Elephant endotheliotropic herpesvirus (EEHV) immunohistochemistry in the Asian Elephant (*Elephas maximus).*

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Abstract

Elephant endotheliotropic herpesvirus (EEHV) infections can lead to a fatal infection in juvenile elephants. To this date no EEHV immunohistochemistry records are available. With this study we tried to localise the virus antigen by using immunohistochemistry. The material used was PCR positive material collected from an elephant from a Dutch zoo. Histological evaluation was hampered by bacterial overgrowth and dissociating epithelium in the slides. We tried various staining protocols to localise the antigen based on other immunohistochemistry stains, other protocols and previous experience. The antibodies used in this study were polyclonal rabbit anti-peptide antibodies against EEHV-1A. Two different antibodies were used: U73 OBP and U41 MDBP. We started out using both antibodies but eventually switched to only testing and varying with the MDBP antibody. We were not able to locate either OBP and MDBP antibodies, however this study can be seen as a starting protocol for future studies and stainings.

Introduction

Elephant endotheliotropic herpesvirus (EEHV) causes acute often fatal haemorrhagic disease in juvenile Asian elephants (*Elephas maximus*) and less frequently in the African elephant (*Loxodonta africana*) and clinical infections account for the majority of their fatalities[1,2]. Since 1995 eight fatal EEHV cases have occurred in the United Kingdom and Ireland alone [3], totally more than 100 fatal cases were reported[4]. The virus is a large 180 kbp DNA virus and has been classified as a member of the Proboscivirus genus within the family of the Betaherpesvirinae[5,6]. To date, multiple subtypes of EEHV have been discovered being 1A, 1B, 2, 3, 4, 5, 6 and 7 with subtype 1 being the most common. The first reported EEHV case was in 1988 when an Asian circus elephant in Switzerland died of a haemorrhagic disease of unknown etiology[7]. Later, in 1999 Richman et al. published about a novel herpesvirus detected in the elephant cases from the years prior[8]. In the next 15 years since the discovery more than 100 cases of EEHV infections in both wild and captive elephants were reported[9]. EEHV has since been one of the major fatal causes of juvenile captive Asian elephants. The clinical symptoms start with vague clinical signs as lethargy, anorexia and more specifically oedema of the head and neck and cyanosis of the tongue[10], progressing to a haemorrhagic disease which ultimately results in a fatal outcome. Infection may combine with reproductive loss in elderly animals. Mortality rate amongst juvenile elephants is 85%[8]. Since the first ever case of EEHV not much information about the pathogenesis has been obtained. Reports about the sudden onset of clinical signs which progresses to a fatal still lack to this date. Immunohistochemistry can play a viable role in understanding this progression and the pathogenesis of infections.

Currently serology and PCR are available, but no immunohistochemical test. Clinically, the presence of EEHV can be detected by qPCR on whole-blood samples when viral DNA is present[1]. A simple enzyme-linked immunosorbent assay-based serology-assay (ELISA) test has been developed by Richman et al. using a synthetic epitope fragment of the gB protein antigen from EEHV1A[11]. Furthermore, trunk washes can be tested using real-time qPCR to detect EEHV species to provide information about quiescent infections and virus shedders[12]. This information can be useful for preventing and treating infections in animals. When able to distinguish an individual that is likely to be infected a treatment can commence very soon, decreasing the chance of a fatal outcome[1].

However, exact viral distribution of the virus in elephants, patients and carriers alike is incompletely known. A study that measured EEHV DNA loads in various organs showed that lung, pancreas, kidney and liver are major target organs in infected individuals[13]. It is believed that infection of endothelial cells plays a major role in the pathogenesis of EEHV. Endothelial cells of large myocardial arteries and the endocardium have a higher presence of singe large basophilic intranuclear inclusion bodies compared to the endothelial cells of the lung, liver or kidney in fatal cases of EEHV infections[14]. To

provide further insight in the pathogenesis of EEHV infections an extension of these studies have to be conducted.

To this date, immunohistochemical localisation of EEHV has not been published. Also no in situ hybridisation studies are currently available. Immunohistochemistry may aid in locating cell types within positive tissues and thus provide more insight into the pathogenesis of EEHV infection. The aim of this research is the set-up of a running EEHV IHC protocol, and subsequent localization of EEHV virus/proteins in elephants, in diseased animals, and in asymptomatic carriers.

