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Screening of Asian elephants (Elephas maximus) in captivity at elephant orphanage, Pinnawala and ETH, Udawalawe for Mycobacterium tuberculosis and elephant endotheliotropic herpes virus type1 (EEHV 1) using direct amplification of pathogen DNA from trunk washes

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***Abstract*— There are many case reports of elephant pathogenic bacteria and viruses that require quick and sensitive diagnostic techniques due to the impact they generate. Out of these the occurrence of TB in elephants, especially in captivity, leading to zoonotic risk for humans who live at the animal-human interface and the different strains of elephant endotheliotropic herpes virus (EEHV) that pose a threat to Asian elephants are of extreme importance. Hence, this study aims to evaluate the PCR based molecular techniques for the rapid and direct detection of TB in captive elephants by primers targeting gene hsp65 and EEHV 1 strain by primers targeting the terminase gene. Serologically positive captive Asian elephants at Elephant orphanage, Pinnawala were screened for TB by specific primer PCR assay for hsp65 gene of *M.tuberculosis* using direct DNA isolates from trunk wash samples. Among 21 trunk washes, only a single amplification was observed, with a size closer to 441bp. Sequencing of this resulted a 415bp fragment which was not responsible for TB. Although, there have been no recorded cases of EEHV in Sri Lanka, many healthy Asian elephants are asymptomatically infected by EEHV1 in the neighboring Indian region. Therefore, asymptomatic Asian elephants in captivity at ETH, Udawalawe were screened for 336bp partial EEHV1 terminase gene using direct DNA isolates from blood, eye swabs and buccal cavity swabs. All tested samples were negative for EEHV1. Since these elephants were closely monitored even after the study and none of them developed classical symptoms of either EEHV or TB, it is difficult to prove the fact that they were originally infected. The nonspecific amplification proves that it is possible to extract microbial DNA from elephant trunk washes.**

***Keywords*— endotheliotropic herpes virus, hsp65, Mycobacterium tuberculosis, Polymerase Chain Reaction (PCR)**

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I. Introduction

Tuberculosis (TB) is a highly contagious bacterial infection caused by organisms in the *Mycobacterium tuberculosis* complex, most notably *M. tuberculosis* or *M.bovis*. In captive elephants, the disease is primarily caused by *M. tuberculosis*, although infection with *M. bovis* has been recorded. Asian elephants (*Elephas maximus*) are more frequently infected with TB compared to African elephants (*Loxodonta africana*) (Payeur *et al*., 2002). Prevalence of TB in Asian elephants was found to be 15% in India and 13% in Nepal (Abraham *et al*., 2010; Mikota *et al*., 2008). Several wild animals act as a store house for the TB organism and spread it to the elephants. However, research investigating the epidemiology of TB in elephants in this part of the world is still in its early stages (Abraham *et al*., 2010). The interest in elephant TB has been increasing over the past years due to its public health threat as well as increased concern for the healthcare and conservation of elephants. During the past 2 decades, infections of captive African and Asian elephants with *M. bovis* and *M. tuberculosis* have been diagnosed worldwide (Angkawanish *et al*., 2010).

According to a study conducted in United States of America for a period of 10 years, female elephants seem to be affected more than the male elephants (Porphyre *et al*., 2008) and also, free ranging wild elephants are believed to be less susceptible to TB than the captive animals, which may be due to the reason that the human pathogen gets transmitted to the elephants in the captive area (Alexander *et al*., 2002).Transmission of TB occurs possibly by aerosolization of the droplets which are infected with mycobacterial organisms (Kaneene and Pfeiffer, 2006). This transport is possible by the way of coughing, trumpeting and trunk spray from the affected animal. Bacterial load, droplet size, length of exposure, closeness of the affected animal and immune status of the exposed animal are the factors which have dominant role in the transmission of organism from one elephant to the other (Fowler and Mikota, 2006). From these elephants it spreads to the elephant handlers, thus playing a significant role in the zoonosis of TB.

