest number of the EEHV-positive samples (Fig. 1). In addition, when weekly sampling's elephants were considered, the total EEHV positive events were 29, which included 25 single positive episodes (86.2%; 25/29) and 4 of two-consecutive week positive episodes with the same type of infection (13.8%; 4/29).

Although EEHV reactivation and shedding were detected, no clinical signs of EEHV HD (e.g., depression, low appetite, facial edema, tongue cyanosis, and bloody diarrhea) were observed during the study period. The average Ct value of EEHV-positive blood and oral swab samples was 37 ± 1.3 and 36.3 ± 2, respectively. Type-specific qPCR detected all three different types of EEHV, EEHV1, EEHV3/4, and EEHV5, in this study. Five elephants (SD, JM, JP, KT, and KK) carried more than one type of EEHV, while three elephants (NT, BL, and PK) had single-type infections (Fig. 1).

In this study, a total of 504 samples were collected, of which 41 samples (8.13%) were EEHV-positive. Of these positive samples, 46% were from blood, 43% from oral swabs, and 11% from both oral and swab samples at the same sampling time. The univariate analysis revealed no significant difference in EEHV detection from blood or oral swab samples (OR=1.17; 95% CI: 0.62–2.22; $P=0.6$).

code	Sampling week																												
	JAN 2018					FEB2018				MAR2018				APR2018				MAY2018					JUN2018				JUL2018		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
SD	n	n	n	n	n	n	$1^{B}5^{S}$	n	n	n	1^{B}	1^{B}	n	5^{B}	n	n	5^{B}	n	n	4^{BS}	n	n	n	n	4^{S}	n	n	n	n
JM	n	n	5^{S}	n	n	n	n	n	n	n	n	n	5^{B}	n	5^{B}	n	n	4^{S}	4^{S}	4^{S}	n	n	n	n	n	4^{S}	n	n	4^{S}
JP	n	n	n	5^{B}	n	5^{S}	n	n	n	n	n	n	n	n	n	n	n	n	4^{BS}	n	n	n	1^{B}	n	4^{S}	4^{S}	n	n	n
KT	4^{B}	-	-	-	-	5^{S}	-	-	-	n	-	-	-	n	-	-	-	n	4^{BS}	n	n	n	4^{B}	1^{B}	n	n	n	n	n
NT	n	-	-	-	-	n	-	-	-	n	-	-	-	n	-	-	-	4^{B}	-	-	-	-	n	-	-	-	-	-	-
KK	n	-	-	-	5^{B}	-	-	-	-	1^{S}	-	-	-	1^{B}	-	-	-	n	-	-	-	-	n	-	-	-	-	-	-
BL	n	-	-	-	-	1^{B}	-	-	-	n	-	-	-	n	-	-	-	n	-	-	-	-	n	-	-	-	-	-	-
MK	n	-	-	-	-	n	-	-	-	n	-	-	-	n	-	-	-	n	-	-	-	-	n	-	-	-	-	-	-
PK	-	-	-	-	-	-	-	-	-	-	-	-	-	n	-	-	-	4^{S}	-	-	-	-	n	-	-	-	n	-	-

code	Sampling week																												
	JUL18		AUG2018				SEPT2018				OCT2018					NOV2018				DEC2018					JAN2019				
	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	
SD	n	n	n	n	n	n	n	n	n	n	n	n	n	5^{B}	n	n	1^{B}	n	n	n	1^{S}	n	1^{S}	n	n	n	n	n	
JM	n	n	n	n	n	n	n	n	n	n	1^{S}	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	
JP	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	
KT	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	
NT	-	-	n	-	-	-	n	-	-	-	n	-	-	-	-	n	-	-	-	n	-	-	-	-	n	-	-	-	
KK	-	-	n	-	-	-	n	-	-	-	n	-	-	-	-	n	-	-	-	n	-	-	-	-	n	-	-	-	
BL	-	-	n	-	-	-	-	-	-	-	n	-	-	-	-	-	-	-	-	n	-	-	-	-	n	-	-	-	
MK	-	-	n	-	-	-	n	-	-	-	n	-	-	-	-	n	-	-	-	n	-	-	-	-	n	-	-	-	
PK	-	-	n	-	-	-	n	-	-	-	n	-	-	-	-	n	-	-	-	n	-	-	-	-	-	-	-	-	

Fig. 1. Sampling schedule throughout the study period (57 weeks), illustrating elephant endotheliotropic herpesvirus (EEHV) detection using qPCR in each sampling week. Blue color shows EEHV-positive, the number indicates the detected EEHV type (1, 4, 5), and the superscript indicates sample type (B, Blood; S, Swab). In addition, (n) indicates negative result and (−) indicates no sample available.

