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Longitudinal molecular and serological surveillance of elephant endotheliotropic herpesviruses through relocation in a captive herd of Asian elephants (Elephas maximus)

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Abstract

Background

Elephant endotheliotropic herpesviruses (EEHVs) can cause fatal haemorrhagic disease in young Asian elephants (*Elephas maximus*), posing a significant threat to the conservation of the species. Understanding the dynamics of EEHV infection within a herd may help to understand risk factors associated with EEHV-HD, however, only few longitudinal EEHV surveillance studies exist.

Methods

The present study documents the presence of EEHV glycoprotein specific antibodies by enzyme-linked immunosorbent assays (ELISA), and presence of virus in blood and trunk wash samples by quantitative PCR (qPCR), in nine Asian elephants over a two-year period spanning relocation from Melbourne Zoo to Werribee Open Range Zoo.

Results

EEHV was more frequently detected in trunk wash and whole blood samples prior to, rather than after relocation, however this was not associated with any identified change in management factors or herd dynamics. Prospective serological testing demonstrated an increase in EEHV5A-specific antibodies in three young elephants with low pre-existing antibodies in response to systemic EEHV5 infection. Trunk wash detection of virus preceded viraemia in four out of five elephants with systemic EEHV5 infection, including two of the animals with low EEHV5A antibodies, thus indicating virus exposure or localised replication.

Conclusions

Serology testing and trunk wash surveillance provide information about susceptibility and exposure to EEHVs, thus adding clinical benefit when included with current EEHV surveillance, including routine haematology and EEHV screening of blood by qPCR.

Background

Elephant endotheliotropic herpesviruses (EEHVs) are endemic in elephant populations, however, primary infection in animals with low or absent EEHV maternal antibody levels can result in haemorrhagic disease (EEHV-HD) and death in Asian elephants *(Elephas maximus*) as well as African elephants (*Loxodonta africana*) [1–3]. Approximately 20% of Asian elephants in captivity develop clinical disease, with a mortality rate of 68–85% [4–6]. Over 160 fatal EEHV cases have been documented, with disease disproportionately affecting young elephants between two and ten years of age [2, 6, 7]. This risk period coincides with waning of protective maternal antibodies and age at primary virus exposure [1, 2]. Fatal EEHV infections in young animals are a major conservation concern, due to the negative impact on survival in *ex situ* populations [8]. There are two known cases of fatal EEHV-HD (EEHV1B and EEHV4) within Australian captive herds [9]. To safeguard health and conservation outcomes, zoological institutions housing Asian elephants have widely adopted EEHV surveillance programs that monitor for virus in circulating blood (viraemia), and viral detection in trunk secretions [7, 10, 11].

Young elephants in larger herds maintain higher levels of EEHV antibodies and are less susceptible to fatal EEHV-HD than counterparts in small captive populations [12], most likely due to continuous exposure to EEHVs during the first years of their lives, where elephants still have protective levels of maternal antibodies. Conversely, elephants that succumb to EEHV-HD typically have low or no antibody levels (normalised $\Delta OD < 0.25$) to the glycoproteins gB and gH/gL of the EEHV-species they are fatally infected with [2]. Prospective monitoring of EEHV serology in a clinical setting provides a novel means of assessing the individualised risk for EEHV-HD, if elephants become systemically infected by EEHVs.

Factors that influence viral dynamics within captive herds still remain relatively unclear [13, 14]. Elephants that develop subclinical infection or survive clinical EEHV-HD become latently infected with EEHVs may intermittently reactivate and shed the viruses even when apparently healthy [10, 15, 16]. Reactivation of herpesviruses in other species may be induced by a broad range of environmental and physiological factors, including unrelated infections, stress, elevated catecholamine levels, and immunosuppression [17]. In horses, transport has been associated with an increase in shedding, transmission and reactivation of some equid-herpesviruses [18]. Several EEHV studies have explored links between physiological stress and EEHV recrudescence, but thus far have not found a significant association between EEHV shedding to serum or urine cortisol levels [13, 19], nor to pregnancy [20]. Examining the correlation of these factors is often hindered by underpowered studies due to small herd sizes. However, some evidence indicates that unrelated illness, social conflict and changes in herd structure can result in an increase in EEHV shedding [13, 14, 20]. Titus et al. (2022) examined the effect of introduction and removal of elephants from herds on viral dynamics, but the effect of transport and change in habitation of an entire herd on herd viral dynamics has not yet been investigated.

The aim of the present study was to document EEHV serology and the frequency of virus detection in trunk wash and whole blood samples in a captive herd of nine Asian elephants in Australia over a two-year period. This study period centred on a major planned disruption to herd routine, encompassing twelve months of data collection before and after relocation of the entire herd from Melbourne Zoo to Werribee Open Range Zoo, located 35 km away. As part of measures to improve EEHV responsiveness to mitigate any risk introduced by relocation, molecular testing capacity was established on-site at the destination facility. It was hypothesised that the relocation of the herd would cause significant physiological stress and thus result in increased shedding of EEHVs within the herd.

Methods

Elephant herd information

The captive herd of nine Asian elephants described in this report, is under the care of Zoos Victoria and is comprised of five adult females (16 years to 53 years of age), one adult male (16 years of age) and three juvenile calves (three years of age) (individual demographic data in Supplementary material 1). Three dams (C50001, C50003, C50004) were nursing calves (C50008, C50006, C50007, respectively) and conceived new pregnancies in approximately the final eight months of the study. All elephants were relocated as part of a management decision to provide greater space for the elephants to improve animal welfare and to accommodate further expansion of the multi-generational breeding herd. To mitigate stress associated with relocation, elephants received 12–18-months of transport crate desensitisation and additional training from the elephant keeper team. Time in transport for the elephants ranged from 4–5 hours from entry into transport crates to exit.

Sample collection

Whole blood and serum samples were obtained from elephants at regular intervals as determined by their veterinary teams for routine haematological analysis and for EEHV surveillance. This included weekly blood sampling in the calves (elephant compliance permitting) and fortnightly sampling in the young adults (C50004 and C50005), or more frequently when indicated by clinical exam or haematology abnormalities, or the detection of EEHV in prior samples. Blood sampling in older adult elephants (C50000 – C50003) was primarily opportunistic collection when sampling for diagnostic purposes other than EEHV surveillance. Samples were collected under trained behaviours from the auricular vein. EDTA whole blood samples were stored at − 20°C until processing for DNA extraction. Blood for serum samples was collected into plain serum separator tubes, with serum separated by centrifugation after clotting at room temperature and stored at − 80°C until processing for EEHV serological analyses.

Trunk wash samples from herd members were routinely and periodically collected for quantitative polymerase chain reaction (qPCR) assessment of EEHV DNA shedding within the herd as previously described [10]. Trunk wash sampling frequency increased from fortnightly to weekly in the three months leading up to and following relocation, resulting in an increased number of trunk wash samples available for testing. Briefly, 50 mL of sterile saline (0.9% w/v NaCl) were instilled via a catheter tip syringe into the nares of each elephant, the proboscis was elevated for approximately 30 seconds, and elephants then expelled the nasal contents into a fresh plastic bag on command. Samples were transferred from these bags into sterile 50 mL screw cap conical centrifuge tubes, centrifuged at 1500 x *g* for 10 minutes at room temperature; the supernatant was discarded, and cell pellets were stored at − 20°C until further processing for DNA extraction. Sample collection and testing within this study was approved by the Zoos Victoria Animal Ethics Committee (project reference number ZV23019).

DNA extraction

All samples were processed and tested on-site within the Werribee Open Range Zoo Veterinary Hospital laboratory. DNA extraction of trunk wash and whole blood samples was performed using the Extracta Plus DNA kit (QuantaBio, Massachusetts, USA). Trunk wash samples were processed as per the manufacturer's tissue homogenate extraction protocol, with 6–12 hour incubation in tissue lysis buffer and proteinase K at 56°C, whereas whole blood samples were processed as per the manufacturer's whole blood extraction protocol.