Therefore, research in the zone of tuberculosis in elephants is required in order to save the elephants and also to prevent the spread of TB to humans and other animals. Currently accepted ‘‘Gold standard’’ laboratory method for detecting and identifying *M. tuberculosis* is a combination of acid-fast bacillus (AFB) smear for initial screening and culture for bacterial isolation and identification. Microscopic examination of AFB smears can yield a result within 24 h

. Unfortunately, smear is neither specific for *M. tuberculosis* nor very sensitive, requiring 10^3 to 10^4 organisms per ml of sputum (Hall *et al*., 1994). Mycobacteria can also be detected and identified directly from sputum specimens by analyzing the mycobacterial mycolic acid by high-performance liquid chromatography. However, the sensitivity of this method is low, and direct detection from sputum is possible only in smear-positive specimens (Jost *et al*., 1995). Bacterial culture is superior to AFB smear and mycolic acid analysis, both in terms of sensitivity and specificity. However, mycobacteria are fastidious organisms with very strict growth requirements. As a result, culture-based diagnostic methods are slow. The need to perform biochemical testing to identify the mycobacterial species adds to the time required to obtain a diagnosis. Even when nucleic acid probes are used for ‘‘rapid’’ species identification from cultures, results are often not available for 2 to 8 weeks. Clearly, a more rapid test for the detection of pulmonary TB and other mycobacterial infections would greatly benefit patient management (Vincent *et al*., 1996).

Therefore, effective TB management requires the rapid detection and identification of the etiologic agent. PCR has proven to be a very useful tool for the rapid diagnosis of infectious diseases, including mycobacteriosis. For the prompt identification of mycobacteria molecular methods are gaining increasing importance due to their rapidity and, in most cases, unequivocal results (Richter *et al*., 1999). Sequencing of the hsp65 gene (Kapur *et al*., 1995), the 16S rRNA gene (Kirschner *et al*., 1993), the spacer region between the 16S rRNA and the 23S rRNA genes, or the 32-kDa protein gene (Soini *et al*., 1994) are powerful techniques for the identification of mycobacterial species. Furthermore, the ITS is suitable for differentiating species of mycobacteria and potentially can be used to distinguish clinically relevant subspecies (Roth *et al*., 1998).

Elephant endotheliotropic herpesviruses (EEHVs) comprehend to the genus Proboscivirus in the subfamily Betaherpesviriniae.

EEHV was first diagnosed in 1995 (Richman *et al*., 1999). This viral infection is referred to as endotheliotropic herpesvirus due to its morphologically visible impact on infected endothelial cells. EEHVs infections lead to significant cause of reproductive failure and death in captive elephants in Europe, the US and Asia (Fickel *et al*., 2001). This can cause acute hemorrhagic disease in endangered Asian and African elephants.

This virus causes decrement to the endothelial cells and often results in pre-acute to acute death within one to five days of the invasion of clinical signs. These include oedema of the head, discrete oral ulceration, lethargy, cyanosis of the tongue and anorexia. Decreasing of white blood cells and platelet count has also been detected (Hardman *et al*., 2011).

Six proboscivirus have been identified as EEHV-1 to EEHV-6. It is believed that these occur only in elephant hosts. Among these types EEHV-1 is the most common type and it is divided into two major variants as EEHV-1A and EEHV-1B. From the worldwide reported clinical cases of EEHV, two deaths were from EEHV 2 and one each from EEHV 3 and EEHV 4 (Latimer *et al*., 2011). Several species of herpesviruses are carried by many animals including humans, throughout their lives and never become clinically ill. These form a latent phase inside a host animal after causing mild symptoms or asymptomatic infection. Many healthy Asian elephants are asymptomatically infected as well (Garner *et al*., 2009). However, still there have been no recorded cases of EEHV in Sri Lanka.

A rapid, sensitive, and specific PCR assay is needed to detect the EEHV subtypes most commonly associated with herpesvirus-associated disease. Such an assay would be useful for the detection of EEHV1 viral DNA in blood samples for the purposes of screening clinically ill animals and monitoring susceptible elephants for early viremia.

The purpose of the study reported here was to introduce a rapid and sensitive method to screen the elephants in captivity at elephant orphanage for TB and to screen the asymptomatic captive elephants at ETH, Udawalawe for EEHV. In this study specific primer PCR was used to screen the elephants separately for *M. tuberculosis* and EEHV1 using direct DNA isolates from the trunk wash samples and eye and buccal cavity swabs.

Captive elephants at Elephant Orphanage Pinnawala and Zoological Garden Dehiwala, require a proper screening method for management of TB both vertically and horizontally. In the case of EEHV, although not still recorded in Sri Lanka, captive elephants can be asymptomatically infected as well. Therefore it is required to have a rapid and sensitive method of identification of TB and EEHV in elephants under captivity.

