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Development and standardization of an in-house indirect ELISA for detection of duck antibody to fowl cholera

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ABSTRACT

Serological tests, such as agglutination and indirect hemagglutination assay (IHA), have been used to identify antibodies against *Pasteurella multocida* in poultry sera, but none are highly sensitive. An enzyme-linked immunosorbent assays (ELISA) has been used with varying degrees of success in attempts to monitor seroconversion in vaccinated poultry, but are not suitable for diagnosis. Commercial ELISA kits are available for chickens and turkeys, but not for ducks. The present study reports development and standardization of an in-house indirect ELISA for detection of duck antibody to fowl cholera. The characteristics of ELISA and IHA were analyzed using a one population Bayesian model assuming conditional dependence between the two diagnostic tests. An in-house indirect ELISA was developed using a heat extract antigen of *P. multocida* strain X-73 as a coating antigen and horseradish peroxidase conjugated goat anti-duck IgG antibody (dIgG-HRP). The checkerboard titration method was done using sera from ducks immunized with *P. multocida* bacterin as positive sera and 1 day old duckling sera as negative sera. The heat extract antigen at 1 μg/ml, sample serum at a dilution of 1:100, and dIgG-HRP 1:2000 were optimal concentrations for the assay. The cut-off value was 0.200. Of the duck sera, 89.05% (244/274) were considered seropositive by ELISA. Estimates for sensitivity and specificity of ELISA were higher than prior values with medians of 94.7% [95% posterior probability interval (PPI) = 89.6–98.2%] and 87.2% (PPI = 68.2–98.3%). Estimates for sensitivity of IHA were lower than prior values (median = 97.6, PPI = 93.2–99.7%) while the specificity was close to the prior value (median = 76.5, PPI = 65.8–85.4%). This finding suggests that an in-house indirect ELISA can be used to detect duck antibody to fowl cholera.

1. Introduction

P. multocida is a gram negative bacterial pathogen responsible for fowl cholera and other diseases of domestic animals. Strains of capsular serogroup A and somatic serotypes 1, 3 and 4 are recognized as the causative agent of fowl cholera (Glisson et al., 2003). The acute form of fowl cholera is associated with high mortality, resulting in a significant economic impact on the poultry industry, while chronic infections and asymptomatic carriers result in the persistence of bacteria within flocks (Glisson et al., 2003). The bacterium can be disseminated within a flock and between houses by secretion and excretion that contaminate the environment (Glisson et al., 2003). Although the history, signs, and symptoms may be used as diagnostic indicators, early detection can reduce morbidity and mortality. However, there are no data concerning seroprevalence in ducks. According to the OIE Terrestrial manual for fowl cholera (2015), chapter 2.3.9, none of the highly sensitive serological tests, such as agglutination or indirect hemagglutination assay (IHA) for detecting antibody against *P. multocida* in serum from avian hosts, are available. ELISA methods have been used to monitor seroconversion in vaccinated poultry, but not for diagnosis. The commercial ELISA kits are available for chickens and turkeys, but not for ducks.

The enzyme-linked immunosorbent assay (ELISA) is a rapid, highly sensitive and specific serological test. There are several different antigens of *P. multocida* that have been prepared for coating ELISA plates, including whole cell antigen, sonicated antigen, potassium thiocyanate extract, lipopolysaccharide, sodium salicylate extract and capsular or heat extract antigen (Afzal et al., 1992). The heat extract antigen was superior to other antigens for preparation and use in ELISA due to its ease of preparation, type specificity, and ability to clearly differentiate

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between protected and unprotected animals (Afzal et al., 1992; Tankaew et al., 2017). The ELISA has been employed to detect the antibody against fowl cholera in previous studies (Marshall et al., 1981; Solano et al., 1983). The ELISA assay was used for investigation of antibody to fowl cholera in avian species, and the results were compared to standard IHA test and microtiter agglutination (MA) test. The sensitivities of those ELISA tests were higher than both IHA and MA tests (Marshall et al., 1981; Solano et al., 1983). This suggested that the ELISA test is a more effective serological test for fowl cholera antibody detection in avian species.

Latent class analysis for diagnostic accuracy studies has been successfully implemented in a Bayesian framework (Kostoulasa et al., 2017). Bayesian latent class analyses are widely used because of their flexibility, the ease of interpretation of their results, and the availability of software for Bayesian data analysis (Lunn et al., 2009). A Bayesian approach is necessary when using non-identifiable latent class analysis, such as the case where characteristics of a diagnostic test that are evaluated by comparison to a gold standard, but unknown factors such as the true prevalence of the disease impair a more classical validation. To solve this problem, a latent class analysis is being increasingly applied for estimation of characteristics, including novel diagnostic tests for many diseases (Drewe et al., 2009; Rahman et al., 2013).

The present study aimed to develop an in-house indirect ELISA method for detection of duck antibody to fowl cholera. The duck antibody titers were also determined via the IHA test as described by Sawada et al. (1982). The characteristics of ELISA and IHA were analyzed using a one population Bayesian model assuming conditional dependence between the two diagnostic tests.

