Original Article

Development of indirect enzyme-linked immunosorbent assay for antibody detection against *Avibacterium paragallinarum*

Panchita Hongprasertkul[1] Wisanu Wanasawaeng[2] Niwat Chansiripornchai[1*]

Abstract

Indirect enzyme-linked immunosorbent assay (I-ELISA) for antibody detection against *A. paragallinarum* serovars A, B and C was developed in separated plates. One hundred Babcock 308 female layer chickens were randomly divided into five groups of 20 each. Groups 1, 2, 3 and 4 were different positive control groups of immunized chickens with commercial trivalent mineral oil vaccine, and prepared bacterins of *Avibacterium paragallinarum* serovars A (221), B (0222) and C (Modesto), respectively. The chickens in Group 5 were assigned as a negative control. Positive and negative control sera from the chickens in Groups 1-5 at 2 weeks after the second vaccination were used to calculate sensitivity and specificity of the newly developed I-ELISA. Forty negative control sera (taken before vaccination) were used to evaluate cut-off values of the I-ELISA against each serovar of *A. paragallinarum* under optimal conditions. The cut-off values of serovars A, B and C, calculated by the mean optical density of all negative sera plus three standard deviations, were 0.334, 0.484 and 0.678, respectively. Efficacy of the developed I-ELISA showed 100% sensitivity for all three serovars of coating antigen, but with low specificity of 30% for all three serovars because of high cross-reactivity among the serovars, while the agreement rate between the I-ELISA and HI assay for serovars A, B and C was around 60%. Nevertheless, the serovar A I-ELISA gave a higher response to serovar A antiserum than to the other two heterologous serovars ($p < 0.05$). In contrast, the I-ELISA results for B and C did not show any significant differences between the homologous and heterologous serovars. This newly developed I-ELISA could be an alternative method for differentiating between *A. paragallinarum*-free chickens and those that have received vaccination, but could not clearly differentiate antibodies among the three serovars of *A. paragallinarum*.

Keywords: antibody, *Avibacterium paragallinarum*, chicken, indirect ELISA, infectious coryza

[1]*Avian Health Research Unit, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand*

[2]*Department of Pathology, Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok 10530, Thailand*

***Correspondence**: cniwat@chula.ac.th*

Introduction

Infectious coryza is an acute respiratory disease which is caused by *Avibacterium paragallinarum*. The disease causes economic loss in the poultry industry worldwide; it leads to 10-40% decrease in egg production in breeder and layer chickens and increases the death rate in broilers (Blackall, 1999). The disease is highly contagious, although typically of a low mortality rate, and infections have continuously been found in chickens in many countries (Abbas et al., 2015; Morales-Erasto et al., 2016). In Thailand, *A. paragallinarum* serovars A, B and C have been found in both commercial and native chickens despite the implementation of proper vaccination programs (Chukiatsiri and Chansiripornchai, 2007; Chukiatsiri et al., 2010). By 2012, 18 isolates that included three serovars (A, B and C) were reported from infected chickens with clinical signs (Chukiatsiri et al., 2012).

Serological monitoring is useful to measure antibody against infectious coryza vaccine and to estimate the vaccine efficacy. The monitoring of chicken's immune response against vaccination can be a potential method for disease control and prevention. In addition, serological test has also been used to investigate the prevalence of disease (Byarugaba et al., 2007). However, serologic diagnosis of infectious coryza has not been conducted because of short incubation period of the disease. At present, the hemagglutination inhibition (HI) test is the best available method for detecting infectious coryza antibody titers (Blackall and Hinz, 2008). However, the HI method for testing infectious coryza involves complicated steps of erythrocyte, antigen and serum preparation. Moreover, the test has a fair degree of ambiguity in the results, which are derived from variations in the individual operator skills and the method assay and sample preparation. An alternative method is the monoclonal antibody-based blocking enzyme-linked immunosorbent assay, or blocking ELISA (Miao et al., 2000; Sun et al., 2007), which is highly specific with an acceptable sensitivity (Zhang et al., 1999). However, this technique is mainly used in research and not in commercial *in situ* based testing. With respect to these limitations, the aims of this study were to develop an indirect ELISA (I-ELISA) assay for antibody detection against *A. paragallinarum* serovars A, B and C in separated plates, particularly estimated immunoresponse against vaccination, and to study the efficacy of the developed I-ELISA with respect to its sensitivity and specificity. Moreover, this study further compared the antibody response against infectious coryza detected by the I-ELISA and HI assay and analyzed agreement between the two methods.