Hence, main objectives of this study were, to screen the elephants in captivity at Pinnawala elephant orphanage for TB and to screen the captive elephants at ETH for EEHV1.

II. Methodology

A. *Sampling for TB*

A total of twenty one trunk wash samples (11 samples from elephant “Mahasen” and 10 samples from elephant “Waruna”) were collected from serologically positive Asian elephants (*Elephas maximus*) at the Elephant orphanage Pinnawala, during a period of three months.

B. *Sampling for EEHV*

A total of six samples (2 blood samples, 2 eye swab samples and 2 samples from the buccal cavity) were collected from selected asymptomatic Asian elephant (*Elephas maximus*) at ETH, Udawalawe.

C. *Collection of trunk wash*

Trunk wash samples containing elephant nasal secretions were collected by means of the standard recommended technique for collection of samples for *Mycobacterium tuberculosis* testing by the USDA.

50 ml of sterile saline solution was poured into the nares of each elephant and the trunk was elevated for 20 to 30 seconds. Then the elephant was instructed to blow the instilled saline solution into a fresh plastic 1 gallon freezer bag. The resulting effluent was transferred to a sterile 50ml conical tube and then chilled on ice until processing.

D. Extraction of DNA

From trunk wash samples for TB: Nasal secretions obtained via trunk washes were processed with QIAamp DNA mini kit (QIAGEN, Germany) using a modified protocol for purification of bacterial DNA.

From body fluids for EEHV: Whole blood samples, eye swabs and buccal cavity swabs were processed using QIAamp DNA mini kit, in accordance with the manufacturer's recommended protocol for the extraction of viral DNA.

E. PCR for amplification of TB genes

hsp 65 : Extracted DNA was amplified by using conventional PCR targeting a 441-bp segment of the hsp65 gene. Forward and reverse primer (Table 1.) were added to the 12.5 µl of Qiagen master mix and 10 µl (50ng) of extracted DNA was added to adjust the PCR mixture to 25 µl.

The thermal cycler was programmed for 35 cycles with initial denaturation at 94°C for four minutes. Each cycle was performed with denaturation at 94°C for one minute, annealing at 64°C for one minute with an extension at 72°C for two minutes. At the end of the last cycle, the mixtures were incubated at 72°C for 10 minutes.

TABLE 1. Specific primers used to amplify selected genes in PCR

Gene	Primer sequence
hsp65	5'-ACCAACGATGGTGTGTCCAT- 3' 5'-CTTGTCGAACCGCATACCCT-3'

F. PCR for amplification of terminase gene of EEHV

Extracted DNA from trunk-wash samples (5 µl) were used with 1.5 µl of each forward (5′-GTGTCGGCTAAATGTTCTTG-3′) and reverse (5′-GTGTCGGCTAAATGTTCTTG-3′) were added to the 12.5 µl of a master mix and volume was adjusted to 25 µl.

PCR amplification of 336bp segment of terminase gene was conducted according to the following condions;40 cycles with initial denaturation at 95^0C for 5 minute, 94^0C for 15 sec., 50^0C for 15sec. and 72^0C for 1 minute followed by 72^0C for 10 min.

G. Visualization of the PCR products

The amplified product was submitted to electrophoresis on a 1% agarose gel in 1X Tris-borate-EDTA(TBE) buffer at pH of 8.6. The gel was stained with 2 µl of ethidium bromide (10 mg/ml) in 100 ml of 1X TBE, and the amplified band was visualized on an ultraviolet transilluminator to check for DNA amplification.

H. Analysis of the sequences

Sequencing was performed by Macrogen Inc. Korea. The resulted sequence was aligned using BLAST with the available species in GeneBank.

Pairwise sequence alignment was carried out using the hsp65 gene of *Mycobacterium xenopi* using EMBL, EMBOSS tool.

