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Evaluation of a Dot ELISA for tuberculosis screening in Asian elephants (*Elephas maximus*)[#]

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Abstract

Banked serum samples of 60 dead captive Asian elephants were used to evaluate a dot ELISA for tuberculosis screening, of which 30 each were taken as cases and controls. The 30 samples that were recorded as positive for Mycobacterium tuberculosis by culture and PCR besides being positive on the DPP® Vet-TB Elephant Assay (DPP) and based on lesions during post-mortem examinations were utlised as known positive controls for this study. The 30 controls were tested negative on DPP and were from elephants that had not shown post-mortem lesions. The dot ELISA was designed on a nitrocellulose membrane template, using Protein A- Horseradish peroxidase as the conjugate and diaminobenzidine tetrahydrochloride as the chromogen substrate. Standardisation of the assay was done using a purified M. tuberculosis antigen, ESAT6-CFP10. Pooled sera samples from live elephants tested recently on the DPP were taken as positive and negative controls. Test results of the dot ELISA were evaluated by comparing the results with the disease status of the cases and controls based on the ante-mortem DPP results and post-mortem lesions. Diagnostic sensitivity (66.67%), specificity (93.33%), positive (90.91%) and negative (73.68%) predictive values were then estimated for the dot ELISA.

Keywords: Tuberculosis, elephant, dot ELISA, ESAT6-CFP10

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Tuberculosis in the wild and captive elephants is now recognised as an emerging zoonotic disease across the world. Most reported cases of tuberculosis in elephants are caused by spillover of various strains of the human tubercle bacilli, *Mycobacterium tuberculosis* (Payeur *et al*., 2002; Singh *et al*., 2017). Until the terminal stages, affected elephants usually do not exhibit clinical signs suggestive of tuberculosis. Hence, ante-mortem diagnosis of tuberculosis in Asian elephants has remained a difficult proposition for field veterinarians (Mikota *et al*., 2015). Serial testing of banked sera samples showed presence of antibodies against the early secretory antigenic target-6 (ESAT6) antigen in sera samples of a diseased elephant 3.5 years prior to isolation of *M. tuberculosis* by trunk wash culture in the same elephant (Lyashchcenko *et al.*, 2006). Later, ESAT6 along with the culture filtrate protein-10 (CFP10) antigen were identified as the major immunodominant antigens in Asian elephants affected with tuberculosis (Lyashchenko *et al*., 2012). The ESAT6-CFP10 antigen is now identified as the immunodominant antigen during the symptomatic as well as asymptomatic phases of tuberculosis in elephants (Ishikawa *et al.*, 2022). The Dual Path Platform Vet-TB Elephant assay kits (DPP), (Chembio Diagnostic Systems Inc., USA) is a rapid immunochromatographic technique that utilises the ESAT6-CFP10 antigen, along with other *Mycobacterium tuberculosis* complex (MTC) antigens, to detect the presence of tuberculosis antibodies in diseased elephants (Lyaschenko *et al.*, 2012). This test is reported to have shown nearly 100 per cent sensitivity and 95 per cent specificity during serological screening of tuberculosis in elephants (Greenwald *et al.*, 2009). Later, DPP was licenced by the United States Department of Agriculture for tuberculosis screening in Asian and African elephants. Test results of the DPP are interpreted as either reactive or non-reactive.

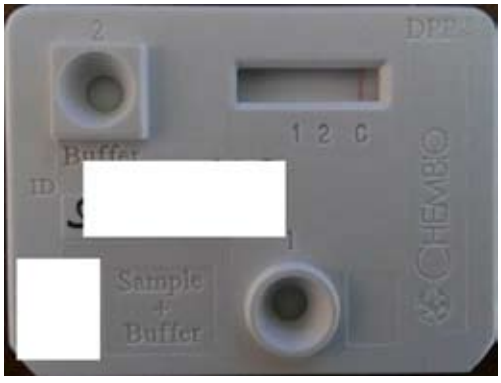
This study describes the standardisation and evaluation of an in-house dot ELISA technique using the ESAT6-CFP10 antigen for tuberculosis screening in Asian elephants. Banked serum samples from 60 dead Asian elephants from Kerala were used for the standardisation and evaluation of dot ELISA in this study. Of these, 30 serum samples available in the laboratory from elephants that had shown reactive results on the DPP during ante-mortem testing and later revealed pulmonary lesions during the post-mortem examinations and shown to be positive for *M. tuberculosis* by culture, and PCR testing were utilised as known positive samples for this study. Another set of 30 samples selected based on the records that elephants had shown non-reactive results on the DPP and did not reveal any post-mortem lesions suggestive of tuberculosis were the known negative controls of this study. The cases and controls were serially numbered from 1 to 30 and were also identified by the individual elephant health record number (EHR) for each elephant. Both the cases and controls included only samples from male elephants and were matched with respect to the estimated ages of the elephants at the time of death. Representative photographs of the reactive (two test lines and one control line visible) and non-reactive (only one control line visible) test results on the DPP for two cases and two controls are shown in Plate 1. Representative photographs of gross lesions in lung parenchyma observed for two cases are shown in Plate 2.