Virus quantification by quantitative PCR

Extracted DNA samples were tested by hydrolysis probe qPCR for the presence of EEHV1, 4 and 5 [10, 21], as well as a host reference gene TNF-α [22]. The presence of elephant TNF-α was used as an assessment of DNA extraction validity with expected quantification cycle (Cq) values of between 21–26 for blood samples, and between 25–35 for trunk wash samples. As the EEHV1 qPCR does not differentiate EEHV1A from EEHV1B, subtyping EEHV1A and EEHV1B qPCR assays were performed if the initial EEHV1 assay returned a positive result [23]. Extracted DNA from trunk wash and whole blood samples were used as template in these PCR assays and amplified using a Mic qPCR thermocycler (Biomolecular Systems, Gold Coast, Australia). Mastermix constituents for each assay included 0.9 µM of each forward and reverse primer, 0.25 µM of FAM-labelled fluorescent probe, 10 µL of PerfeCTa® qPCR ToughMix® UNG (QuantaBio, Massachusetts, USA), 5 µL of DNA template and the remaining volume to 20 µL made up with molecular biology grade water. Negative control reactions contained no DNA template. Thermal cycling was performed with a modified protocol from that published by Stanton, Zong [10], with holds at 45°C for 5 minutes, and 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. Sample amplification curves were compared to standard curves generated by means of qPCR on serial 10-fold dilutions of a known quantity of synthetic DNA constructs of the target region as previously

described [9] with a positive control included for each EEHV target in every qPCR run. Standard curves were generated from 2 x 10^6 to 2 x 10^0 viral genome equivalents (VGE)/µL and separate limit of detection assays were performed. Any samples with detectable fluorescence below this lower limit of detection (trace virus detection) were considered negative for purposes of analysis. Calculated viral loads within blood samples were determined by multiplying the calculated VGE/µL by 2,000 to account for a 1 in 2 sample dilution during extraction process (where 100 µL of blood is used and 200 µL of elution is obtained) and a 1000-fold unit conversion (µL to mL) to standardised viral concentration reporting (VGE/mL of blood). Trunk washes are reported as VGE/µL (of PCR reaction) and were not normalised to the cell pellet mass or volume due to the common inclusion of food material and sand debris in samples. The detection of EEHV1B has previously been reported for a small subset (n = 60) of trunk wash samples from six elephants (C50000 – C50005) in the first six months of this study [9].

Serological analysis by enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) were used to determine EEHV serology by measuring antibodies to EEHV1A glycoprotein B (gB), and the glycoprotein H and L heterodimer complex (gH/gL) for each of EEHV1A, 1B, 4 and 5A, as previously described [2, 24]. The EEHV1A gB detects antibodies cross-reactive to multiple EEHV species and thus acts as a pan-EEHV serology marker [2]. Briefly, 100 µL of the target antigens were coated onto 96-well Maxisorp Nunc-Immuno ELISA plates (Thermo Fisher Scientific, Scoresby, Australia) at either 5 ng/well (gB) or concentrations equimolar to 40 ng EEHV1A gH/gL (all gH/gL antigens), and plates were incubated at 4°C overnight. Unbound antigen was then discarded, and wells were washed with Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific, Scoresby, Australia) with 0.05% v/v Tween-20 (PBST; Thermo Fisher Scientific, Scoresby, Australia). Blocking buffer, consisting of DPBS, 3% w/v bovine serum albumin (Thermo Fisher Scientific, Scoresby, Australia) and 0.1% v/v Tween-20, was added to wells and the plate was incubated at room temperature for two hours. At this stage, ELISA plates were stored at − 20°C for no longer than 12 months prior to testing. Wells were washed with PBST and coated with 100 µL of elephant serum diluted 1/100 in blocking buffer and incubated for one hour. The plate was washed and 100 µL of Protein A/G-HRP (Thermo Fisher Scientific, Scoresby, Australia) diluted 1/5000 in blocking buffer was added and plates were incubated at room temperature for one hour. The plate was washed and 100 µL of substrate solution TMB (Surmodics, Eden Prairie, USA) was added, the plate was incubated for 10 minutes in the dark, and the reaction was stopped with 100 µL of 12.5% (w/v) H_2SO_4 (Thermo Fisher Scientific, Scoresby, Australia). Absorbance was read at 450 nm using an ELISA plate reader (Multiskan FC; Thermo Fisher Scientific, Scoresby, Australia). Each ELISA assay contained a 'no serum' negative control using DPBS only, as well as a 'no antigen' negative control for each sample, and a positive control serum, using a sample from one of the adult elephants. The difference in optical density (ΔOD) was calculated for each sample by subtracting the measured OD of the 'no antigen' well from the measured OD of the various target antigen wells. Wells that reached saturation of optical density (> 4.005) were manually corrected to have the highest detectable OD (4.005). Normalised ΔODs were determined by dividing test sample ΔODs by the ΔOD of the positive control as previously described [12]. All samples in the first 12 months were normalised to elephant sample C50004 29/12/2023, and all samples in the following 12 months were normalised to C50004 05/12/2024. The two positive controls had comparable ΔODs when compared within the one assay (normalised ΔOD between positive controls ranged from 0.93 to 1.33 across the target antigens).

Data analysis

Data were imported into R v4.4.0 [25] for further analysis with the 'summaryTools' package v1.1.4 [26] and visualisation with the 'ggplot2' package [27]. Statistical analyses were performed in R using the 'rstatix' package v0.7.2 [28] and GraphPad Prism v10.5.0 [29]. The frequency of virus detection between study periods was compared by Fisher's Exact Test. A non-linear mixed effects model was fitted to compare the magnitude of viral load over time using Wood's equation to allow statistical comparison of the time to peak viraemia, peak height and rate of decay between different antibody levels (high or low EEHV5-antibodies). Wood's curve was selected as it is a highly flexible Weibull-type growth-decay curve commonly used to fit non-linear biological patterns such as lactation curves in dairy cattle [30]. The model is described in full in Supplementary material 2.

The model was fitted using restricted maximum likelihood with the R package 'nlme' v3.1 [31], assessing convergence via the relative change in the objective function and parameter estimates. Models were built iteratively, gradually increasing complexity, acknowledging the limitations of the study size, with selection choices guided by Akaike Information Criterion (AIC) [32], parameter identifiability, convergence, and biological interpretability. The residuals of the final model were checked for skew, and model predicted curves were compared to the observed data. Then 95% confidence intervals (CIs) were estimated for the modelled parameters and sieve residual bootstrapping [33], which respects the temporal correlation structure and is less prone to bias with smaller sample sized datasets, was used to estimate confidence intervals for calculated outputs from the model (formed from combinations of model parameters), including estimates of the timing and height of the model peak by antibody level grouping and confidence intervals of their differences.

Results

Detection of EEHV1A, 1B and 5 within the herd over two years

A total of 772 trunk wash samples were tested for EEHVs by qPCR, with 299 of these in the 12 months prior to relocation, and 473 of these in the 12 months post-relocation (Table 1). Samples with no detectable elephant DNA (3.1%, n = 24) were excluded from analysis. Samples (11.4%, n = 85) with trace EEHV detection (single replicate positive or estimated viral load below the lower limit of detection) were considered negative for purposes of analysis. The proportion of positive trunk wash samples differed significantly between the two study periods. In total, 216 trunk wash samples were positive for any EEHV (28.9%), with a higher proportion of positives in the samples prior to relocation (57.0%, n = 163/286), compared with the post-relocation period (11.4%, n = 53/462; Fig. 1a). A positive trunk wash was approximately 9.3 times more likely in the pre-relocation period than the post-relocation period (Fisher's Exact Test: OR = 9.33, 95% CI: 6.42, 13.69, $p < 0.001$).

Table 1
EEHV detection in trunk wash and whole blood samples from nine elephants. Samples are separated by test period (prior to and after relocation of the elephant herd).

Parameter	Trunk wash						Whole blood					
	Pre-relocation	%	Post-relocation	%	Total	%	Pre-relocation	%	Post-relocation	%	Total	%
Samples tested	299		473		**772**		398		296		**694**	
Excluded	*13*	*4.3%*	*11*	*2.3%*	**24**	*3.1%*	*0*	*0%*	*0*	*0%*	**0**	*0%*
Samples analysed	286	95.7%	462	97.7%	**748**	96.9%	398	100%	296	100%	**694**	100%
Any EEHV positive	163	57.0%	53	11.4%	**216**	28.9%	100	25.1%	1	0.3%	**101**	14.6%
EEHV1 positive	30	10.5%	8	1.7%	**38**	5.0%	0	0.0%	0	0.0%	**0**	0.0%
EEHV1A	0	0.0%	1	0.2%	**1**	0.1%	0	0.0%	0	0.0%	**0**	0.0%
EEHV1B	21	7.3%	3	0.6%	**24**	3.2%	0	0.0%	0	0.0%	**0**	0.0%
EEHV1 subspecies not classified	9	3.1%	4	0.8%	**13**	1.7%	0	0.0%	0	0.0%	**0**	0.0%
EEHV4 positive	0	0%	0	0.0%	**1**	0.1%	0	0.0%	0	0.0%	**0**	0.0%
EEHV5 positive	145	50.7%	57	12.1%	**202**	27.0%	100	25.1%	1	0.3%	**101**	14.6%
Multiple EEHVs positive	9	3.1%	2	0.4%	**11**	1.5%	0	0.0%	0	0.0%	**0**	0.0%

The most frequently detected virus in trunk washes was EEHV5, identified in 202 samples (27.0% of all samples) (Fig. 1a). The second most prevalent virus was EEHV1, detected in 38 samples (5.0%), with sub-type qPCRs confirming 24 of these positives as EEHV1B (3.2%) and a single sample as EEHV1A (0.1%). The EEHV1 subtype could not be determined for 13 samples (1.7%), all of which were low level positives on EEHV1 assay and likely below the limit of detection for the EEHV1A/1B subspecies qPCR assays. EEHV4 was not detected in any sample. Concurrent shedding of multiple EEHVs was observed in 11 samples (1.5%), all of which were simultaneous detection of EEHV1B and EEHV5. At the individual level, all animals within the study tested positive for EEHV5 in at least one sample, while seven of the nine elephants also tested positive for EEHV1B in at least one sample (Fig. 2). During the early pregnancies of three adult female elephants (C50001, C50003 and C50004), no EEHVs were detected in blood or trunk wash samples (Fig. 2).