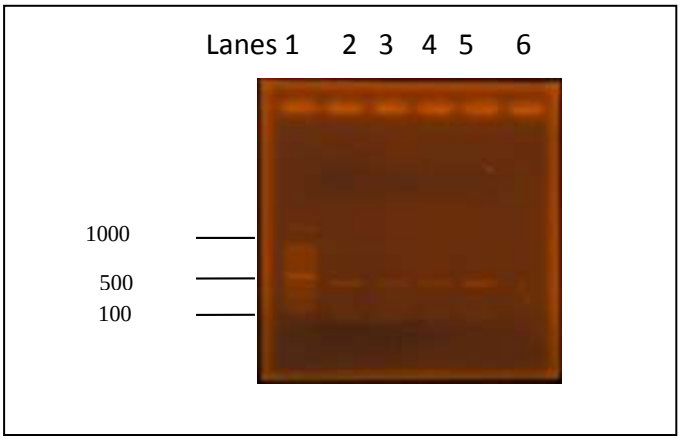
III. Results and Discussion

This report describes the use of a specific primer PCR to screen for TB and EEHV1 in clinical samples from healthy Asian elephants in captivity. This is the first time where a PCR based molecular method has been used to screen the captive Asian elephants for TB and EEHV1 in Sri Lanka. One of the main purposes was to screen the elephants to prevent unmanageable treating of captive elephants with high dose of antibiotics for TB. The importance of EEHV1 in Asian elephants has been emphasized by the number of mortalities the disease has caused since the index case in 1995 (Richman *et al*., 1999).

A. Amplification of hsp65 gene

The PCR assay was performed on twenty one direct DNA isolates (from trunk wash samples) which were collected during the period of three months. Molecular study of MTB infection was based on detection of the presence of hsp 65 gene (441 bp) of *M. tuberculosis*. Positive amplification (a band with approximately 400bp in size) was given by only a single sample, collected from elephant "Waruna" (trunk wash collected on 11.07.2014) [Fig.1].

Fig. 1. PCR product (DNA extract of the sample collected on 11.07.14).Lane 1 : 100bp DNA ladder, Lanes 2 to 5 : replicates(D1-D4) of direct DNA isolates.



B. Sequence of the PCR product (with the primers correspond to hsp65 gene)

The PCR product of isolated DNA was sequenced [Fig.2] and compared with available DNA sequences using the NCBI-BLAST program. Results showed that, there was no similarity with the MTB complex.

Fig. 2. Consensus sequence of the hsp65 gene obtained by sequencing

1	TTAAACGAGCTGATTCGACAGTATC	25
26	GAGAGATCGGTGCTGAGCTGGTTAA	50
51	AGAAGTAGCGAAGAAGACCGATGAT	75
76	GTTGCTGGTGACGGTACCACCACCG	100
101	CAACCGTGCTCGCACAGGCGCTAGT	125
126	ACGCGAGGGTCTGCGTAATGTGGCA	150
151	GCAGGCGCTGATCCAATTAGCCTCA	175
176	AGCGCGGTATCGAATACGCTACTGA	200
201	GGCGATCACCAAGCAGCTGCTGGCT	225
226	AACGCAAAAGAAATCGAAACCACCG	250
251	ATGAAATCGCGGCAACCGCTTCTAT	275
276	TTCCGCTGCTGATCCTGAGATCGGT	300
301	CGCCTAATTGCCGAGGCAATTGAAA	325
326	AGGTTGGTAAAGAGGGCGTTGTGAC	350
351	CGTTGAGGAATCCAACACCTACGGC	375
376	ACCGAGCTTGAACTAACCGAGGGTA	400
401	TGCGGTTCGACAAGA	415

Table 2. The BLAST results for the automated sequence of PCR product, correspond to primers which were used to amplify hsp 65 gene.

Bacterial type	Query cover	Identity	E value	NCBI Gene bank Accession number
Rothia dentocariosa ATCC 17931	93%	78%	5e-57	CP002280.1
Corynebacterium suicordis strain CIP 108201	83%	78%	2e-50	EF028799.1
Corynebacterium imitans strain DSM 44264	71%	75%	1e-27	CP009211.1
Bifidobacterium breve S27	28%	83%	8e-20	CP006716.1
Bifidobacterium breve 689b	28%	83%	8e-20	CP006715.1
Bifidobacterium breve NCFB 2258	28%	83%	8e-20	CP006714.1
Metarhizium acridum CQMa 102	12%	92%	1e-08	XM_007817723.1

Nucleotide Blast of identified sequence resulted different types of bacterial species as mentioned above. The best match was the *Rothia dentocariosa* with 93% of query cover and 78% identity to the amplified sequence. It is a species of gram positive, round to rod shaped bacteria which is part of the normal community of microbes residing in the mouth and respiratory tract.

