Journal of Microbiological Methods 134 (2017) 30–34

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Evaluation of an In-house indirect ELISA for detection of antibody against haemorrhagic septicemia in Asian elephants

Pallop Tankaew [a,b], Thawatchai Singh-La [c], Chatchote Titaram [b], Veerasak Punyapornwittaya [c], Preeyanat Vongchan [d], Takuo Sawada [a,e], Nattawooti Sthitmatee [a,*]

[a] Department of Veterinary Bioscience and Veterinary Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Muang, Chiang Mai 50100, Thailand
[b] Center for Excellence in Elephant Research and Education, Faculty of Veterinary Medicine, Chiang Mai University, Muang, Chiang Mai 50100, Thailand
[c] Department of Food Animal Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Muang, Chiang Mai 50100, Thailand
[d] Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand
[e] Laboratory of Veterinary Microbiology, Nippon Veterinary and Life Science University, Musashino, Tokyo 180-8602, Japan

ARTICLE INFO

Article history:
Received 2 November 2016
Received in revised form 28 December 2016
Accepted 10 January 2017
Available online 12 January 2017

Keywords:
Haemorrhagic septicemia
Asian elephant
Indirect ELISA
Bayesian analysis

ABSTRACT

Pasteurella multocida causes haemorrhagic septicemia in livestock and wild animals, including elephants. The disease has been reported in Asian elephants in India and Sri Lanka, but to date there have been no reported cases in Thailand. ELISA or indirect hemagglutination assays (IHA) have been demonstrated to be able to detect the antibody against the disease in cattle, but no data are available for elephants. The present study reports a novel in-house indirect ELISA for antibody detection of haemorrhagic septicemia in Asian elephants, and evaluates the sensitivity and specificity of the method using a Bayesian approach. The characteristics of ELISA and IHA were analyzed using a one population Bayesian model assuming conditional dependence between these two diagnostic tests. The IHA was performed as recommended by the World Organization for Animal Health (OIE) manual for haemorrhagic septicemia. An in-house indirect ELISA was developed with a heat extract antigen of *P. multocida* strain M-1404 (serovar B:2) as a coating antigen and rabbit anti-immunoglobulin G conjugated with horseradish peroxidase (eIgG-HRP). The checkerboard titration method was done using elephant sera immunized with *P. multocida* bacterin and negative sera from colostrum-deprived elephant calves. The concentrations of heat extract antigen (160 μg/ml), sample serum (1:100), and eIgG-HRP (1:1000) were optimal for the assay. The calculated cut-off value was 0.103. Of the elephant sera, 50.59% (43/85) were considered seropositive by ELISA. The sensitivity of the ELISA test was higher than that of the IHA test [median = 86.5%, 95% posterior probability interval (PPI) = 52.5–98.9%] while the specificity was lower (median = 54.1%, PPI = 43.6–64.7%). The median sensitivity and specificity of IHA were 80.5% (PPI = 43.8–98.0%) and 78.4% (PPI = 69.0–87.0%), respectively. These findings suggest that our in-house indirect ELISA can be used as a tool to detect the antibody against haemorrhagic septicemia in Asian elephants.

1. Introduction

Haemorrhagic septicemia is caused by *Pasteurella multocida* serovar B:2 or E:2. The bacteria are pathogenic in cattle, elephants, and horses (Ahmed, 2001; Chandrasekaran, 2002; Singh, 2002). The disease results in high morbidity and high mortality rates, particularly in young animals. Stress factors including immunosuppression, shipping, weaning, inclement weather and viral infections enhance colonization of the respiratory tract with the bacterium (Fowler and Mikota, 2006). The bacterium can be transmitted easily through secretion and excretion. Seroprevalence of haemorrhagic septicemia in Asian elephants has been reported from India and Sri Lanka (Harish et al., 2002; Yadav et al., 2012). However, there are no data concerning seroprevalence in elephants from Thailand or Southeast Asia. The OIE Terrestrial manual for haemorrhagic septicemia (2008), chapter 2.4.12, recommends the hemagglutination inhibition test as a method for monitoring antibodies to the pathogen. This method has been used extensively to monitor antibody response to *P. multocida* (Joseph and Hedger, 1984; Nagy and Penn, 1976). However, the titers measured in that assay are not directly correlated with levels of disease protection (FAO, 1979). The enzyme-linked immunosorbent assay (ELISA) has been used for the determination of specific antibodies in various human and animal diseases. The previous studies employed the heat extract antigen of *P. multocida* strains as a coating antigen for detection of antibody against haemorrhagic septicemia in cattle including elephant by the indirect ELISA method (Afzal et al., 1992; Vitoorakool et al., 1998). Vitoorakool et al. (1998) demonstrated the use of indirect ELISA in order to detect

* Corresponding author.
E-mail address: drneaw@gmail.com (N. Sthitmatee).

http://dx.doi.org/10.1016/j.mimet.2017.01.008
0167-7012/

elephant antibody against haemorrhagic septicemia vaccination in Asian elephants. The previous method employed a polyclonal antibody against protein G conjugated with horseradish peroxidase (HRP) as a secondary antibody, and could be used to identify the elephant antibody post-vaccination (Vitoorakool et al., 1998). Recently, a specific antibody against elephant immunoglobulin G (eIgG) has been produced (Vongchan, 2013). The eIgG possesses the potency and specificity to be applied for immunological studies in elephants.

Normally, characteristics of a diagnostic test are performed by comparing to a gold standard; in the present case such a standard is lacking, and the true prevalence of the disease is unknown. To solve this problem, latent class analysis is being increasingly applied for estimation of characteristics, including a novel diagnostic test in many diseases (Drewe et al., 2009; Rahman et al., 2013).