Materials & Methods

Antibodies
Antibodies were kindly provided by G.S. Hayward (John Hopkins School of Medicine, Baltimore, USA) and consisted of two different antibody sera (undiluted). Both sera were polyclonal rabbit anti-peptide antibodies to EEHV1A lytic cycle nuclear antigens. The first antibody serum is U41 Major DNA binding protein (MDBP) and the second is U73 UL9-like Origin binding protein (OBP)[15]. These were generated against expected to be hydrophilic antigenic epitopes 15 to 18 aa in length tailed with Y and SC for conjugation (Gary Hayward, personal communication, May 29, 2017).

Tissues
All the tissues used for this study originated from a four year old Asian elephant from a Dutch Zoo. It died on December 7, 2015 after a short clinical course.
Necropsy was performed on the 8th of December 2015 at the Veterinary Pathology Diagnostic Centre (VPDC) at the University of Utrecht, The Netherlands.
Macroscopical investigation revealed an animal in an advanced state of decomposition.
Macroscopical lesions consisted of ulcerations on the inside of the lip, discolouration and extensive post mortal formation of gas in the thorax and abdomen. The heart, liver and pancreas were autolytic and the mucosa of the trachea could not be evaluated.
For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, routinely paraffin embedded, cut in 3 micron slides and stained by haematoxylin and eosin stain (HE).
Tissue samples from several organs including heart, liver, spleen, kidney, lung and lip were frozen at minus 20°Celsius

Histological evaluation was hampered by extensive post mortal artefacts: autolysis and bacterial overgrowth. The selected paraffin blocks that we used for this study were heart (C.13), lip (C.17) and spleen (C.9). In the HE slide made of liver tissue one vague structure was visible that could be interpreted as an inclusion body which could indicate the presence of EEHV-1. Remaining slides revealed no inclusion bodies.

Presence of EEHV-1 virus particles was confirmed by PCR performed on frozen samples (Table 1) by Katharina Seilern-Moy at the Animal and Plant Health Agency, department of Virology in New Haw, Addlestone, Surrey in the United Kingdom.

Table 1

Tissue	EEHV-1 copyno. /Microgram DNA
Heart	2.56E+07
Spleen	3.69E+06
Liver	2.16E+06
Lung	1.10E+06
Blood	8.00E+05
Kidney	2.07E+05

Immunohistochemistry

In the course of this study, five staining protocols were tested (Table 2).

Table 2.

	Used antibodies	Organ	Date
1.	MDBP & OBP	Heart	31-05-2017
2.	MDBP & OBP	Heart	06-06-2017
3.	MDBP	Lip	12-06-2017
4.	MDBP	Spleen	22-06-2017
5.	MDBP	Lip	06-07-2017

The tissue used for the first staining was the heart (cassette 13)
Before commencing the first IHC-staining (performed on 31-05-2017) the tissue was cut in 3µm thin slides and was mounted on silan-coated microscope slides. These slides were dried overnight at a temperature of 55°C. The next day the slides were deparaffinised and rehydrated by respectively being soaked in xylene (2x5 minutes), 100% alcohol (2x5 minutes), 96% alcohol (2x5 minutes), 70% alcohol (2x3 minutes) and distilled water (2x3 minutes). During the process of deparaffinisation the antigen retrieval (AR) step was already being prepared. 750 mL of citrate buffer pH 6.0 was preheated in an 850 Watt microwave on 100% power level for 10 minutes. Thereafter the slides were incubated in the buffer for 5 minutes at a 50% power level. The bowl of citrate with the slides was then removed and put on the table to cool down for 20 minutes. The time expired and the slides were rinsed in phosphate buffered saline (PBS). The next step in the process is the endogenous peroxidase activity block. In order to block the endogenous peroxidase the slides have to soak in a 1% H_2O_2 dilution in methanol for 30 minutes. After this the slides were rinsed in PBS on an orbital shaker for 3x5 minutes. When the slides were rinsed they were to be incubated with the primary antibody. The available antibodies were MDBP and OBP. The dilutions that were used in the first protocol were 1:25, 1:50, 1:100 and 1:200. These were made by combining 4µl antibody with 96 µl Normal Antibody Diluent (NAD) for the 1:25 dilution, 2µl antibody with 98µl NAD for the 1:50 dilution, 1µl antibody with 99µl NAD for the 1:100 dilution and finally 1µl antibody together with 199µl NAD for the 1:200 dilution. The dilutions were made in duplo: one dilution series for the OBP antibody and one for the MDBP antibody. In total 10 slides were made for the first staining, four diluted slides and one negative control for each of the dilutions. The slides were placed in a humid chamber so they could be incubated overnight. The next morning the slides were rinsed in PBS/Tween on an orbital shaker for 3 times 5 minutes. Followed by incubation with Brightvision® (Immunologic, Duiven, The Netherlands) for 30 minutes in a humid chamber at room temperature, then washed with PBS (without Tween) again for 3 times 5 minutes. Subsequently, the slides were incubated with AEC in the humid chamber for 20 minutes, washed in distilled water to block the effects of AEC, then rinsed in running tap water for 5 minutes. Slides were soaked in haematoxylin for 45 seconds, then rinsed for 15 minutes in running tap water. Finally the slides are covered with a glass and some Aquatex (Merck Chemicals, Amsterdam, The Netherlands).