2. Materials and methods

2.1. Duck sera and animal ethics

Two hundred and seventy-four samples of duck sera were collected in order to be classified as positive or negative for fowl cholera by indirect ELISA and IHA test (Sawada et al., 1982). Sera from ducks immunized with *P. multocida* bacterin and 1 day old duckling sera were used as positive and negative sera, respectively. The animal welfare committee of the Faculty of Veterinary Medicine, Chiang Mai University, approved the use of the laboratory animals, in accordance with the laboratory animal ethics guidelines (license number R15/2558). The sera were separated and kept at − 20 °C until use.

2.2. Heat extract antigen preparation

The heat extract protein of *P. multocida* strain X-73 (serovar A:1, ATCC15742) was used as a coating antigen. Briefly, *P. multocida* strain X-73 was grown in brain heart infusion broth (BHI; Merck, Darmstadt, Germany) at 37 °C for 6 h. Then, 0.1 ml of the bacterial suspension was spread onto a blood agar plate and incubated at 37 °C for 18 h. Bacterial colonies on one plate were harvested with a sterile L-shaped spreader and resuspended in 5 ml of phosphate-buffered saline (PBS, pH 7.2) then placed in a boiling water bath for 1 h. After boiling, the suspension was centrifuged and washed three times at 10,000 × *g* for 20 min. The supernatant was collected and concentrated with carboxymethyl-cellulose sodium salt (Sigma-Aldrich). The amount of total protein was measured with the bicinchoninic acid (BCA) method using the BCA protein assay kit (Pierce®, Rockford, IL, USA) following the manufacturer's instructions. Stock antigen preparations were stored at − 20 °C until use.

2.3. ELISA procedure

The immunoplate (Nunc-immuno™, Denmark) was coated with the heat extract antigen in the coating buffer, 0.05 M carbonate buffer (pH 9.6) then incubated at 4 °C overnight. Unbound antigen was discarded, and the wells were washed three times with washing buffer (PBS plus 0.05% Tween: PBST). Non-specific bindings were blocked by adding blocking buffer (1% bovine serum albumin (BSA) and 1% skim milk (Difco) in PBS pH 7.2) to each well and incubating the plate at 37 °C for 1 h. After the blocking buffer was removed, the plates were again washed with PBST. A 1:100 dilution of duck serum was added in triplicate then incubated at 37 °C for 1 h. Horseradish peroxidase (HRP)-conjugated goat anti-duck IgG antibody (KPL, Gaithersburg, MD, USA) was used as the secondary antibody at a 1:2000 dilution, and the plate was then incubated at 37 °C for 1 h. After washing three times, tetramethylbenzidine (TMB; KPL) was added as a substrate. After incubation for 20 min, the reaction was terminated by addition of 2 M sulfuric acid. The optical density at 450 nm (OD_{450}) was measured using an automatic ELISA plate reader (AccuReader, Metertech, Taipei, Taiwan R.O.C.). All samples and controls were run in triplicate, and each experiment was performed at least twice.

2.4. Standardization of the indirect ELISA method

The indirect ELISA conditions were optimized by checkerboard titration using serial dilutions of heat extract antigen tested against serial dilutions of positive and negative duck sera (Crowther, 2001). Briefly, the heat extract antigen was coated to the plate using concentrations of 0.5, 1.0, 2.5, 5.0 and 10.0 μg/ml. Duck serum concentrations were diluted with blocking buffer at dilutions of 1:50, 1:100, 1:200 and 1:400. The Horseradish peroxidase (HRP)-conjugated goat anti-duck IgG antibody was also diluted with blocking buffer at dilutions of 1:500, 1:1000, 1:2000 and 1:5000. The checkerboard titration method was conducted using sera from ducks immunized with fowl cholera bacterin as positive samples and 1 day old duckling sera as negative samples. The optical density values derived from the method were calculated and showed as the signal to noise (S/N) ratio. Signal to Noise Ratio calculation (S/N ratio) = Mean OD of positive sample / Mean OD of negative sample.

2.5. Calculation of cut-off value

The cut-off value was obtained by determining the optical density (OD) at a wavelength of 450 nm (OD_{450}) calculated from the mean negative control plus three standard deviations, as described previously (Crowther, 2001; Tankaew et al., 2017). For the interpretation, any duck sera that had an OD value greater than the cut-off value were classified as seropositive. Duck sera with an OD value lesser than the cut-off value were classified as seronegative.

2.6. Indirect hemagglutination assay (IHA)

The IHA test was performed via the method as described previously (Sawada et al., 1982). Duck sera titer at 1:160 or higher by IHA were considered as suggestive of the disease.

2.7. Sensitivity and specificity estimation

A latent class analysis was performed using a Bayesian model for estimation of sensitivity and specificity of the two tests (ELISA and IHA) as described elsewhere (Gardner et al., 2000; Branscum et al., 2005). Since both the ELISA 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). Thus, a Bayesian model for the two diagnostic tests and one population was implemented. The prevalence of the disease was assumed based on expert opinion. Prior information on test performance and prevalence was introduced in 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 information obtained from previous studies (Bowersock et al., 1992; Takada-Iwao et al., 2007; Dogra et al., 2015;