A total of 694 whole blood samples were tested for EEHVs by qPCR, including 398 samples prior to relocation and 296 samples after relocation (Table 1). Of these samples, 101 tested positive to any EEHV, all of which were EEHV5 positives (14.6%; Fig. 1b). Blood samples (9.1%, n = 54) with trace EEHV detection (single replicate positive or estimated viral load below the limit of detection) were considered negative for purposes of analysis. The proportion of positive blood samples differed significantly between the study periods, with a markedly higher proportion of positives observed pre-relocation (25.1%, n = 100/398), compared with the post-relocation period (0.3%, n = 1/296). A positive blood sample result was approximately 98.6 times more likely in the pre-relocation period compared with post-relocation (Fisher's Exact Test: OR = 98.6, 95% CI: 17.1, 3876.3, $p < 0.001$).

Molecular testing was performed on-site to facilitate rapid intervention in response to EEHV detection. The analytical sensitivity was equivalent to the published assays, with a lower limit of detection of 0.6 VGE/µL or 1200 VGE/mL of blood for each assay [10]. The time from sample collection to the laboratory to qPCR result readout was approximately 3–4 hours, allowing prompt clinical response and real-time monitoring of viraemias.

Series of EEHV5 viraemias in three elephant calves

The difference in frequency of virus detection in whole blood and trunk washes between study periods was largely driven by a series of EEHV5 infections. During September Year 1 of the study, a marked EEHV5 viraemia (191,600 VGE/mL) was detected in one of the elephant calves (C50008, 1 year and 8 months old) during routine EEHV surveillance (Fig. 3). This elephant had low antibody levels for EEHV5A (normalised ΔOD = 0.08) on routine serology (Fig. 4a). Upon detection, treatment including the administration of antiviral medications and plasma transfusions was initiated immediately, and EEHV surveillance frequency was increased. The peak of viraemia in this elephant was observed between two and four days after first detection (242,000 VGE/mL). Following this viraemia, high levels of EEHV5 shedding in trunk wash samples were observed in this elephant, peaking at 1,330 VGE/μL nine days after viraemia was first detected and persisting for approximately four months. Simultaneously, EEHV5 was detected in trunk wash samples from all members of the herd, resolving following relocation in all but two elephants (Fig. 2). In the month following the EEHV5 viraemia in the first elephant calf (C50008), two other elephant calves with low antibody levels for EEHV5A (C50006, C50007, both 1 year and 11 months old; normalised ΔOD = 0.16, 0.06 respectively; Fig. 4a) developed EEHV5 viraemias, which were both preceded by detection of EEHV5 in trunk wash samples of each individual. Viraemia was lower at the time of first detection in both these animals than the initially infected elephant (approximately 3,500 VGE/mL in both animals), possibly due to increased testing frequency, but viral loads rose rapidly in the following days. Both animals received antiviral treatment and plasma transfusions, as well as an increased frequency of EEHV testing. Viraemia peaked at 82,000 VGE/mL and 66,000 VGE/mL for elephants C50006 and C50007 respectively.

EEHV5 viraemia was concurrently detected in two young adult animals (C50004 and C50005), the first of which was the dam of the affected calf (C50007) (Fig. 3). These animals had high antibody levels for EEHV5A prior to viraemia (C50004, C50005; normalised ΔOD = 1.0, 0.88 respectively; Fig. 4a). Both of these viraemias were similarly preceded by EEHV5 detection in trunk washes for several weeks prior to detection of virus in circulating blood. Viraemia was detected in the dam (C50004) three days after onset of viraemia in her calf, initially at low levels (1,200 VGE/mL) rising to 61,000 VGE/mL within a week and peaking at 75,000 VGE/mL. She remained persistently viraemic, while continuing to shed EEHV5 in trunk washes for approximately four months. Viraemia in the adult male (C50005) was detected 12 weeks after the first calf developed viraemia, with a low level viraemia peaking at 18,700 VGE/mL eight days after initial detection. This animal was monitored without therapeutic intervention. Of all the affected individuals, this animal maintained the longest period of ongoing viral shedding, with EEHV5 regularly detected in trunk washes for the following six months. The dams (C50001 and C50003; normalised ΔOD = 0.81, 0.68 respectively) of the other two calves had no detectable viraemia but were not as frequently screened during this period. The amount of virus detected in trunk wash samples was comparable between viraemic animals and non-viraemic animals (Fig. 5a). There was a trend towards higher viraemic loads in the three animals with low EEHV5-specific antibodies (Fig. 5b).

Viral load dynamics of EEHV5 viraemia in five elephants were modelled by the elephant's levels of EEHV5-specific antibodies (Fig. 6). The model that provided the best balance of fit, and parsimony (lowest AIC) included individual random effects on the intercept for each individual (i.e. the viral load at first detection) and fixed effects of EEHV5A-specific antibody levels on the growth (b) and decay rates (c) of viral load, as well as the time to viral load peak (d). When comparing elephants that had low EEHV5A-specific antibodies with those that already had high antibody levels to this virus subspecies, the elephants with low antibodies reached peak viral load 7 days earlier (6.2 days [95% CI 3.5, 8.0] compared with 13.1 days [95% 8.4, 17.9]; Table 2). Animals with low antibody levels showed a trend towards more rapid viral load decay (14% faster decay; 95% CI between 4% slower and 35% faster), and a trend towards a higher viral load with an estimated 2.0-fold (95% CI 0.75, 4.9) higher peak compared with animals that had high antibody levels prior to viraemia. The remaining model outputs are included in Supplementary material 2.

Table 2
Calculated model outputs with their 95% confidence intervals determined from 1000 bootstrap replicates.

Parameter	Estimate	95% Confidence interval
Peak height (high EEHV5- antibody levels)	3.5×10^4 VGE/mL	$(1.7 \times 10^4, 8.5 \times 10^4)$
Peak height (low EEHV5- antibody levels)	7.1×10^4 VGE/mL	$(4.2 \times 10^4, 1.3 \times 10^5)$
Peak height difference (fold-difference)	2.0 times	(0.75, 4.9)
Peak time (high EEHV5- antibody levels)	13.1 days	(8.4, 17.9)
Peak time (low EEHV5- antibody levels)	6.2 days	(3.5, 7.9)
Peak time difference between groups (days difference)	-6.9 days	(-12.6, -2.3)
Viral load decay rate (multiplicative difference)	1.06	(0.98, 1.14)
Supplementary material 1 (.xlsx): Elephant demographic data.		
Supplementary material 2 (.docx): Non-linear mixed effects modelling methods and results.		

EEHV5A-specific antibodies increased in response to systemic EEHV5 infection

Relative antibody levels to each of the EEHV glycoprotein antigen targets were much lower in the three calves compared with other elephants of the herd, prior to EEHV5 viraemias being detected (Fig. 4a and 7). The normalised ΔOD for EEHV5A gH/gL for the three calves ranged from 0.06 to 0.16 in the serum samples closest to the onset of viraemia (Fig. 4a). Serial serum samples demonstrated a marked increase in normalised ΔOD for EEHV5A gH/gL and EEHV1A gB for each of these elephants within one week of viraemia, with antibody levels persistently elevated for at least three months. A moderate increase in antibody levels to EEHV1A, 1B and 4 gH/gL was concurrently observed in all three elephant calves. As part of their treatment, each of the calves received plasma transfusions from donor elephant C50000 (Fig. 4b). While one calf was concurrently shedding EEHV1B (C50006), no concurrent viraemias with any other EEHV were observed in the three calves. Interestingly, all antibody levels declined in the calves over the following year, with EEHV5 gH/gL normalised ΔODs of 0.36–0.44 approximately a year after EEHV5 viraemias. In contrast, both of the adult elephants maintained high levels of EEHV5A gH/gL antibodies before and after the onset of viraemia.

Discussion

This study describes a two-year prospective, longitudinal study of EEHV viral dynamics by molecular detection, serology and clinical outcomes in an *ex situ* herd of nine Asian elephants in Australia, before and after relocation of the herd. Contrary to our hypothesis, molecular detection of EEHVs was far more common in the pre-relocation period compared with post-relocation. This study is novel in the utilisation of serology data in conjunction with molecular detection of EEHVs, in the clinical management of elephants. It documents the levels of circulating EEHV1A, 1B, 4 and 5A antibodies in the nine elephants, with high levels in six adults and low levels in three calves, followed by systemic EEHV5 infection in these three calves and two young adults, and a subsequent increase in EEHV5A-specific antibodies in the calves. Moreover, it addresses the potential benefits in including EEHV serology testing and trunk wash qPCR surveillance, in addition to routine haematology and PCR screening for viraemia in young, susceptible elephants.