For the second staining (performed on 06-06-2017) the exact same protocol was used because of logistics reasons the first staining could not be considered as a validate staining. However there was one difference between the first staining and the second staining being that the series of dilutions were separated for both the antibodies. The dilutions and negative control for the OBP antibody were in a different humid chamber than the dilutions and negative control for the MDNP antibody. After being incubated and separated all the following steps were separated, including the washing and rinsing process.

For the third staining (12-06-2017) a different organ was used, instead of heart we switched to lip tissue (cassette 17). Also the protocol was changed. We tried a different approach to the antigen retrieval. Instead of incubating the slides at 50% power level for 5 minutes we now tried 2 different incubation times. In total twelve slides were made of lip tissue. These slides were split in 4 groups. The twelve slides were split in two series, with the difference between them being the solution the slides were rinsed in and the antibody was diluted in: one series of six slides being washed in TBS and TBS/Tween and one series in PBS and PBS/Tween. Within these two series we also varied in incubation time in the citrate for antigen retrieval: three slides were incubated for 20 minutes at a power level of 50% and the other three were incubated for 40 minutes also at a power level of 50%. Before incubating the slides with the antibody we incubated each slide with 100µl 10% normal goat serum for 20 minutes. When these 20 minutes expired we did not rinse the slides but tilted the slides so the serum could run off. Hereafter we incubated the slides with dilutions of the MDBP antibody. The dilutions we used in this staining were 1:200, 1:300 and 1:400. Besides the previously mentioned steps the protocol for the next day remained the same for this staining.

The fourth staining (22-06-2017) was a totally different approach compared with the previous three stains. This time the tissue we used was that from the spleen. The deparraffinisation and rehydration steps were the same corresponding to previous staining-protocols. This time we used the same amount of time of antigen retrieval for all the slides: 40 minutes incubation at a power level of 50% and 20 minutes of cooling down on an orbital shaker. All rinsing and washing was done with PBS or PBS/Tween this time. Also the slides were blocked with 10% normal goat serum like the previous staining. The dilutions used this time were 1:300, 1:400, 1:600, 1:800 and 1:1000 and were made in duplo because we wanted to try two different blocking processes (ABC and Brightvision®) . 100µl of dilution was incubated on the tissue per slide. The slides were put in a humid chamber and were left to incubate overnight at room temperature. The next day we wanted to split up the slides for the previous blocking processes. However due to logistics reasons we switched to trying out ABC and ABC-elite for the staining.

