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Elephant endotheliotropic herpesvirus gB-specific antibody levels in sera of Asian elephants (*Elephas maximus*) in Japanese zoos

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ABSTRACT. Prevalence of elephant endotheliotropic herpesvirus (EEHV) infections in Asian elephants in Japan was assessed by determination of EEHV gB specific antibody levels. Among 28 healthy Asian (sub) adult elephants from 11 zoos, 27 animals exhibited intermediate to high antibody levels. Like elsewhere worldwide, this suggested exposure of Asian elephants in Japan to at least one EEHV (sub) species. Longitudinal observations of two elephants monitored from birth to 30-month of age showed consistent high antibody levels. Another juvenile showed antibody levels that decreased to undetectable levels prior to death at 13 months of age. This fatal case supports earlier reports that low antibody levels are a risk factor for development of EEHV hemorrhagic disease.

KEYWORDS: antibody level, Asian elephant (*Elephas maximus*), elephant endotheliotropic herpes virus (EEHV), elephant endotheliotropic herpes virus gB specific antibody, Japanese zoo

Elephant endothelial herpesviruses (EEHVs) may cause acute fatal hemorrhagic disease (EEHV-HD) in Asian (*Elephas maximus*) and African (*Loxodonta* spp.) elephants. EEHV-HD primarily occurs in young elephants, commonly between one and ten years of age, and is the single greatest cause of mortality in elephants under human care [8, 14, 15]. In recent years, research and case reports have highlighted the importance of routine monitoring and early initiation of treatment [25]. Consequently, the survival rate has improved slightly [14, 15]. In Asian elephants the majority of EEHV-HD cases has been caused by EEHV1A infections. Although early diagnosis and initiation of treatment may positively impact disease prognosis, the time between disease onset and death is often limited (24 to 48 hr). It is therefore strongly recommended to evaluate whether a young elephant is at risk of EEHV-HD prior to potential disease development [21].

The relation between EEHV-specific antibody levels and EEHV-HD has previously been investigated [6, 7]. To detect EEHV-specific antibodies, enzyme-linked immunosorbent assays (ELISAs) were developed using recombinant EEHV antigens produced in *Escherichia coli* and in mammalian cells. The recombinant EEHV gB antigen expressed in mammalian cells was found to be superior in sensitivity, and results of the ELISA correlated better with the risk of developing EEHV-HD [2, 7]. Serological surveys of

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large populations of elephants in Laos, Sri Lanka, and European zoos have already been conducted using the mammalian gB-based ELISA and from these studies it was concluded that latent EEHV infections are essentially omnipresent in adult elephants [6, 7] and young elephants if kept in a large herd. Multiple recent studies indicate that a low or undetectable level of EEHV-specific antibodies is a risk factor for developing EEHV-HD, supporting the postulate that fatal cases mainly occur in elephants experiencing a primary EEHV infection [5, 7, 20].

In Japan, there are no free-living Asian elephants. As of 2022, 81 Asian elephants are being held in various zoos throughout the country in relatively small groups of 2–3 elephants average. Two cases of mortality due to EEHV-HD have been reported so far, yet evaluation of EEHV prevalence using serological assays has not been conducted in Japan. Accurate assessment of EEHV prevalence could contribute to risk assessment for EEHV-HD development in young elephants in Japan. Clarifying levels of EEHV-specific antibodies in individual juvenile elephants will provide important insights into whether an elephant is still at risk for EEHV-HD or not.

Eleven zoos from various regions of Japan participated in the present study. Serum samples from 28 Asian elephants, aged 1 to 44 years, were collected in 2021. Blood samples were taken aseptically from an ear vein by veterinary staff at each zoo, and serum samples were stored at −20°C. Additionally, 32 frozen serum samples of three individual elephants from a single zoological collection (Elephant A-C), aged 0 to 3 years, were also used for retrospective longitudinal assessment of EEHV gB specific antibody levels. Elephants A and B were tested using serum samples collected at three-month intervals from birth to 30 months of age. Elephant C was tested using serum samples collected at one-month intervals from 4 months to 13 months of age. This animal was hand-reared using raw goat milk (Kimura goat farm, Oita, Japan) and artificial elephant milk (Morinyu Sunworld, Tokyo, Japan) after receiving only a small amount of colostrum due to rejection by its mother, and eventually died at 13 months of age due to EEHV-HD [23]. As of 2024, elephants A and B are still alive and healthy, with samples from their respective ages of 4 and 3 years included in the aforementioned survey of 28 individuals.