Factors associated with EEHV detection

Hematological values: The hematological values of each elephant were monitored with EEHV monitoring. The fluctuation of hematological values in each elephant was noted. However, the average blood values were within the normal range when compared to the reference levels [21]. In addition, we noted increased red blood cell count and pack cell volume in male elephants. The average lymphocyte level during the EEHV-positive period was also more likely to be higher than the average level during the EEHV-negative period (Table 2). In addition, approximately 49% EEHV-positive samples showed an elevated lymphocyte count when compared to an individual average level.

SCLs: SCL fluctuation within and between individual elephants was observed throughout the study period. Thus, the average SCL of each elephant was calculated and used as the reference value for each individual (Table 3). Most elephants showed high SCLs during 42nd–44th weeks; however, only one event of EEHV reactivation was detected on 43rd week. Moreover, when the SCLs from EEHV-positive elephants were compared, the SCL at EEHV-positive event was higher than the average SCL of the individual at only 16% events (Table 3), and 26% EEHV-positive events occurred one week after increased SCL. Other EEHV-positive events occurred when the SCL was average. Additionally, the highest SCL in each elephant was not always associated with EEHV detection, as no EEHV DNA was detected in either blood or swab samples at the time.

Management and environmental changes: Medical records revealed no major health issues during the study period. At 34th week, JM showed mild abdominal pain that correlated with high SCL at that time; however, no EEHV reactivation or shedding was observed. At 41st week, JM gave birth to a female calf, correlating with the high SCL of a group of female elephants, and no EEHV detection was recorded. From 45th to 53rd weeks, PK was in the musth period; however, no elevation in SCL was observed and EEHV was undetected during this period. Changes in management including elephant barn reconstruction, introduction of new mahout, and changing the holding area of one elephant (BL) was recorded; however, no EEHV was detected during this period. Regarding the season, the percentage of EEHV-positive events in summer, rainy, and winter seasons were 43%, 24%, and 33%, respectively.

Univariate logistic regression was used to determine the association of factors including age, types of sample, season, hematological values, management changes, medical records, environmental temperature, and SCL with EEHV detection. Results revealed a significant association of seasons, SCL, and lymphocyte count with EEHV detection ($P<0.2$). Further analysis using multivariate logistic regression showed that only season was significantly associated with EEHV detection ($P<0.05$). Bonferroni's method was then used to compare the number of EEHV-positive samples in three different seasons, which revealed that summer was significantly related to EEHV detection in this study (Fig. 2).

DISCUSSION

To evaluate the hypothesized variables associated with EEHV reactivation and shedding, this longitudinal study considered hematological values, seasons, and stressful events in a captive Asian elephant herd in Thailand. The herd was selected based on the presence of previous EEHV clinical cases, close contact among herd members that could facilitate transmission, and suitably

Table 2. The average (± SD) and range of lymphocyte levels ($\times 10^3$ cells/µl) of each individual elephant throughout the study period, comparing between the average levels during elephant endotheliotropic herpesvirus (EEHV)-negative and EEHV-positive periods

Elephant code	EEHV-negative period		EEHV-positive period	
	Average ± SD	Range	Average ± SD	Range
SD	4.64 ± 2.12	2.17–15.08	7.15 ± 3.88	3.1 0–15.08
JM	4.53 ± 1.69	1.36–9.36	4.58 ± 1.14	1.36–9.36
JP	4.15 ± 1.82	2.01–10.6	4.45 ± 1.40	2.83–6.01
KT	4.61 ± 1.84	2.24–9.76	4.95 ± 1.01	3.74–6.46
NT	6.19 ± 1.74	3.03–8.54	6.41 [a]	- [a]
KK	5.93 ± 2.07	2.8–12.44	6.11 ± 1.09	5.15–7.31
BL	5.47 ± 3.03	2.17–12.15	9.07 [a]	- [a]
MK	4.66 ± 1.38	2.79–8.15	- [b]	- [b]
PK	4.70 ± 3.50	2.51–16.51	6.32 [a]	- [a]

a) Single EEHV-positive period; b) No EEHV-positive periods.