The last staining was performed on 6-7-2017. This time we tried to vary in antigen retrieval methods. Instead of using citrate we now switched to four different substances. In total twelve slides were made, using lip tissue. These twelve slides were split up in four different groups, each group being three slides. The first group was incubated with proteinase K for 5 minutes at room temperature. The second group was incubated with 0,1% pronase for 10 minutes at room temperature. The third group was incubated with trypsin for 10 minutes in a stove at 37°C. The last group was incubated with pepsin for 15 minutes in a stove also at 37°C. The steps following the antigen retrieval were identical for all the slides. When the time had expired the slides were rinsed in PBS. Thereafter the endogenous peroxidase was blocked by soaking the slides in 1% H_2O_2 in methanol for 30 minutes. When these minutes expired the slides were yet again rinsed in PBS and incubated with 10% normal goat serum for 20 minutes. We did not rinse the slides after incubating them with the 10% NGS but instead drained them on a tissue and immediately incubated the primary antibody for the dilutions of 1:500, 1:1000 and the negative control slide was incubated with PBS. The slides were then placed in a humid chamber and left to incubate overnight at room temperature. The next day the slides were rinsed in PBS/Tween for two times 5 minutes and incubated with the secondary antibody being biotinylated anti rabbit IgG made in goat in a dilution of 1:125 for 30 minutes. The slides were then rinsed in PBS (without Tween) and incubated with the ABC Elite Vector® pk-6100 kit for 30 minutes. The slides were then rinsed again in PBS and AEC was incubated for 20 minutes. The AEC was discarded on a wet tissue and the slides were rinsed in running tap water for 5 minutes. The slides were then put in haematoxylin for 50 seconds to stain the nuclei in the tissue. Finally the slides were rinsed in running tap water for 15 minutes and mounted with coverslips using Aquatex.

Results

Results per series are summarised below.

Results of the first staining, are described in table 3. All results were interpreted as negative.

1st staining Organ: Heart		**Dilution**			
Antibody	Negative control	1:25	1:50	1:100	1:200
OBP	Negative.	No visible staining, lot of rotting bacteria in the tissue.	As 1:25.	Strong background colour, some of the bacteria are stained as well.	Weaker background colour compared to 1:100, sporadically some nuclei are visible.
MDBP	Negative.	Negative.	As 1:25.	No macroscopic visible staining.	No macroscopic visible staining.

Table 3.

Results of the second staining are described in table 4.

2nd staining Organ: Heart		**Dilution**			
Antibody	Negative control	1:25	1:50	1:100	1:200
OBP	Negative.	Collagen is incidentally stained, overall negative.	Red coloured, granular tissue visible at the edge of the slide: interpretation is negative.	Negative.	Negative.
MDBP	Negative.	Negative.	Negative.	Negative.	Non-specific colouring on basal membrane and some cells have a non-specific staining of the cytoplasm. Some small nerves are slightly stained. This might be considered as a signal.

Table 4.

The third staining, performed on 13-6-2017 was performed by using the MDBP antibody because of results of previous stains. For this staining we switched to lip tissue as this could be considered more fresh and had a considerable less amount of bacteria throughout the tissue. The results are shown in table 5.

3rd staining Organ: Lip	Dilution		
Substance used for rinsing and incubation time of antigen retrieval.	1:200	1:300	1:400
PBS; 20 minutes AR	Slightly visible background staining, basal membrane of venules and nerves stained.	Two red circular shapes visible in an artery between several erythrocytes. Around the same size as an erythrocyte.	Epithelium of the lip is stained light pink. Interpretation is non-specific.
PBS; 40 minutes AR	Epithelium is stained throughout the whole slide. Red circular shapes visible within disintegrated epithelia cells.	Epithelium is also stained throughout the whole slide. However, it is a slightly more light than previous dilution. Skeletal muscles are stained and also a visible staining perineural.	A large light pink coloured venule with erythrocytes, other cells and red circular shapes are visible.
TBS; 20 minutes AR	Negative.	Epithelium is lightly stained (non-specific).	Inside an artery some red circular cells visible between the erythrocytes.
TBS; 40 minutes AR	Red shapes visible in disintegrating epithelium.	As 1:200.	Some red staining within the vascular structures throughout the slide.

Table 5.

The results from this staining were dubious because we still could not define a clear signal

Because we were not satisfied with the results yet we tried another staining method. In the previous protocols we used Brightvision® for the staining. In hope of getting a better result we tried to vary in the staining method: Instead of using Brightvision® we used an avidin biotin complex this time. We tried two different kits. The result from the fourth staining are described in table 6.