The dot-ELISA was performed as described by Ariffin *et al.* (2020), with modifications in the basic procedure. Optimum concentrations of the coating antigen, ESAT6-CFP10 (ProSpec Tany Technogene Ltd. Israel; catalogue no. PRO-2737) (400 ng/dot), test serum (1:10), and Protein A-HRP conjugate (Sigma; catalogue no. 18-160) (1:1,000) were estimated after checkerboard titrations. For the positive and negative controls, pooled serum samples from live elephants recently tested on the DPP, which respectively gave reactive and nonreactive results, were used.

Strips of nitrocellulose membrane (NCM) (Himedia; catalogue no. SF108A) (1 cm x 0.5 cm), were attached with adhesive (Fevibond, Pidilite Industries, Ltd.) to a plastic template. Two microlitres of antigen (containing 400 ng) were deposited on the NCM and allowed to dry at room temperature. The unbound sites were blocked with five per cent solution of skimmed milk powder (Amul India Ltd.) for one hour. The membrane was rinsed three times in one per

1a: Case 14 (E440)
Reactive Result

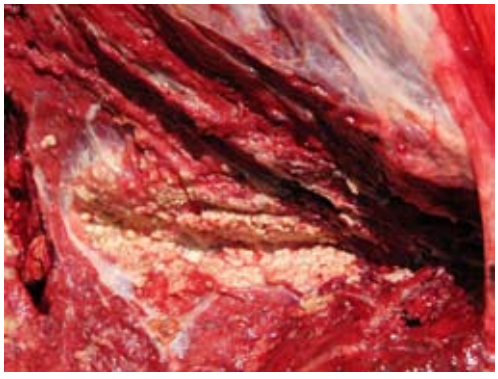
1b: Case 23 (E039)
Reactive Result



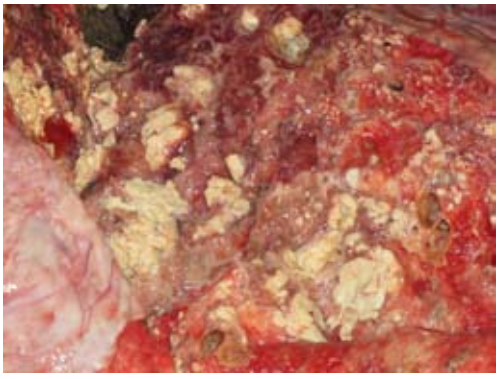
1c: Control 02 (E591)
Non-Reactive Result

1d: Control 12 (E415)
Non-Reactive Result

Plate 1: Representative test results on the DPP® Vet-TB Elephant assay showing reactive and non-reactive results for the two cases and two controls selected for the study.



2a: Case 20 (E019) showing early stage gross lesions



2b: Case 01 (E429) showing late stage gross lesions

Plate 2 : Gross pathology lesions of pulmonary tuberculosis observed during post-mortem examinations of the two cases selected for the study.

cent phosphate buffered saline with tween-20 (PBST-20) and incubated at 37 °C for one hour in sera of the cases and controls diluted in one per cent bovine serum albumin with PBST-20. The membrane was again rinsed as before and incubated in Protein A-Horseradish peroxidase conjugate (Sigma; catalogue no. 18-160), diluted in BSA-PBST, at 37 °C for 30 minutes. It