All the similar sequences available in the database have relatively higher E-values. Usually lower the E-value or closer it is to zero more significant the match is. However, virtually identical short alignments have relatively higher E-values. These higher E-values make sense because short alignments have higher probability of occurring in the database. When compared with all the hits from BLAST, *Corynebacterium imitans* strain DSM 44264 complete genome has lowest E-value and therefore, it has higher similarity to the automated sequence.

Corynebacteria are a heterogeneous group of gram-positive, high-G+C content, rod-shaped bacteria that belong phylogenetically to the *Actinobacteria*. They are found in a broad range of environments (such as dairy products, soil, sediments and aquatic sources), but particularly in man and other animals. Some corynebacteria are well-established pathogens of man and animals, whereas many others occur as part of the normal flora of skin and mucous membranes. *Corynebacterium suicordis* also may be part of the normal micro-flora in elephant mucous membranes while *Corynebacterium suicordis* sp. nov. has been isolated from respiratory infections of pigs (Vela et al., 2003). Pair wise sequence comparison revealed similarity of 68.0 % with the *Corynebacterium suicordis* strain CIP 108201 65kDa hsp65 gene, partial cds.

C. Similarity to hsp65 gene in Mycobacterium africanum

Pairwise sequence alignment of the sequenced gene of isolated bacteria with the *Mycobacterium africanum* hsp65 gene partial cds (GenBank Accession number: FJ617583.1) resulted 66.3% similarity and 15.1% gaps with score of 1067.5. Hence we can state that, the amplified gene from direct DNA isolates from trunk wash sample have high similarity with the hsp65 gene of *Mycobacteria* spp.

The target gene hsp65 is the most conserved gene among the mycobacterial species. In this study, sequence analyses showed that DNA extracted from trunk wash sample is not related to the MTB complex, although there was high similarity to the hsp65 in *Mycobacterium africanum* when subjected to pair wise alignment.

D. Amplification of terminase gene of EEHV1

The study on twenty five direct DNA isolates from blood, eye swab, buccal cavity swab and trunk wash, gave negative results for PCR (Table 3).

Table 3. Summary of the PCR result of EEHV1 amplification

Sampling site	Number of samples	% of positive EEHV1
Blood	02	00
Eye swab	02	00
Buccal cavity swab	02	00
Trunk wash	19	00
Total	**25**	**00**

Multiple samples of blood, eye swabs, buccal cavity swabs and trunk washes were tested to increase the likelihood of viral DNA detection. Though onset of symptoms of diseased elephant at ETH appeared as EEHV infection, some of the other diseases such as encephalomyocarditis in elephants can have same symptoms.

E. Use of direct DNA isolates in PCR

For the PCR amplification direct DNA isolations from trunk wash samples was used in the case of TB, without using the isolated bacterial DNA cultured in selective media for mycobacteria such as Löwenstein–Jensen medium (LJ medium). With trunk-wash tests, sensitivity has been reported to be poor and affected by collection and processing methods as well as the degree of contamination of the collected samples.

Identification of non tuberculosis bacterial DNA from trunk wash samples illustrate the ubiquitous presence of environmental bacteria. None of the pathogenic bacteria were detected and there were number of soil bacterial sequences identical to the target gene, such as *Ralstonia pickettii, Pseudgulbenkiania spp., and Cuprriavidus necator.*

On the other hand, when elephants were screened at ETH for EEHV1, DNA was extracted from blood, eye swab and buccal cavity swab and directly subjected to PCR. Successful amplification of mycobacterial DNA targets is a challenge when biological specimens contain several inhibitors of the PCR reaction. In this study, the PCR technique was evaluated for the detection of limited mycobacteria cells, from trunk wash samples, by using the target gene *hsp65*.

IV. Conclusion and Suggestions

Although elephants showed positive result for serological tests conducted for TB it was not confirmed in by the *hsp 65* PCR. Under stress conditions elephants may give positive results for TB when the serological tests are conducted. And also the screened captive elephants at ETH, Udawalawe turned negative for EEHV1. The amplification proves that it is possible to extract microbial DNA from elephant trunk washes.

Combined use of both the Stat-Pak assay and TB PCR will be useful to detect active and latent TB infection in captive elephants, continued monitoring of the health of elephants, particularly serologically positive elephants, and repeated examination of trunk washes are required to confirm TB infection in elephants. It is important to determine the most reliable site for screening for use in populations in the where minimal samples can be taken. Where possible, multiple samples would increase the likelihood of viral DNA detection in an individual, especially if the sampling can only be done once.

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