4th staining Organ: Spleen		Dilution				
Staining kit used	Negative control	1:300	1:400	1:600	1:800	1:1000
Vector ABC® pk-4000	Negative.	Negative.	Negative.	Non-specific background staining on bacteria, red aspect on germination centres. Interpretation: negative.	Negative.	Negative.
Vector Elite ABC® pk-6100	Negative.	Strong background colour. Bacteria are stained as well: false-positive because of non-specific staining.	Strong background colour. Some nuclei are stained light red. Some macrophages seem to have a red colour in their cytoplasm. These might be erythrocytes.	Background colour is less intensive compared to previous dilutions. Bacteria are still stained non-specific.	Negative.	Colour visible in coil-shaped cells. Bacteria are visibly stained.

Table 6.

Yet again, a lot of the dilutions did not provide a signal of the antibody and were interpreted negative. The Vector Elite ABC® pk-6100 kit showed better results compared to the Vector ABC® pk-4000 kit.

For the final staining we tried yet something different. Taking the results of the previous staining in account we used the Vector Elite ABC® pk-6100 kit for staining. We have varied a lot in dilutions and staining but did not vary the antigen retrieval, besides the length that is. For the final staining we tried different methods of antigen retrieval including pepsin, trypsin, 0,1% pronase and proteinase K. For the length for each of these antigen retrievals we consulted other protocols that included the use of these techniques and prolonged them for our staining. The exact length is explained in the materials & method of this paper. The results for the final staining are described in table 7.

5th staining Organ: Lip		Dilution	
Substance used for Antigen retrieval	Negative control	1:500	1:1000
Proteinase K	Negative.	Epithelium stained lightly, interpretation: non-specific. Intravascular some red granula up to 1µm in width. Some erythrocytes have a light red colour.	Epithelium stained non-specific. Overall: negative.
0,1% Pronase	-	Non-specific background staining. The cytoplasm of some Mast cells is lightly stained.	No background staining. Cytoplasm of mast cells is stained identically to the 1:500 dilution.
Trypsin	Negative.	Non-specific background staining. Perivascular structures lightly stained red. Some erythrocytes are stained red; this might be considered as positive. Bacteria are also stained but this is interpreted as non-specific. Some intravascular monocytes are stained red, might be considered as positive.	Non-specific background staining of epithelium.
Pepsin	Negative.	Both perivascular and perineural structures are stained identically to staining of 6-6-2017 MDBP 1:200 dilution. Strong non-specific background colour on collagen and skeletal muscles.	Negative.

Table 7.

Discussion

EEHV-1A antigen OBP and MDBP were not located in this study. As a guideline for our immunohistochemistry, a CD3 staining protocol used in other studies from the same laboratory (dept. pathobiology, Faculty of Veterinary Medicine, University of Utrecht)[16] was used and modified following the results to lead to a final protocol for EEHV immunohistochemistry. We expected the EEHV-1 antigen to stain like a regular CD-3 protocol. Furthermore we also used Chiou et al. 2002 as guideline for our immunohistochemistry[17]. The first staining of this study was performed identically to the CD-3 staining of Mekonnen et al. 2007[16]. The results from this staining did not get any positive results, likely due to the fact the slides were dried out during the process of staining. This can affect the slides in a negative manner and therefore the staining was repeated more careful on the 6th of June. The results are respectively shown is table 2 and table 3. Both antibodies OBP and MDBP did not give the desired results however while evaluating the slides we considered the MDBP antibody to be more likely to lead to a successful result and also taking into account that focusing on one antibody and not two at the same time is logistically more favourable. Hence all future staining were performed using the MDBP antibody and not the OBP antibody. The organ used in our first staining was heart, because of all tested organs this yielded the highest amount of EEHV-1 copyno./Microgram DNA. The heart tissue however, had a lot of bacterial overgrowth and this did not favour our staining. For that reason we switched to another organ for our third staining. In this

case we used tissue gathered from the lip of the elephant because this was histologically the best preserved available PCR positive tissue.