Materials and Methods

Bacterial strains and medium: *A. paragallinarum* serovars A (221) and C (Modesto) were provided by the Bacteriology Section, National Institute of Animal Health, Bangkok, Thailand. *A. paragallinarum* serovar B (0222) was provided by Queensland Primary Industries and Fisheries, Animal Research Institute, Australia. These strains were recovered on blood agar, cross-streaked with *Staphylococcus aureus* supplied V-factor, and then incubated at 37°C with 5% (v/v) CO_2 for 24-48 h. A single colony was picked up and grown on GC agar base supplemented with 2% (w/v) soluble hemoglobin powder and vitox (Oxoid, Cambridge, UK) within a candle jar at 37°C for 24-48 h. The bacterial isolates were confirmed by biochemical and polymerase chain reaction (PCR) tests as described previously (Chen et al., 1996).

Chickens: One hundred female layer chickens (Babcock 308) were obtained from a commercial hatchery (Kerd Charoen, Chachoengsao, Thailand). The chickens were raised in the animal experimental facility, Faculty of Veterinary Science, Nakorn Pathom, Thailand until 12 weeks of age. The guidelines and legislative regulations of Chulalongkorn University, Bangkok, Thailand on the use of animals for scientific purposes were followed as certified in permission no.1431025. Feed and water were provided *ad lib*. At 13 weeks old, all chickens were randomly divided into five groups of 20 each to produce a positive sera control for a commercial trivalent mineral oil vaccine (Coryza Oil-3®, Zoetis, Animal Health, Campinas), prepared bacterins of *A. paragallinarum* serovars A, B and C, and a negative control, respectively. Before vaccination, HI test was performed on all the chickens to confirm their *A. paragallinarum*-free status.

Immunization tests and vaccine preparation: Positive control sera in Group 1 were obtained from the immunized chickens with Coryza Oil-3® that contained *A. paragallinarum* at 10^8 colony forming units (CFU)/ml, while the positive control sera in Groups 2, 3 and 4 were obtained from the chickens immunized with prepared bacterins of *A. paragallinarum* serovars A (221), B (0222) and C (Modesto), respectively. Each strain was grown for 18 h in supplemented test medium broth (TMB; Blackall and Reid, 1982), collected by centrifugation at 1,400 x g, 30 min using a high speed centrifuge (Eppendorf, Germany) and then washed three times in pH 7.2 phosphate-buffered saline (PBS). The bacterial cells were rechecked for contamination and resuspended to a final concentration of 10^9 CFU/ml. The bacterial cells were then inactivated with 0.2% (w/v) formalin for 48 h and prepared with Freud's complete and incomplete adjuvant (antigen: adjuvant (w/w) ratio of 1:1) for the first and second immunizations, respectively. The vaccines were given by 0.5-ml intramuscular breast injection at two weeks apart. All vaccinated birds in each group were separated in each experimental room. The chickens in Group 5 were assigned as the negative control and immunized with PBS. Blood samples were collected at 0, 2, 4, 6 and 8 weeks after the first vaccination. Forty serum samples taken before vaccination (at 13 weeks old) presented no antibody response against *A. paragallinarum* by the HI test and were used to determine cut-off values of the developed ELISA kit.

Hemagglutinin antigen preparation: Antigens for the HI test were prepared as previously described (Sun et al., 2007) with some modifications. Briefly, the bacterial cells of strains 221, 0222 and Modesto were cultured in GC agar base (Oxoid, Hampshire, UK), harvested and washed three times in sterile PBS. Then, the antigens

were treated with potassium thiocyanate-saline solution (0.5 M KSCN, 0.425 M NaCl, pH 6.3) with stirring at 4°C for 2 h. The bacterial suspension was then sonicated for 4 min 40 sec with an ultrasonic cell disruptor (Vibra Cell™, Sonics) for two cycles. The sonicated bacteria were harvested, washed three times in PBS and resuspended in PBS containing 0.01% (v/v) thimerosal. These antigen preparations were kept at 4°C for 4 days before testing.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): The purified bacterial cells of each serovar were analyzed for molecular weight of protein by SDS-PAGE to confirm purity of the antigen protein, using 12% (w/v) acrylamide resolving gel and 4% (w/v) stacking gel (Laemmli, 1970). Protein was loaded at approximately 100 µg per well (10 µl), and resolved by electrophoresis at a constant voltage (120 V) in the Protean II electrophoresis cell (BioRad Laboratories, Richmond, CA) for 50 min. The gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 for 1.5 h and destained with destaining dilution until protein bands appeared.