Differences in virus detection rates between study periods are largely attributable to subsequent EEHV5 infections approximately eight months into the study. This clustering of positive results introduces significant skew into the dataset relative to the background rate of infrequent virus detection. There were no identified changes to animal management or training routines, nor any concurrent health events that may have precipitated EEHV5 shedding within the herd and subsequent viraemia in the five animals. After relocation of the herd, an anticipated major stressor to the elephants, there was markedly less frequent detection of EEHV shedding across the herd. This may have related to the fact that the transport distance was relatively short (35 km, 4–5 hours in transport) and hence only a minor stressor. It is possible that longer distances of travel may be more likely to result in EEHV shedding, similar to observations in other host species [18].

Compared to other EEHVs, EEHV5 is rarely reported in the scientific literature, moreover not commonly associated with haemorrhagic disease [34]. However, there are at least two reports of fatal EEHV5 infection [35, 36]. A threshold for clinical intervention with systemic EEHV5 infections and a high viraemia is not well established. Comparatively, an EEHV1 viraemia exceeding 5,000 VGE/mL may prompt initiation of EEHV treatment, particularly if demonstrating clinical signs, haematology abnormalities or known to be lacking specific EEHV1-specific antibodies [2, 37]. While all six adult elephants maintained high levels of EEHV-antibodies, the cohort of three calves experiencing systemic infection had demonstrably low EEHV5-specific antibodies (normalised ΔOD < 0.25) and treatment was provided according to specialist advice and clinician judgement. It should be noted that the precise risk of developing EEHV-HD in the affected animals was still uncertain. The previously established cutoff value for assessing protection against EEHV-HD (normalised ΔOD > 0.25), was based on antibody levels detected in 22 EEHV1-HD cases and a single EEHV5-HD case [2]. The EEHV5-HD cases tested in that study was one of the previously mentioned EEHV5-HD fatalities, and this animal had no detectable antibodies for all EEHV (sub)species at the time of infection. Therefore, the level of antibodies considered protective for haemorrhagic disease in EEHV5 infections has not clearly been defined. Further studies are required to determine antibody levels considered protective for haemorrhagic disease associated with EEHV4 and EEHV5 infections.

The present study shares similarities to several previous longitudinal studies, although is of a longer duration and thus contains a greater sample number than these prior studies (1,417 samples tested compared with a median of 151, range 87–504 samples). [10, 13, 14, 16, 38]. A slightly higher frequency of EEHV-positive samples was reported in this study (21.6%, n = 355/1417, 9 elephants), compared with similar studies testing for EEHV1, 4 and 5, with 19.6% (n = 11/56, 7 elephants) [16] and 8.1% (n = 41/504, 9 elephants) [13] EEHV-positive in these prior studies. Other longitudinal studies did not include EEHV5 in their testing approach due to the lower case fatality rate attributed to EEHV5 [5, 39]. These studies reported EEHV detection rates of 60% (n = 82/136; EEHV1 only, 4 elephants) [20], 31% (n = 27/87; EEHV1 only, 5 elephants) [10], 9.7% (n = 16/165; EEHV1 and EEHV4, 5 elephants) [14] and 4.2% (n = 12/289; EEHV1, 6 elephants) [38]. The shedding frequency in our study exceeded that described in a larger retrospective cross-sectional study spanning 12 institutions in Europe [11], which found that 13.9% (n = 397/2,863, 78 elephants) of predominantly trunk wash samples were positive for any EEHV. In that study, the majority of positive qPCR tests were EEHV1 (13.7%, n = 391/2,863 samples), the remainder EEHV4 (0.6%, 6/995); and no reported EEHV5 positives, albeit in a considerably smaller sample size (n = 0/59) [11]. The wide distribution of virus detection rates across studies is likely influenced by herd size and demographics, animal relocations associated with breeding programs, as well as study duration and sample type. Despite the larger sample size, this study still faces the common challenge of longitudinal EEHV studies, where sporadic primary infections which result in high viral loads and much more frequent virus detection, occur against a background of endemic latent infections which are infrequently shed [14, 16]. Ultimately, longitudinal studies on relatively small

herd sizes (median 6 elephants, range 4–10) lack the statistical power to determine epidemiological associations between viral shedding and clinical or management events. A collaborative, multi-institutional, prospective cohort study with standardised testing may provide more robust data for determining association between management and health factors, and EEHV viral dynamics.

Pregnancy was not associated with virus detection in blood or trunk wash in three animals that were in approximately their first trimester of pregnancy. This is consistent with some prior literature which documented no clear association between pregnancy and EEHV shedding [20] but differs from other studies which described trends towards increased frequency of EEHV detection in pregnant elephants [10, 38]. In some human herpesviruses, shedding of virus in pregnancy is typically highest in the third trimester, in the peri-parturient and post-parturient periods [40, 41]. Documenting EEHV shedding through the entirety of several elephant pregnancies will further elucidate the effect of pregnancy on EEHV viral dynamics.

Concurrent active infections with multiple EEHVs have previously been documented in both subclinical affected healthy animals [42] and in fatal cases [43]; however, the rate of concurrent detection of EEHVs has not previously been reported. The present study detected concurrent shedding of EEHV1B and EEHV5 in 11 trunk wash samples (1.5% of all samples) from two animals, with one elephant calf (C50006) concurrently shedding both viruses for several weeks. Concurrent infection with two or more viruses is a prerequisite for herpesvirus recombination. Previous genomic analyses have suggested that variability within hypervariable regions of the EEHV genome is driven by recombination [9]. Genomic analysis from animals concurrently shedding multiple EEHVs may further clarify whether inter-species recombination can occur in EEHVs.

In this study, on-site implementation of diagnostic testing facilitated a rapid interval from routine sampling to reporting of viraemia. This could be particularly advantageous in cases of EEHV1-HD cases, which often progress rapidly from mild disease to death [37]. Additionally, on-site testing allowed for a tailored testing routine during this series of EEHV5 viraemias, with daily monitoring of viral loads providing real-time information about progression of infection. With increasing portability and operational simplicity, on-site and point-of-care molecular diagnostic assays are increasingly being implemented in human medicine with improved clinical outcomes [44–46]. This present study, documenting the prompt reporting of a series of EEHV5 infections, suggests that veterinary patients, including zoo animals which are often serviced by on-site veterinary laboratory facilities, may similarly benefit from rapid on-site molecular testing capacity [47].

In four of the elephants that experienced EEHV5 infection, virus was detected in trunk wash samples two to four weeks prior to the development of viraemia, including in two of the calves with low EEHV5 antibodies. Shedding of EEHVs in trunk secretions is considered the likely route of transmission between herd-mates [10, 48]. Despite this, trunk wash surveillance recommendations and implementation are non-standardised, with only 35% of facilities routinely testing samples for EEHVs in one study of European zoos (n = 31 institutions) [11]. The findings of the present study support the implementation of routine trunk wash surveillance, as trunk wash samples may provide early evidence of virus exposure or localised infection prior to the development of viraemia, particularly in animals with low antibodies. This was observed for EEHV5 in four animals (C50004, C50005, C50006 and C50007), although in another individual (C50008) systemic infection was detected prior to localised infection. While the viral dynamics observed in the calves, with low EEHV5 antibodies and high viraemias are suggestive of primary infection, it is alternatively possible that detection in trunk washes prior to blood samples reflects individual viral reactivation. More broadly, the temporal relationship between viraemia and trunk wash shedding during primary infections and viral reactivation events remains unknown, and these dynamics may differ between EEHV species. Trunk wash surveillance should therefore be additional to existing surveillance practices of screening for viraemia and haematology abnormalities, rather than replace these methods. Detection of EEHVs in trunk wash samples as an early indicator may prompt heightened monitoring and increased frequency of blood sampling for EEHV screening and haematological analysis.

While virus was detected in trunk washes prior to viraemia in four animals, it was simultaneously detected in trunk washes of four other elephants which did not develop viraemia. Each of these elephants had high antibody levels for EEHV5, and thus likely mounted a sufficient immune response to prevent systemic infection. The detection of virus in trunk washes, with similar trunk wash viral loads to viraemic animals is suggestive of localised infection. Similar findings were observed in a study of EEHV5 subclinical infections by Atkins, Zong [34], where several elephants had virus detected in trunk washes despite the absence of viraemia. Interestingly, there is a considerable change in shedding patterns immediately following relocation, with EEHV5 detected in the trunk washes of all nine elephants in the month prior to relocation, and detected in only two elephants immediately after relocation. This may simply represent the natural senescence of infections, combined with a temporal gap in sampling after relocation. Alternatively, some of the viral detection by trunk washes could represent environmental contamination and a subsequent reduction in environmental exposure to EEHV5 after relocation. There is a lack of data about environmental persistence of EEHVs, but other herpesviruses can persist in water sources for up to 21 days [49]. Environmental sampling for EEHVs during periods of high shedding would further explore whether virus may persist in the environment of elephants in captivity.