Whilst evaluating the slides from the first and second staining we thought of possibilities that could influence the outcome of the process. It is known that certain antibodies can be affected by the substance the slides are rinsed in after incubation steps. To identify if this could be the case in our protocol we split the protocol into two different protocols: one with PBS and PBS/Tween for rinsing and one with TBS and TBS/Tween for rinsing. The MDBP antibody was diluted differently this time: for the PBS protocol the antibody was diluted in PBS instead of NAD and for the TBS protocol it was diluted in TBS. The second thing we varied in was the length of the antigen retrieval. This was done to see if the variation of the incubation length of the antigen retrieval affected the results. Half of the slides were incubated for 20 minutes and the other half were incubated for 40 minutes. Both groups were cooled down for 20 minutes after the incubation. As we assumed the length of the antigen retrieval did have its consequences on our antigen. The slides that were incubated for a longer period of time, in this case 40 minutes, appeared to be more colourful compared to the slides that were incubated for 20 minutes. Even though the non-specific background colour also became relatively more present the result inclined to be more positive. Identically to the variation of antigen retrieval the MDBP antigen was also influenced by the difference in rinsing substance and antibody diluent. TBS was interpret as less useful then its PBS counterpart because overall the slides were less stained, besides some red circular shapes that seemed to be rounded off too sharp and was therefore more on top of the tissue rather than actually amongst the cells in the tissue.

For the fourth staining we used the spleen as organ. The lip tissue did not give us any indication of the presence of the antigen besides some non-specific staining in arteries and venules. Until now we did not have any discovery about the localisation of the EEHV antigen in our own slides. In the HE slides that were made of several organs before this study commenced we did find a clue however. In the spleen and liver slide some deviating structures were seen present. In the liver there was an inclusion body visible. This is why we considered using spleen or liver as our next sample. Taking into account that just like the heart the liver slide was overgrown with bacteria we decided to use the spleen as it was less contaminated with bacteria. This time we decided to vary in staining method because previous stains did not have the desired result. Instead of using Brightvision® we now switched to using an avidin and biotin complex. There were 2 kits available for use and these were the Vector ABC® pk-4000 kit and the Vector ABC Elite® pk-6100 kit. When evaluating the slides we discovered that the slides stained with the Vector ABC Elite® pk-6100 kit showed a better result compared to its counterpart however, it was still not the result we were looking for.

In the fourth series, antigen retrieval buffer varied: trypsin, pepsin, proteinase K and 0,1% pronase were used each with their corresponding incubation time. All previous antigen retrievals were performed in a citrate buffer. The incubation length for each antigen retrieval was taken from their corresponding standard protocol. The slides that were treated with trypsin as antigen retrieval gave the best results compared to other antigen retrieval methods. The staining of the tissue was similar to one of our first stains with citrate as antigen retrieval. Pepsin, proteinase K and 0,1% pronase all had a high non-specific background colour on skeletal muscles and collagen making them less viable for future use with the MDBP antibody.

Due to unavailability of EEHV immunohistochemistry records, decisions and steps were based on other immunohistochemistry stains, previous experience and other staining protocols. This trial-and-error process has not yet led to a final EEHV-1 staining protocol. For future research about EEHV IHC this study can be used as starting point. Following the results of this study the most promising steps to lead to a result are using trypsin as antigen retrieval method and using the dilutions 1:500 to 1:1000 on lip tissue, because the lip organ was in this case the least histological hampered. When histologically better preserved material will be available in the future for IHC, heart, kidney, liver or spleen will be preferred as these organs contain the highest viral load.

In this study we did not conduct a staining on a PCR negative elephant as negative control. This is due to the fact we only had material of a PCR positive animal and there was no material available of a PCR negative tested animal. Moreover, this study only used antibodies against EEHV-1A and not the other subtypes of the virus. If this study was able to locate EEHV-1A, the localisation of other subtypes could be achieved using this study as starting point and by using antibodies that were generated against other EEHV-subtypes since they behave alike.

Immunohistochemistry of EEHV is of an importance to discover at post mortal investigation asymptomatic carriers within a population of elephants. This way a lot of individual elephants can be treated from the sudden onset disease and reduce the mortality in the populations. Although other detection techniques exist, such as PCR or serological tests, these do not provide information about the localisation of the virus and therefore a working protocol for immunohistochemistry is required. Also there is a lack of records about a successful cultivation of the virus for the use of in situ hybridization.

In conclusion, this study did not yet result in a working IHC protocol for EEHV on formalin fixed tissue. This study can be used as a basis for future IHC studies of EEHV. The importance of EEHV IHC is relevant compared to other techniques as IHC staining can provide information about the localisation of the virus in an animal which helps understand pathogenesis and thus may be viable for future cases.

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