The assay was carried out as described previously with minor adjustments [7]. Briefly, purified recombinant EEHV1A gB (diluted in PBS; 100 µL/well) was coated overnight on microplates (Nunc MaxiSorp (R) high protein-binding capacity ELISA plates, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, plates were washed three times with PBST (PBS containing 0.05% Tween-20) and incubated with blocking buffer (PBS containing 0.1% Tween 20 and 3% BSA [w/v]) for 2 hr. Next, plates were washed four times with PBST and 100 µL of serum diluted 1:100 in blocking buffer was added to the wells for 1 hr. Plates were washed, and incubated with 100 µL HRP-conjugated recombinant Protein A/G (0.5 µg/mL diluted in blocking buffer (Pierce, Thermo Fisher Scientifics, USA), previously reported to bind elephant IgG [9, 17], for 1 hr. After washing four times with PBST, 100 µL/well TMB Substrate (Sigma-Aldrich, Merk, Germany) was added, the plates were incubated for 10 min in the dark, and the reaction was stopped by adding 100 µL 12.5% H_2SO_4. Optical density (OD) was measured at 450 nm in a Microplate Reader (Multiskan FC, ThermoFisher Scientific). For each sample, the antigen-specific signal (signal in wells coated with antigen) and serum-specific background signal (signal in wells without antigen) were assessed simultaneously, and the ΔOD value (difference between OD value of antigen coated well minus OD value uncoated well) was determined. The statistical analysis was performed using EZR software [11] and the graphical user interface for R (R Foundation for Statistical Computing (R), Vienna, Austria). *P*-values <0.05 were considered significant. The test results compared the antibody levels of all elephants by the number of elephants kept at each facility (4 or more, 3, 2, 1). Additionally, the antibody levels were compared across four groups: calves (under 1 year old), juveniles (1–5 years old), subadults (5–15 years old), and adults (15 years and older). The three juvenile elephants were screened for viremia weekly by conventional PCR and loop-mediated isothermal amplification (LAMP) methods, as previously reported [13, 22]. This study complied with the ARRIVE (Animal Research: Reporting In Vivo Experiment) guidelines 2.0 [18], the ethical guidelines for the code of ethics issued by the Japanese Association of Zoos and Aquariums (JAZA) [10], and the Japanese act on welfare and management of animals (No. 105) [16].

Figure 1A shows the antibody levels of all elephants categorized by the number of animals kept at each facility (4 or more, 3, 2, and 1). Differences observed were found to be non-significant as assessed by the Kruskal-Wallis test. Figure 1B shows the antibody levels of all elephants divided into four groups: calf (under one-year-old), juvenile (1–5-years-old), subadult (5–15-years-old), and adult (over 15-years-old). Antibody levels of two healthy elephants (Elephant A and B) tested longitudinally were also included as references in calf group (samples at 3 and 9 months of age; shown in grey) and juvenile group (samples at 2 years of age; shown in grey), respectively, yet excluded from statistical analysis since these data are not independent. All elephants were seropositive for EEHV, with no significant differences (*P*=0.212) between the mean antibody levels (without reference values) of the groups as evaluated by the Kruskal-Wallis test. One adult elephant displayed a low antibody level (Δ OD of 0.839).

Figure 2 shows changes in EEHV antibody levels from birth to 30-momth of age in elephants A-C. The antibody levels of one healthy young elephant (Elephant A) remained high throughout the complete study period, while weekly EEHV-specific PCR and LAMP tests were negative. For Elephant B, housed at the same facility, a reduction of the antibody levels was observed after 15 months of age, followed by increasing antibody levels from 22 months onwards. Presence of EEHV as assessed by PCR and LAMP was only detected at 22-month of age. Elephant C were already relatively low at first sampling (<6 months of age) and decreased to non-detectable levels measured just before death at 13 months of age.

In Fig. 2, it is shown that the antibody levels of healthy juvenile elephants were maintained at high levels, while the antibody levels of the juvenile elephant that contracted and died from EEHV-HD were low. These observations suggest that healthy elephants were exposed to EEHV when still protected by maternal antibodies and is in line with previous observations that young elephants with low antibody levels are at a risk of developing EEHV-HD [6, 7]. Additionally, these results suggests that from an earlier age before the study period, Elephant C had only low levels of maternal antibodies. Elephant C, unlike the other two juvenile elephants, was abandoned shortly after birth and raised artificially, therefore, the amount of colostrum ingested was minimal [12]. Instead of maternal milk, the elephant was hand reared with commercially available formula milk. Although the low level of antibodies is not strong evidence