In this study, longitudinal monitoring of serology data informed EEHV-HD risk, documenting a relative lack of EEHV-antibodies to a range of EEHV (sub)species including EEHV5A, and subsequently systemic EEHV5 infections in the three elephant calves. The calves had some detectable antibodies to each of the EEHVs which likely reflect prior transfer of maternal antibodies, although immune responses to prior infections cannot be excluded. Following EEHV5 infection, each of the calves demonstrated an increase in antibody levels to EEHV5A gH/gL, as well as to the pan-EEHV gB antigen, which is known to be cross-reactive across EEHV species [24]. Given that detection of EEHVs in trunk wash samples may indicate primary infection or reactivation of latent virus in an animal with prior exposure, combined monitoring of trunk wash samples alongside

antibody levels may help distinguish between these scenarios and reveal the underlying viral dynamics. This highlights the utility of including serology in the clinical management of EEHVs within captive herds.

Pre-existing EEHV5A-specific humoral immunity in two adult elephants did not preclude viraemia in those animals. However, differences were observed in the EEHV5 viral load dynamics between elephants with different antibody levels. The higher peak viraemia observed in animals with low antibody levels may have resulted from the lack of pre-existing adaptive immunity to dampen initial viral replication [1, 2]. The reasons for the earlier peak and more rapid rate of decay of viral load in the elephants with low antibody levels are less clear, but may relate to later initial detection in the first EEHV5 infection (C50008), differences in therapeutic interventions or attributed to random chance. Given the small sample size and otherwise inconclusive evidence on the efficacy of antiviral therapy, the effect of treatment on viral dynamics remains unclear [5, 7]. The pattern of viraemias observed is consistent with observations in surviving cases of EEHV-HD, with a viral peak between 3 and 7 days after first detection [22, 50–52]. Conversely, animals succumbing to EEHV-HD demonstrated escalating viraemias with later peaks of viraemia [53, 54], possibly suggesting a failure to respond to therapeutic interventions or to mount an appropriate immune response. Non-linear mixed-effects modelling approach, as used for comparison of antibody levels in this study, may provide a valuable approach for investigating the effects of therapeutic intervention and serostatus on viral load progression over time with consolidated datasets.

Each of the elephant calves developed transient increases in detectable antibody levels to each of the other target antigens (gH/gL complex of EEHV1A, 1B and 4). One elephant calf (C50006) was concurrently shedding EEHV1B during this time, which could account for some of the increase in her EEHV1B antibodies. For the other increases in antibody levels, in the absence of detection of these viruses in the calves, these changes may be attributable to these animals receiving plasma transfusions from an elephant with confirmed high EEHV-antibody levels (Fig. 4B). The magnitude of normalised ΔOD for EEHV4 gH/gL increase was greater than that observed for EEHV1A and EEHV1B gH/gL, which may reflect the high EEHV4 antibody titres in the plasma donor. Given that optical density saturation was observed in the positive control animal, it is feasible that real differences in transfused antibody levels exist between the EEHV (sub)types which cannot be quantified. Alternatively, it is possible that concurrent EEHV4 infection occurred in the calves alongside EEHV5 infection without detection in blood or trunk wash samples. Cross-reactivity between antibodies to EEHV5A gH/gL and EEHV4 gH/gL is unlikely based on the initial serology validation studies [2].

Conclusions

Transport and relocation of the elephant herd was shown not to be associated with an increase in viral shedding, although the transport distance and duration was relatively short. Low levels of EEHV antibodies were observed in three elephant calves, followed by systemic EEHV5 infection in each animal, and a subsequent marked increase in EEHV5A specific antibodies. EEHV5 viraemia was simultaneously observed in two young adults with high antibody levels. Trunk wash samples were EEHV5 positive prior to blood samples in four out of five elephants experiencing viraemia. On-site molecular testing facilitated rapid test turnaround time and thus prompt reporting of viraemia during routine surveillance. The findings of the present study support the integration of serology and real-time trunk wash surveillance into existing EEHV surveillance protocols, which include testing of blood for haematology and molecular detection of EEHVs. These complementary tools collectively enable individualised risk profiling for systemic EEHV infection, early identification of virus exposure and localised infection, and thus can facilitate improved EEHV surveillance.

Abbreviations

AIC
Akaike Information criterion
DPBS
Dulbecco's phosphate buffered saline
EDTA
Ethylenediaminetetraacetic acid
EEHV
Elephant endotheliotropic herpesvirus
EEHV-HD
Elephant endotheliotropic herpesvirus haemorrhagic disease
ELISA
Enzyme-linked immunosorbent assay
gB
Glycoprotein B
gH/gL
Glycoprotein H and L heterodimer complex
HRP
Horseradish peroxidase

OD
Optical density
PBST
Phosphate-buffered saline with Tween-20
qPCR
Quantitative polymerase chain reaction
TNF-
Tumour necrosis factor alpha
VGE
Viral genome equivalents

Declarations

Ethical approval:

Sample collection and testing within this study was approved by the Zoos Victoria Animal Ethics Committee (project reference number ZV23019).

Competing interests:

The authors declare that they have no competing interests.

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Author Contribution

JW, PV, AL, CH, NR and JD contributed to study and project administration. SG, BM and NR collected clinical samples and provided clinical oversight. WS, VR, CdH, and TH developed ELISA methodology and provided necessary antigens. JW and PM performed sample preparation, molecular and serological testing and data curation. JW, PV, AL, CH, NR, JD and SF analysed the data. JW wrote the manuscript and all authors have read and approved the final manuscript.

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Data Availability

The dataset supporting the conclusions of this article is included within the article and its additional files.

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Figures

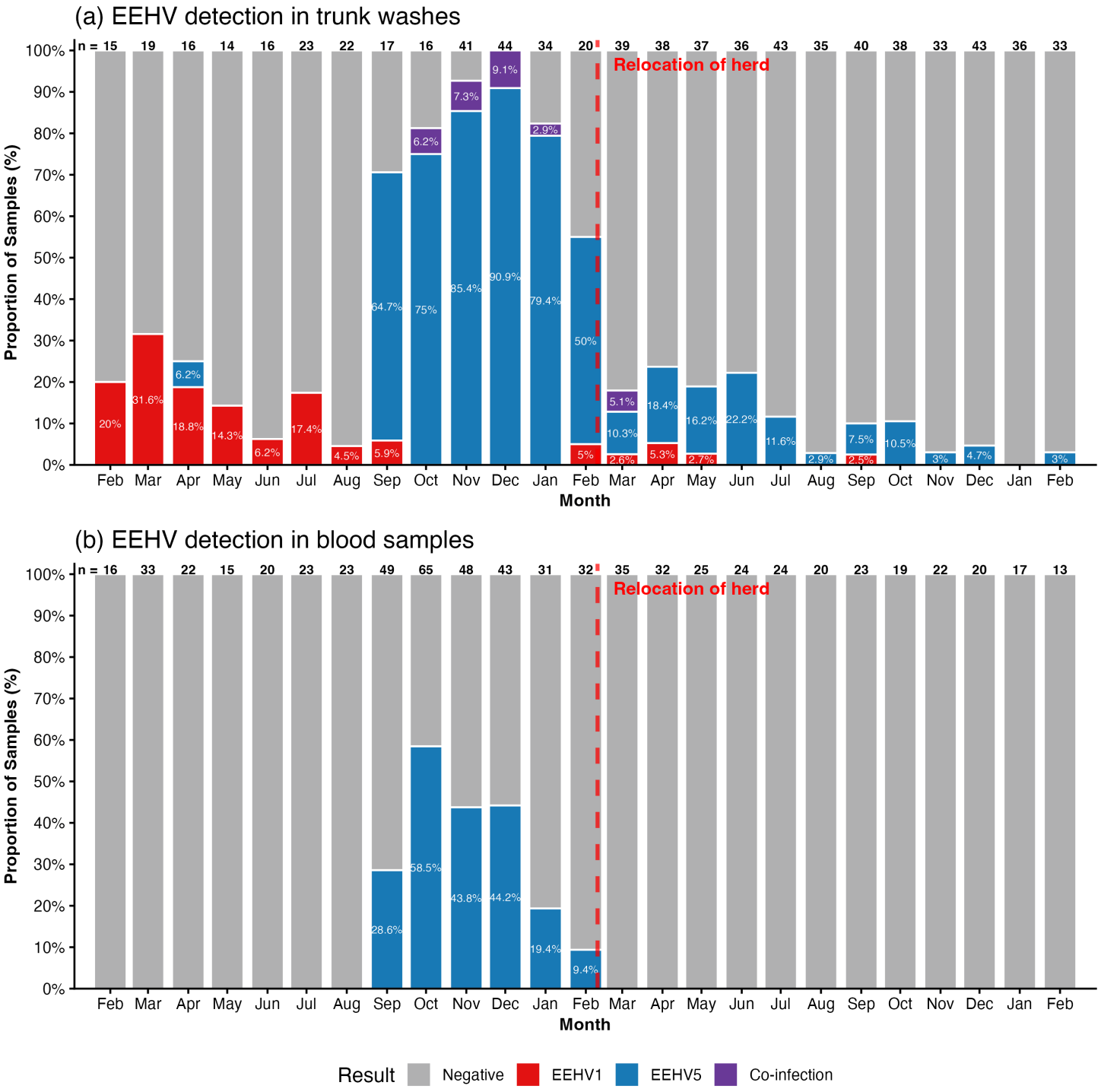


Figure 1

Proportion of EEHV detection in (a) trunk wash samples and (b) whole blood samples. Samples are categorised by month and EEHV species, where bar fill represents the proportion of outcomes for samples tested in each month (grey = no EEHV detected, red = EEHV1, blue = EEHV5,

purple EEHV1 and EEHV5 concurrently detected). The percentage of positive samples for each EEHV species is indicated by white text annotation on each bar and the number of samples tested in each month is indicated above each column.