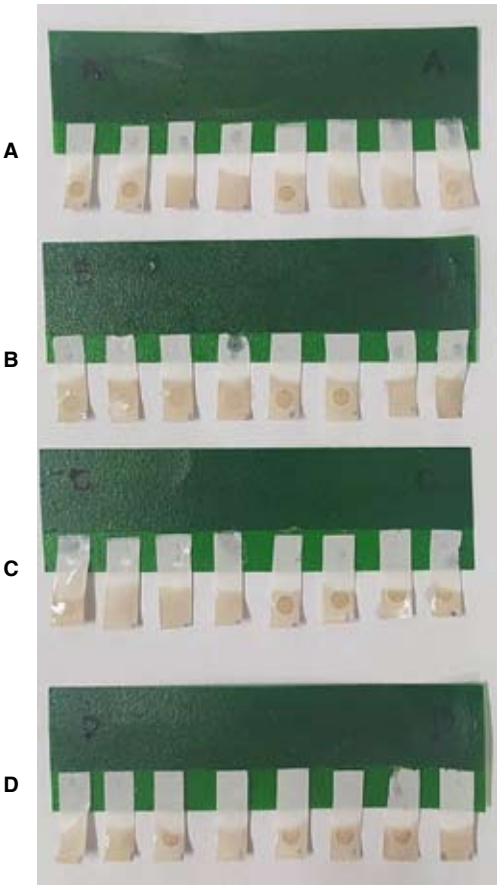


Plate 3: Dot ELISA results of the 30 cases showing 20 dots

(D7-Positive Control & D8-Negative Control)

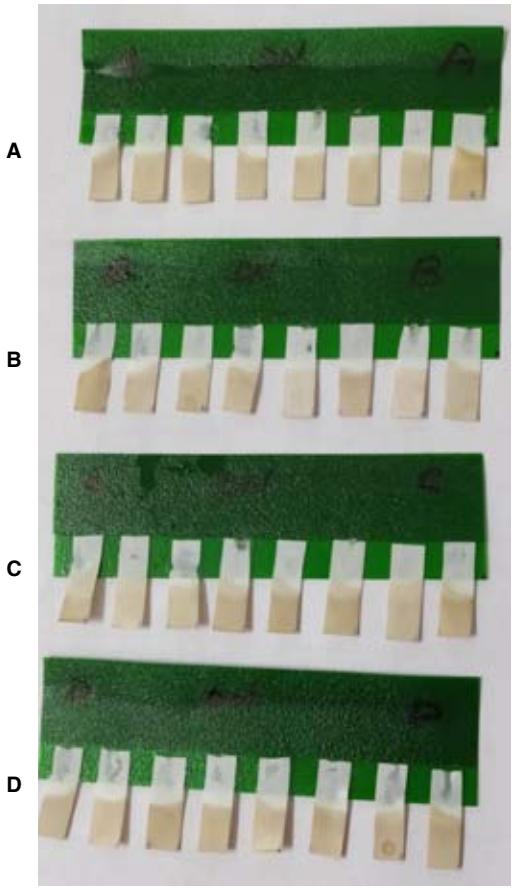


Plate 4: Dot ELISA test results of the 30 controls showing 2 dots

(D7-Positive Control & D8-Negative Control)

was rinsed and immersed in diaminobenzidine tetrahydrochloride chromogen substrate solution (Sigma; catalogue no. 8059S) for 2-3 minutes and the reaction was stopped by rinsing the membrane in phosphate buffered saline.

Among the 30 cases (dead-positive elephants), 20 serum samples (66.67 per cent) produced dots clearly visible with the naked eye, while the remaining 10 samples (33.33 per cent) did not produce visible dots. Among the 30 controls (dead-negative elephants), 28 serum

Table 1: Test results of dot ELISA on the 2x2 table

		Disease status with gold standard	
		Positive	**Negative**
Dot ELISA test result	**Positive**	**a** 20 (True Positives)	**b** 2 (False Positives)
	Negative	**c** 10 (False Negatives)	**d** 28 (True Negatives)

samples (93.33 per cent) did not produce any visible dot, while two samples (6.67 per cent) produced visible dots. Photographs of the dot ELISA results of the cases and controls are shown in Plates 3 and 4. Based on the test results on dot ELISA, a standard 2x2 table was prepared as given in Table 1.

Estimates of diagnostic sensitivity, specificity, positive and negative predictive values, and likelihood ratio for the dot ELISA were calculated as given in Table 2.