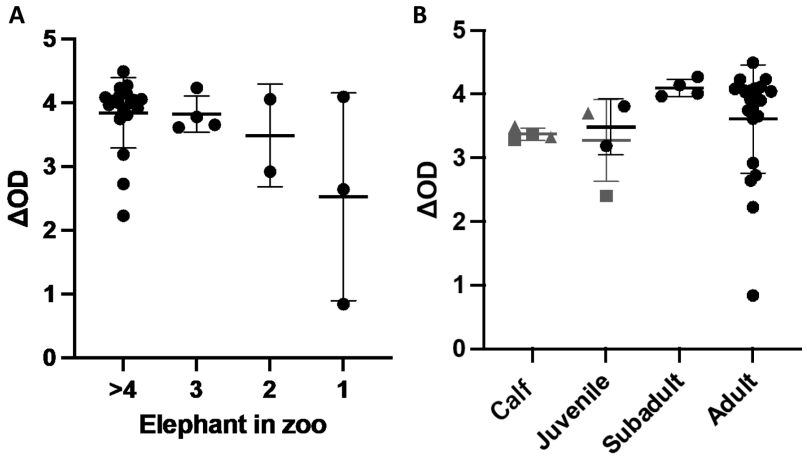

Fig. 1. Elephant endotheliotropic herpesvirus antibody levels of managed Asian elephants in 11 Japanese zoos. **A**) Elephants were grouped based on the number of Asian elephants kept in each zoo (=herd size). **B**) Elephants were grouped based on their ages: calf (under one-year-old), juvenile (1–5-year-old), subadult (5–15-year-old), and adult (over 15-year-old). Antibody levels of two elephants that were also tested longitudinally were plotted with the same symbols used in Fig. 2 (triangle: elephant A and square: elephant B) in grey.

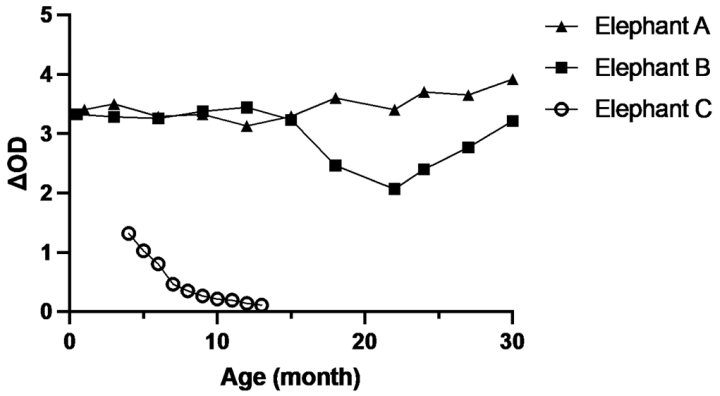

Fig. 2. Elephant endotheliotropic herpesvirus (EEHV) antibody levels of Asian elephant calves over the time. Elephants A and B are healthy individuals. Elephant C died of EEHV-hemorrhagic disease at 13 months of age.

for the presence of antibodies in the milk of elephants, the low EEHV-antibody levels in this artificially-raised elephant suggest that antibodies present in the dam's milk contribute to a prolonged presence of antibodies in the calf than seen in other mammalian species.

The results of this study support the notion that almost all adult elephants have been infected with and are thus likely carriers of one to multiple EEHV species [6, 7]. Latently infected elephants may occasionally experience a reactivation of the virus, leading to viral shedding through trunk and mouth secretions and hence potential transmission to young elephants within the same herd. It is believed that under the protection of EEHV-specific maternal antibodies, EEHV infection and protective immunity may be established in young elephants without apparent clinical abnormalities [6, 7]. However, if young elephants with low to non-detectable EEHV-specific (maternal) antibodies are infected with EEHV, they may not be able to sufficiently control the virus infection, resulting in EEHV-HD development as observed for Elephant C. Lower levels of antibodies in a calf may be due to a dam not passing on sufficient EEHV-specific antibodies in the period around and after birth [1, 6]. Monitoring EEHV-specific antibody levels at 9, 12 and 15 months of age, as recommended by the EAZA elephant TAG in 2022, can provide insight in the natural decline of the maternal antibodies during the period in which the calf should seroconvert by natural exposure to EEHV [4]. Consequently, protocols for managing elephants with low EEHV antibody levels under zoo settings need to be established.

Efforts are underway to devise and validate countermeasures, including vaccine development [3, 19]. In captive Asian elephants, particularly in herd settings, social changes due to management practices may trigger reactivation of EEHV in Asian elephants [24]. Other causes of EEHV reactivation are unknown at this time. Considering the risk of missed opportunities for antibody acquisition due to lack of reactivation and transmission in small population groups, management in larger groups may be more desirable [6]. In Japanese zoos, smaller population groups and individual management are common, and elephants often move between facilities for future breeding opportunities. To improve EEHV antibody induction rates and reduce the incidence of EEHV-HD, it is recommended to manage elephants in larger, more natural, herd sizes. This strategy increases the likelihood that young elephants are exposed to EEHV

before their maternal antibody levels drop, enabling the build-up immunity and thereby reducing the risk of developing EEHV-HD upon primary infection.

CONFLICT OF INTEREST. No author has any conflict of interest.

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