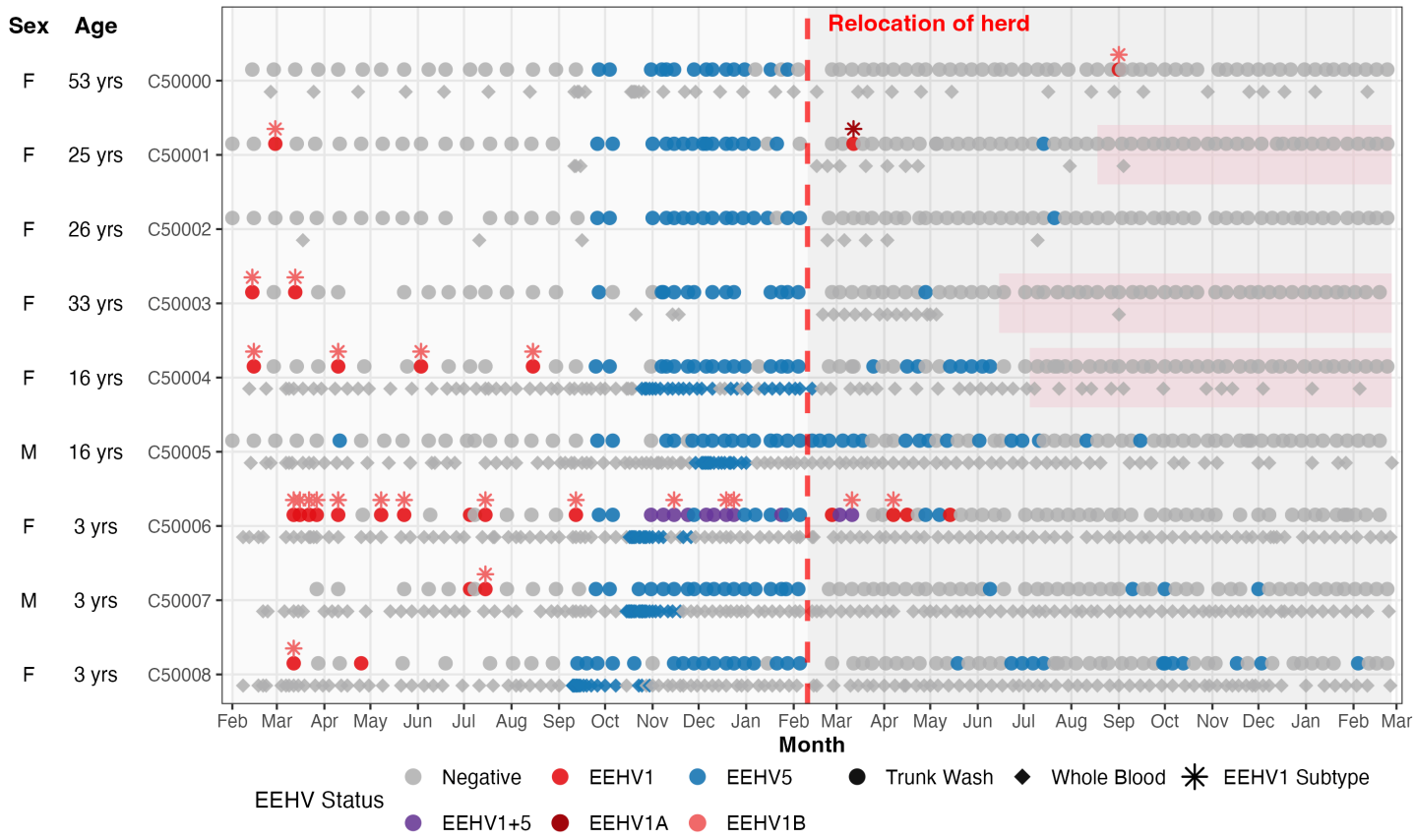


Figure 2

Detection of EEHVs in trunk wash and whole blood samples over the study period. Individual demographic data, including sex, age at the end of the study period and unique identifier is presented on the left. Trunk wash samples are represented by circles, and whole blood samples are represented by diamonds. The fill of each symbol represents the test outcome for each sample (grey = negative for all EEHVs, red = EEHV1 positive, blue = EEHV5 positive, purple EEHV1 and EEHV5 positive). EEHV4 was not detected within the study period. EEHV1A (maroon) and EEHV1B (pink) subtyping are overlaid as asterisks on EEHV1 positive results for samples with subspecies confirmed by qPCR. Approximate period of pregnancy for three elephants is indicated by the pink box overlay.

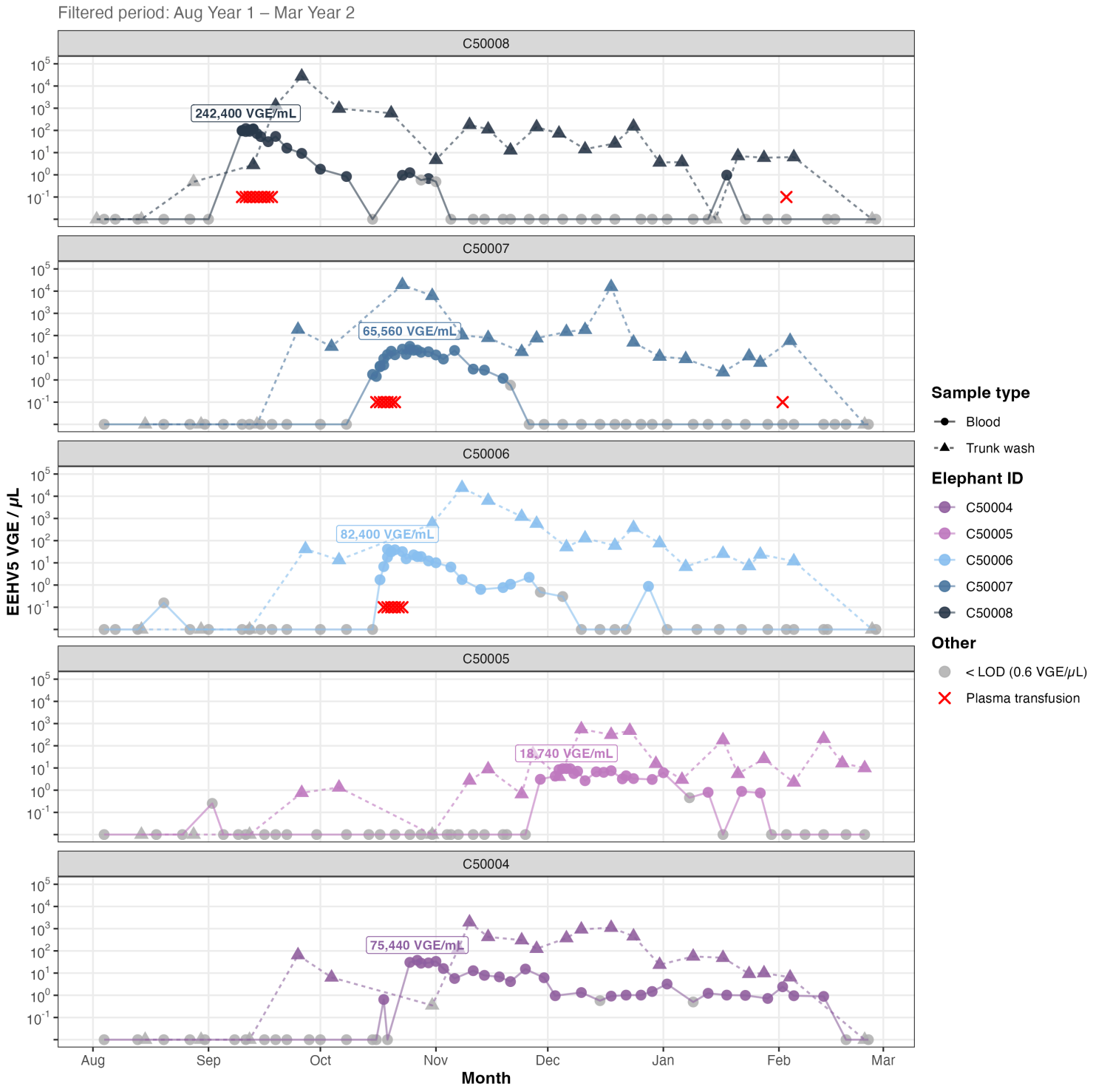


Figure 3

Detection of EEHV5 in samples from five elephants during a series of systemic infections. Infectious load (VGE/µL) is presented for whole blood samples (triangles and dotted line) and trunk wash samples (circles and solid line) for each elephant separated by ID. Negative results including values below the lower limit of detection (0.6 VGE/µL) are greyed out. Viral load per mL of blood is calculated by multiplying VGE/µL x 2000, and peak viral loads are labelled for each elephant. Plasma transfusions in the three elephant calves are indicated by red crosses.