Table 3. The average serum cortisol levels (SCL) and the number of elephant endotheliotropic herpesvirus (EEHV)-positive samples

Elephant code	SCL ± SD (ng/ml)	Number of sampling week	Number of EEHV-positive samples [a]	Number of high SCL periods	Number of EEHV-positive samples during high SCL period
SD	4.4 ± 4.7	57	11	6	2 (33%)
JM	8.9 ± 4.9	57	9	6	0 (0%)
JP	9.7 ± 5.1	57	6	6	1 (17%)
KT	15.1 ± 4.3	44	5	4	0 (0%)
NT	15.1 ± 6.3	12	1	2	1 (50%)
KK	25.3 ± 11.2	12	3	1	0 (0%)
BL	68.2 ± 25.2	11	1	2	1 (50%)
MK	25.4 ± 9.2	12	0	2	0 (0%)
PK	15.7 ± 9.6	9	1	2	0 (0%)
Total				31	5 (16.13%)

a) Includes both EEHV-positive blood and swab samples in each sampling week.

trained elephants and veterinarians to permit sample collection. A total of 504 samples were collected over 57 weeks, and the EEHV detection percentage was 8.13%. None of the EEHV-positive elephants in this study developed EEHV HD-related signs during the study period. Therefore, the positive events in this study may be referred to as subclinical or asymptomatic EEHV infection [30].

During the study period, EEHV was detected in eight out of nine elephants. The EEHV detection frequency was highly variable, and no pattern of viral shedding was observed. A higher frequency of viral detection was found in SD and KK, the two youngest members of the herd. This result is similar to that of a previous study, which showed that subclinical EEHV infection was more likely to be detected in young elephants when compared to other age classes [26]. In addition, a previous study [13, 30] found an increased frequency of EEHV shedding during pregnancy. However, in our study, JM was pregnant at 14 months of age when samples started to collect, but no increase in the shedding frequency was found. This finding agreed with a previous study that reported no difference in the frequency of EEHV detection throughout the pregnancy period [3]. Moreover, one episode of high viral shedding was detected in SD (Ct=32); however, no consistent clinical signs were observed, and no virus was detected in the blood collected on the same day. This high level of viral shedding could potentially be a risk factor for virus transmission to other elephants. However, since EEHV is yet to be cultured [14, 19], studies on viral physiology, pathogenicity, and infectious dose (*in vitro*) are limited. A low percentage of EEHV detection (11%) from both sampling sites at the same sampling time was reported, suggesting the independence of viral reactivation from shedding. This finding was similar to that of previous studies showing that viral shedding did not necessarily correlate with viremia [26, 30]. Moreover, this study found no significant difference in the detection of EEHV from different types of samples, similar to the findings of Sripiboon *et al*. [26].

It is common for hosts to be infected with multiple types of herpesvirus simultaneously [9, 10] and mixed EEHV infection has previously been reported in both healthy and clinically ill elephants [11, 25]. Mixed infection was also found in five elephants in this study. These and other findings suggest that infection with one type of EEHV is unlikely to protect elephants from infection by other types, and also raises the possibility of ineffective cross-immunity between each type of virus. Thus, type-specific molecular tests are recommended to confirm EEHV infection and analyze epidemiological relatedness, which will further improve health management.

It is recommended that standard blood profiles of each individual elephant should be established in each elephant facility, given the

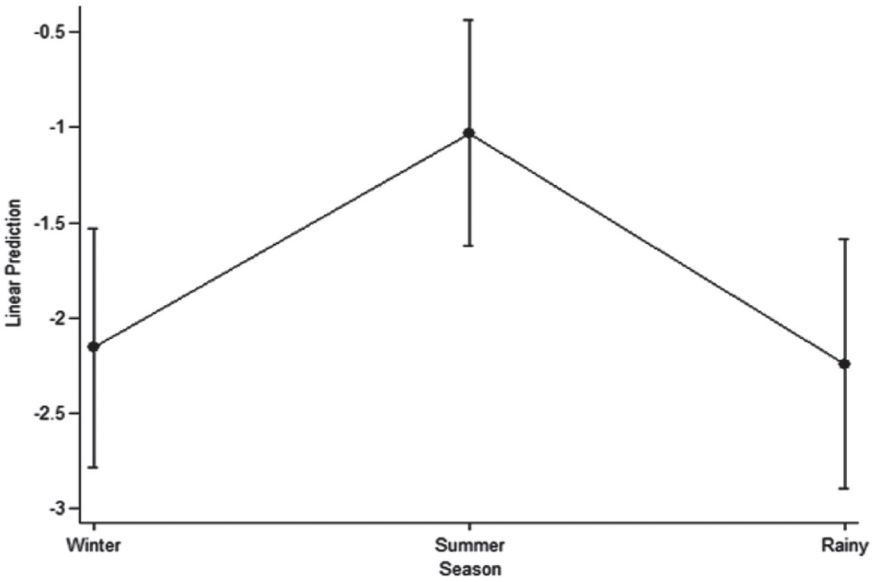

Fig. 2. The plot shows the relationship between season in Thailand and elephant endotheliotropic herpesvirus (EEHV) detection, when Bonferroni's method was used for comparison. EEHV was significantly detected during summer in Thailand (mid-February to mid-May).