Table 2:Epidemiological metrics of the dot ELISA

Parameter	Value
Sensitivity	66.67 %
Specificity	93.33 %
Positive Predictive Value	90.91 %
Negative Predictive Value	73.68 %
Likelihood ratio	10

In this study, the estimates of sensitivity and specificity of the dot ELISA was observed to be lesser than those described for the DPP. In this dot ELISA, only one *M. tuberculosis* antigen, ESAT6-CFP10, was used while several other purified antigens of *M. tuberculosis* and *M. bovis* were used in the DPP. Another reason could be the possible reduction in antibody levels in the banked serum samples, during extended storage. Further optimisation of the dot ELISA can be achieved by using greater number of fresh serum samples from live elephants with known disease status. In addition, standardised combinations of different *Mycobacterium tuberculosis* complex antigens can help to improve the accuracy.

Tuberculosis among captive elephants has been reported in southern India. In a previous study, seroprevalence of tuberculosis among captive Asian elephants in southern India was estimated to be nearly 15 per cent (Abraham *et al.*, 2008). Validation of the dot ELISA method described in this study can offer great help to field veterinarians in the ante-mortem diagnosis of tuberculosis in elephants.

Summary

Preliminary results from this dot ELISA indicate the potential of this method to develop field-based serological tests for tuberculosis screening in elephants. With further optimisation, the dot ELISA using ESAT6-CFP10 antigen has potential for addressing the challenges of ante-mortem tuberculosis screening in elephants.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Abraham, D., Cheeran, J. V., Sukumar, R., Mikota, S. K., Rao, S., Ganguly, S. and Varma, S. 2008. *Health assessment of captive Asian elephants in India with special reference to tuberculosis, A preliminary report submitted to Project Elephant, Ministry of Environment and Forests, Government of India*. Asian Elephant Research and Conservation Centre, A Division of Asian Nature Conservation Foundation, Bangalore, India, 2p.

Ariffin, N., Yusof, N.A., Abdullah, J., Abd Rahman, S.F., Ahmad Raston, N.H., Kusnin, N. and Suraiya, S., 2020. Lateral flow immunoassay for naked eye detection of *Mycobacterium tuberculosis*. *J. Sensors*: 1-10.

Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J.H., Ball, R., Dumonceaux, G., Schmitt, D., Moller, T., Payeur, J.B., Harris, B., Sofranko, D., Waters, W.R. and Lyashchenko, K.P. 2009. Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. *Clin. Vaccine Immunol*. **16**: 605-612.

Ishikawa, S., Ozeki, Y., Suga, S., Mukai, Y., Kobayashi, H., Inouchi, E., Kaboso, S.A., Gebretsadik, G., Dewi, D.N.S.S., Nishiyama, A., Tateishi, Y., Takihara, H., Okuda, S., Yoshida, S., Misawa, N. and Matsumoto, S. 2022. Monitoring IgG against Mycobacterium tuberculosis proteins in an Asian elephant cured of tuberculosis that developed from long-term latency. *Sci. Rep*. **12**: 4310.

Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Mikota, S., Miller, M., Moller, T., Vogelnest, L., Gairhe, K.P., Robbe-Austerman, S., Gai, J. and Waters, W.R. 2012. Field application of serodiagnostics to identify elephants with tuberculosis prior to case confirmation by culture. *Clin. Vaccine. Immunol*. **19**: 1269-1275.

Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Olsen, J.H., Ball, R., Dumonceaux, G., Dunker, F., Buckley, C., Richard, M., Murray, S., Payeur, J.B., Andersen, P., Pollock, J.M., Mikota, S., Miller, M., Sofranko, D. and Waters, W.R. 2006. Tuberculosis in elephants: antibody responses to defined antigens of Mycobacterium tuberculosis, potential for early diagnosis, and monitoring of treatment. *Clin. Vaccine. Immunol*. **13**: 722-732.

Mikota, S.K., Lyashchenko, K.P., Lowenstine, L., Agnew, D. and Maslow, J.N. 2015. Mycobacterial infections in elephants, In: Mukundan, H., Chambers, M.A., Waters, W.R., Larsen, M.H. (ed.) *Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals: The Many Hosts of Mycobacteria*. pp. 259-276.

Payeur, J.B., Jarnagin, J.L., Marquardt, J.G. and Whipple, D.L. 2002. Mycobacterial isolations in captive elephants in the United States. *Ann. N. Y. Acad. Sci*. **969**: 256-258.

Singh, A., Gupta, A.K., Gopinath, K., Sharma, P. and Singh, S. 2017. Evaluation of 5 Novel protein biomarkers for the rapid diagnosis of pulmonary and extra-pulmonary tuberculosis: preliminary results. *Sci. Rep.* **7**: 44121. ■