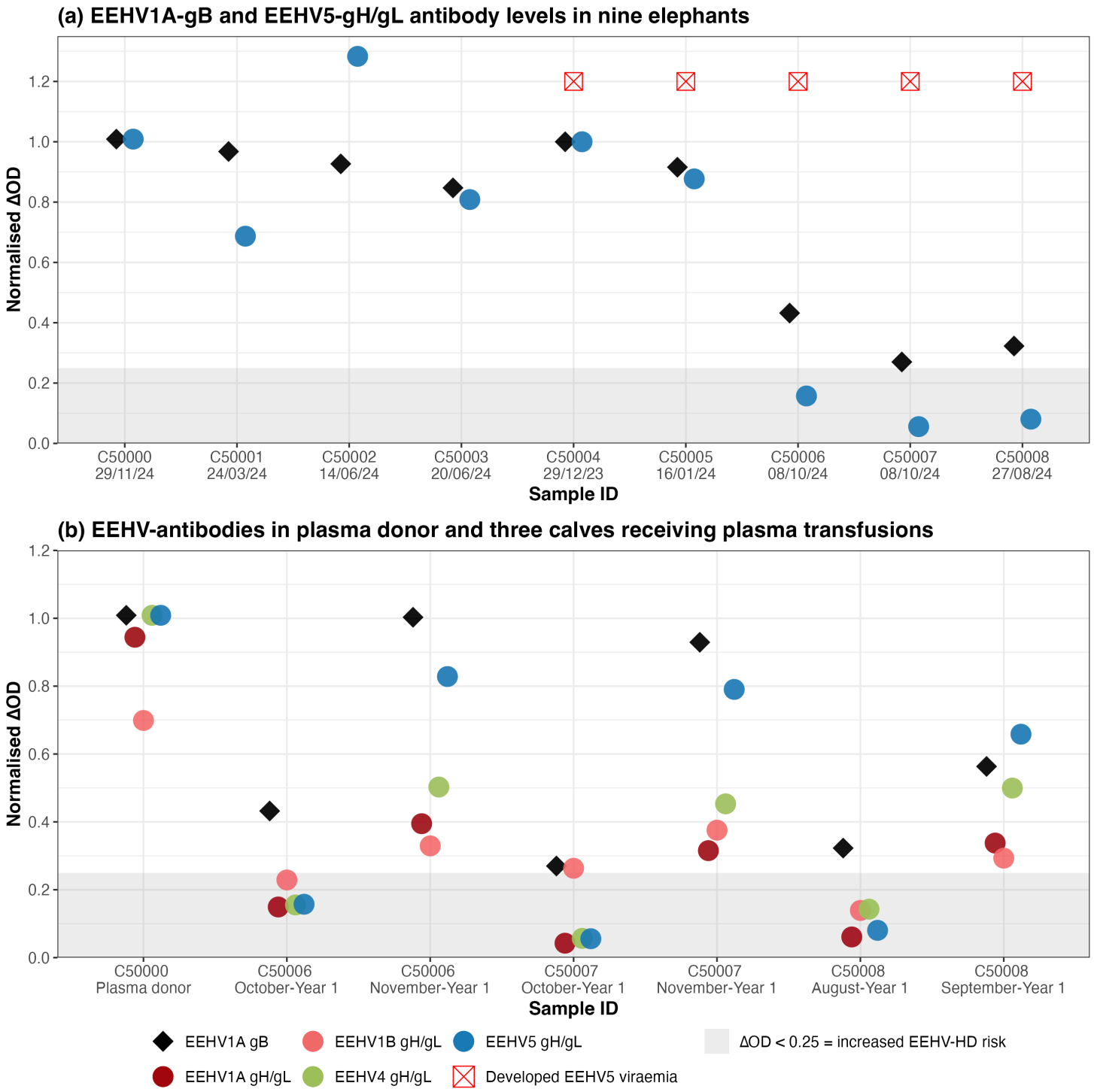


Figure 4

Individual elephant antibody levels to EEHVs determined by normalised difference in optical density with ELISA. (a) EEHV1A-gB (pan-EEHV marker) and EEHV5-gH/gL antibody levels in all nine elephants in samples closest available in date to the series of EEHV5 infections. Animals that subsequently developed EEHV5 viraemia, including two adults (C50004 and C50005) and three calves (C50006, C50007 and C50008) are indicated by red crossed squares. (b) Antibody levels to each of the EEHVs for the plasma donor and the three plasma recipient elephant calves, with samples approximately two weeks prior to and one week after the onset of EEHV-5 viraemia, encompassing a series of transfusion administrations for each calf. All samples are normalised to the optical density of a seropositive adult (sample ID: C50004 29/12/23).

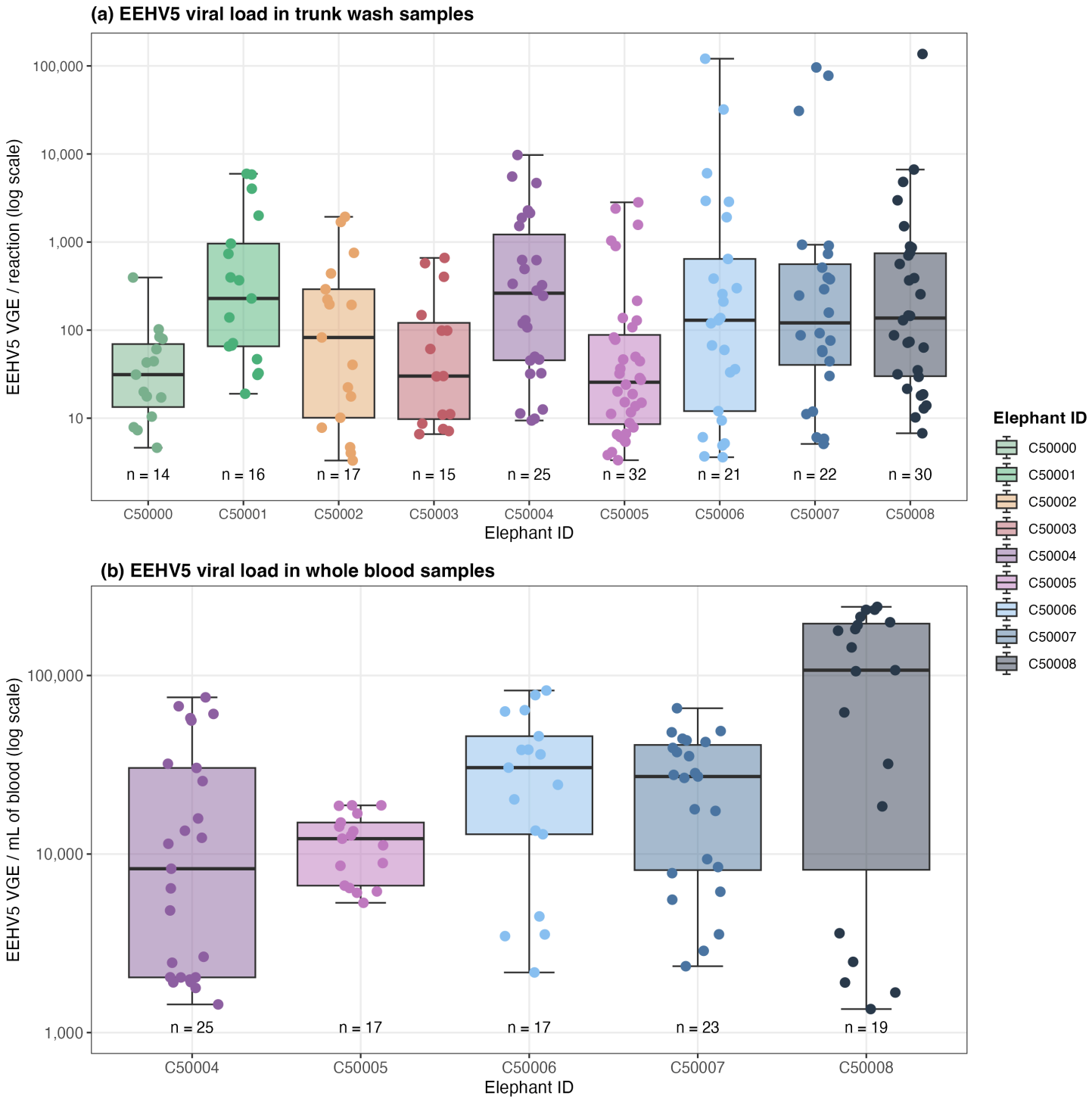


Figure 5

Average viral load by elephant for EEHV5-positive (a) trunk wash and (b) whole blood samples. Box plots show the median, the interquartile range, and whiskers show the range; scatterplots show the individual observed points.