variation in hematological values among individuals and age classes [34]. It is better to compare blood results to individual standardized values than to the age-specific normal reference ranges. Our study showed that the lymphocyte count increased insignificantly during positive events (P<0.2). A previous study has also reported that the lymphocyte count increased toward the onset of clinical signs, due to immune response to the infection [34]. We suggest that basic hematological values could be used as rule-in criteria in EEHV-suspected cases and to monitor disease progression. Further studies are necessary, but the outcome in this study is valuable for clinical application.

Here, we hypothesized that EEHV infection is likely to be reactivated by stressful events, such as pregnancy, weaning, husbandry, and management changes, with such stress impeding immune function [17]. In this study, SCLs were analyzed to determine the occurrence of any stressful events. A higher SCL was noted in the male elephants than that in the females. This was probably due to the difference in management system, in which male elephants were in constricted areas most of the time, to prevent any injuries from aggressive behavior, which could lead to high cortisol levels. SCLs were highly varied, which was similar to the wide SCL range (7.59–73.54 ng/ml and 11.13–51.55 ng/ml in non-cycling (n=6) and cycling (n=22) Asian elephants, respectively) reported by a previous study [4]. Therefore, this study used each individual average SCL to determine the change in SCL during the study period. A previous study reported a lack of correlation between stress levels and EEHV detection frequency [3]. The findings from this study also reported an inconsistent correlation with an increasing level of the stress hormone, suggesting that EEHV is spontaneously reactivated and sporadically shed, even in the absence of stress. However, whether long-term elevation of stress hormones can increase EEHV reactivation frequency remains unknown, and requires further investigation.

While considering the environmental factors associated with EEHV detection, we found no association between management changes and EEHV detection during the study period. However, it was possible probably due to no extreme management changes, such as weaning, training, and introducing new member during this study period. Continued long-term monitoring during potential stressful events should be conducted to evaluate this relatedness. The only factor found to be significantly associated with EEHV detection in this study was season; EEHV was likely to be reactivated and shed during summer (mid-February to mid-May), especially between end-April and mid-May. This finding could imply to the limitation of food sources during summer. Wild Asian elephants have different preferences for food sources at different times of the year [8, 16]. Their preference choice is generally based on the levels of protein and fiber in food [16]. However, food sources are limited and only provided by humans for captive elephants. The limitation of food sources, together with the low nutrient content during summer, could affect the immune function of the elephants. In humans, herpesvirus reactivation was shown to be reduced by nutritional supplementation. Virus-specific T cells are important to control viral reactivation, and glucose and some particular protein (glutamine) are needed to reactivate T cell functions [33]. As forage quality is generally low during summer [18, 22], this could imply high viral reactivation during this period. In addition, a previous study showed a rise-and-fall pattern of IgG antibodies against EEHV1 infection in captive Asian elephants in European zoo, in which one particular elephant showed a decrease in IgG levels before EEHV was detected in trunk wash; the antibody titers increased after being EEHV-positive up to eight weeks, when EEHV was not detected using PCR [31]. Results from this study, together with those from previous studies, suggest that data regarding nutrient composition, immune function, and EEHV reactivation should be considered for further studies.

In conclusion, this is the first longitudinal study conducted in an EEHV-positive herd in Thailand. The study revealed variation in EEHV detection frequency in each elephant, with the highest EEHV detection frequency being detected in the two youngest

elephants in the herd, which were less than 15 years old. A lack of correlation between stress levels and EEHV detection was observed; thus, it is suggested that EEHV can be spontaneously reactivated and shed. In this study, EEHV was frequently detected during summer, which could be due to the limited food source and low nutrient content that affect immune function. This study was based on one particular herd of captive Asian elephants in Thailand; whether the results of this study could refer to other herds requires further investigation. Longitudinal EEHV monitoring provides information crucial for risk management of the disease and should be conducted in each herd to obtain a better understanding of viral dynamics within a herd.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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