The objective of present study was to determine and standardize a horseradish peroxidase conjugated rabbit-anti elephant IgG based in-house indirect ELISA method for measuring Asian elephant antibody associated with haemorrhagic septicemia. The sensitivity and specificity of the method were evaluated using a Bayesian approach.

2. Materials and methods

2.1. Elephant sera and animal ethics

Fifty samples of positive elephant sera against haemorrhagic septicemia were kindly provided by the Department of Livestock Development, Ministry of Agriculture and Cooperative and the Center for Excellence in Elephant Research and Education, Chiang Mai University. Thirty colostrum-deprived neonatal elephant sera were used as negative control sera to haemorrhagic septicemia. In addition, 85 samples of elephant sera, obtained from the Center for Excellence in Elephant Research and Education, Chiang Mai University, were also used in this study. These elephants were raised at government or private elephant camps in Chiang Mai or Lampang, Thailand. There had been no haemorrhagic septicemia vaccinations or disease outbreaks within the past 3 years. Elephant sera were tested by indirect hemagglutination assay (IHA; Sawada et al., 1982) in order to be classified sera as positive or negative sera to haemorrhagic septicemia. The IHA was performed by the method as described in the OIE Terrestrial manual for haemorrhagic septicemia, 2008, chapter 2.4.12 and titers at 1:160 or higher by IHA in surviving in-contact animals were considered as suggestive of the disease. The experiments and animal use protocols were approved by the Faculty of Veterinary Medicine, Chiang Mai University Animal Use and Care Committee (License No. S7/2558).

2.2. Preparation of the heat extract antigen

A heat extraction protein of *P. multocida* strain M-1404 (serovar B:2), which was provided by Professor Dr. Takuo Sawada, Laboratory of Veterinary Microbiology, Nippon Veterinary and Life Science University, Tokyo, Japan, was prepared and used as a coating antigen (Afzal et al., 1992). *P. multocida* strain M-1404 was grown in brain heart infusion broth (BHI; Merck, Darmstadt, Germany) for 6 h at 37 °C. Then, 0.1 ml of bacterial suspension was taken and spreaded onto a blood agar plate. Plates were incubated at 37 °C for 18 h. Colonies on plates were harvested with a sterile L-shaped spreader and resuspended with NaCl to a concentration of 0.85% (5 ml/plate). The heat extract antigen was prepared by heating at 100 °C for 1 h followed by centrifugation three times at 10,000 × *g* for 20 min. Pellets were discarded and supernatants were transferred to a new tube. The amount of total protein in the supernatant was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA) and the eluted protein was kept at −20 °C until use.

2.3. Preparation of horseradish peroxidase conjugated rabbit-anti elephant IgG

Rabbit-anti elephant IgG (eIgG) was prepared as described previously (Vongchan, 2013). Then, rabbit sera was conjugated to peroxidase using the EZ-Link™ Activated Peroxidase Antibody Labelling Kit (Pierce) and kept as horseradish peroxidase conjugated rabbit-anti elephant IgG (HRP-anti-eIgG) at −20 °C until use.

2.4. Indirect ELISA

The ELISA plate (Nunc-Immuno Plate MaxiSorp, Intermed, Roskildes, Denmark) was coated with heat extract antigen and incubated by adding elephant serum followed by eIgG-HRP. Immunoplates (Nunc-immuno™ plate, Denmark) were coated with the heat extract antigen in the coating buffer; 0.5 M carbonate buffer (pH 9.6), and then incubated at 4 °C overnight. Plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). Non-specific bindings were inhibited with blocking buffer; 1% skim milk (Difco) in PBS (pH 7.2). Elephant sera or eIgG-HRP were diluted in blocking buffer and added as primary or secondary antibodies. Then, tetramethylbenzidine (TMB; KPL, Gaithersburg, MD, USA) was used as a substrate and the optical density (OD) read at 450 nm using an automatic ELISA plate reader (AccuReader, Metertech, Taipei, Taiwan, R.O.C.).

2.5. Standardization of the indirect ELISA method

A checkerboard titration was conducted to optimize the indirect ELISA reaction (Crowther, 2001). The heat extract antigen were coated to plate with different concentrations; 20, 40, 80, and 160 μg/ml. Serum concentrations were diluted with blocking buffer at different dilutions; 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600. The HRP-anti-eIgG were also diluted with blocking buffer at different dilutions; 1:1000, 1:2000, 1:3000; 1:4000, 1:5000, and 1:6000. The checkerboard titration method was conducted using positive and negative samples as described above.

2.6. Calculation of cut-off value

The cut-off value was obtained by measuring the optical density (OD) at a wavelength of 450 nm. The cut-off OD values were calculated from the mean of the negative control plus 3 standard deviations, as described previously (Crowther, 2001). For the interpretation, any elephant sera that had an OD value greater than the cutoff value were considered as seropositive. Elephant sera with an OD value lower than cutoff value were considered as seronegative.

2.7. Estimation of sensitivity and specificity

A latent class analysis was performed using a Bayesian model for estimation of sensitivity and specificity of the two tests (ELISA test and IHA test) as described elsewhere (Branscum et al., 2005; Gardner et al., 2000). Because both the ELISA test and IHA tests are based on detection of antibody response, their results were considered to be conditionally dependent on each other (Gardner et al., 2000). We thus employed a Bayesian model for two diagnostic tests and one population. The prevalence of the disease was assumed based on expert opinion. Prior information on test performance and prevalence was introduced into the analysis using probability distributions (prior distributions). Sensitivity and specificity prior values for the ELISA and IHA tests were modeled as beta distributions based on expert opinion and information obtained from the study of El-Jakee et al. (2016). As there was a lack of prior information concerning both tests, priors were set conservatively with wide distributions. The characteristics of the tests from this information were selected as the most likely values, while a 95% lower limit for the