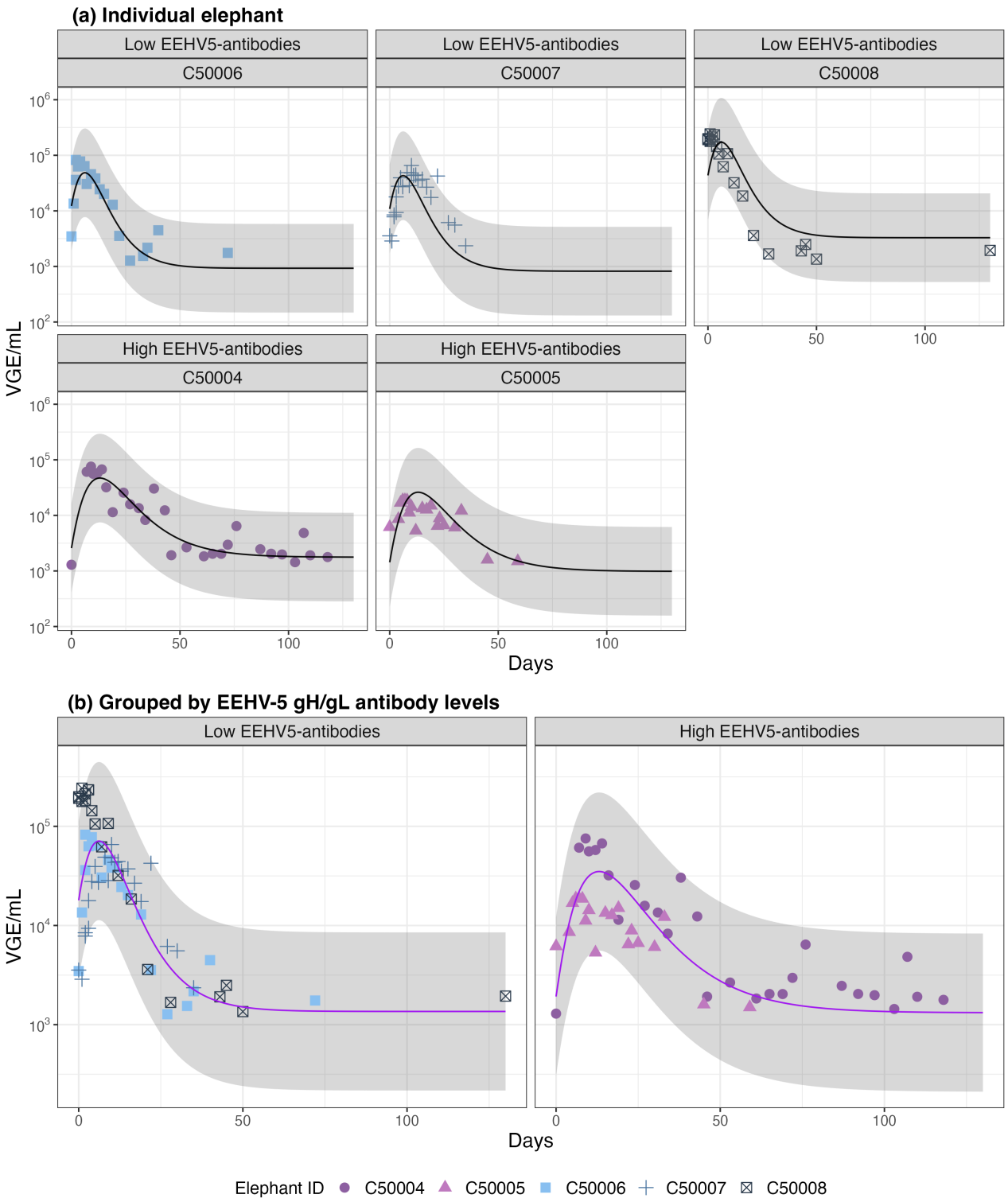


Figure 6

Viraemia in five elephants with viral load modelled by EEHV5-antibody levels. (a) Observed viraemia over time and individual fitted curves for individual animals. (b) Data grouped by animals levels of EEHV5-specific antibodies (high or low) with group-specific fitted curve. Curves were fitted by non-linear mixed effects modelling using Wood's equation and the 95% confidence interval is indicated by grey banding.

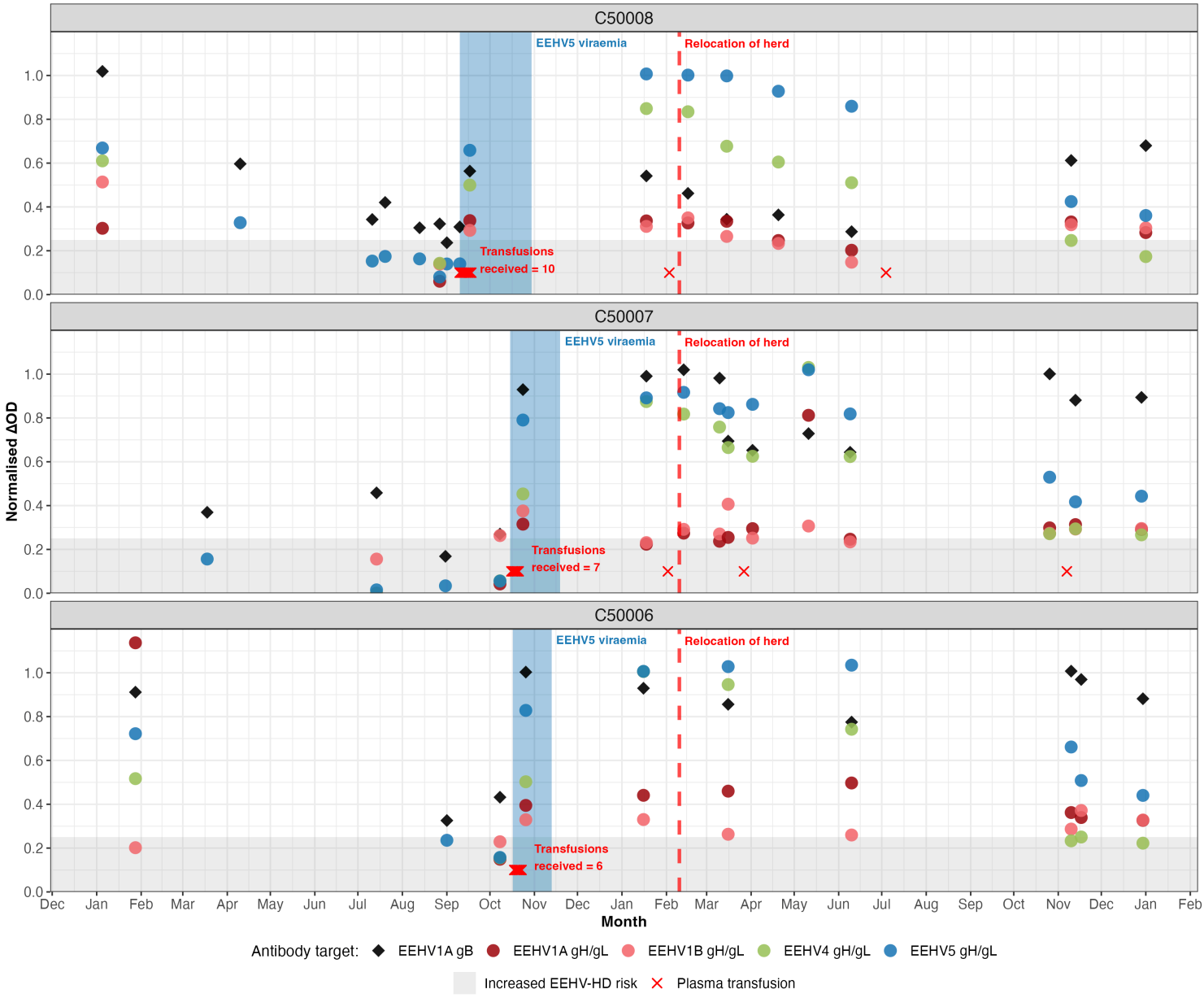


Figure 7

Measurement of EEHV glycoprotein antibodies in three elephant calves over time. Antibody levels determined by normalised difference in optical density (ΔOD) measured by enzyme-linked immunosorbent assay (ELISA). Serum samples were analysed for antibodies to EEHV1A gB (pan-EEHV marker: black diamond) and gH/gL antibodies (EEHV species-specific: circles) of EEHV1A (maroon), 1B (pink), 4 (green) and 5 (blue). Plasma transfusion treatments are indicated by red cross symbols. Individual periods of viraemia are highlighted for each animal with dark blue shading, and previously identified antibody level associated with increased risk of EEHV-HD (normalised ΔOD < 0.25) is outlined by grey shading.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- S1Elephantdemographicdata.xlsx
- S2NLMEModelMethodsResults.docx