Establishment and optimization of I-ELISA: Checkerboard titration of the I-ELISA was performed by adjusting the (i) antigen concentration level used for coating the plate, (ii) serum dilution and (iii) conjugate concentration (Goat anti-chicken IgG (H+L) horseradish peroxidase, KPL, USA). The coating antigen of each serovar was two-fold serially diluted from 25 to 0.78 µg/ml. The positive and negative sera were optimized by two-fold dilution from 1:50 to 1:800. The optimal conjugate was chosen from 1:500, 1:1000 and 1:2000 in accordance with the manufacturer's recommendation. All samples were tested in duplicate and measured at a wavelength of 405 nm (OD_{405}). The highest positive/negative ratio (P/N) values were considered optimal.

Validation of the method: The 40 negative control sera were used to evaluate the cut-off values of the developed I-ELISA against each serovar of *A. paragallinarum* under optimal conditions. These cut-off values were calculated following the mean of the total negative OD values plus three standard deviations (SD) as reported (Shen et al., 2015). Results were considered as positive for the serum sample when the OD value was higher than the cut-off value. Repeatability of the I-ELISA against each serovar of *A. paragallinarum* was evaluated by using coated antigens from the same and different batches to test with positive (n = 3) and negative (n = 3) serum samples. Coefficient of variation (CV) was derived from three replicate tests with the intra- and inter-assays. Specificity was determined by antigenic cross-reactivity tests using reference positive control serum (Synbiotics Corporation, USA) against various chicken pathogens, including infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS) and *Pasturella multocida* (PM). In addition, antigenic cross-reactivity was tested by the three different serovars of *A. paragallinarum* (A, B and C). Results from all control serum samples at 2 weeks after the second immunization were used to calculate the sensitivity and specificity of the newly developed I-ELISA. Diagnostic specificity and sensitivity of the I-ELISA method were calculated by qualitative method validation. Results of the I-ELISA were compared with control serum. The sera from Group 1 were defined as positive for all three serovars, while the sera from Groups 2, 3 and 4 were defined as homologous positive to serovars A, B and C, respectively. The sera from Group 5 were defined as negative to all serovars. Moreover, concordance between the I-ELISA and HI assay was considered from the agreement rate, [true matched positive samples + true matched negative samples / total samples] x 100 (Shen et al., 2015).

I-ELISA: Ninety-six-well microplates (SPL Lifescience, Korea) were coated with 100 µl per well of sonicated cells of the respective serovars, A (221), B (0222) or C (Modesto), in separate plates for detection of serum antibodies against each of the *A. paragallinarum* serovars. The antigen concentration was prepared by using the Qubit® Protein Assay Kit (Qubit® 2.0 Fluorometer, Invitrogen, USA) and adjusted to approximately 25 µg protein/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.4 (Sigma, CA), and incubated overnight at 4°C before being washed three times with 300 µl of washing buffer. The plates were incubated with 300 µl blocking solution (KPL, USA) for 1 h to minimize non-specific interactions and then washed three times with washing buffer. After that, 100 µl of each diluted serum sample (1:800 for serovar A, 1:400 for serovars B and C) was transferred into duplicate wells and the plates were incubated for 30 min at room temperature. After washing three times, 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-chicken IgG (1000-fold dilution) in milk diluent solution (KPL, USA) was added and then incubated for 30 min. Following washing, 100 µl of ABTS peroxidase substrate (KPL, USA) was added to each well and incubated for 7 min. Finally, a stop solution (KPL, USA) was added and OD was measured at 405 nm with an ELISA plate reader (Biotek Instrument, USA).

HI test: All samples were tested by the HI method modified from that reported by Chukiatsiri et al. (2009). A sample with an HI titer of 1:5 or higher was interpreted as a positive result, because previous studies have shown that vaccinated chickens with an HI ratio of greater or equal to 1:5 can be protected from *A. paragallinarum* challenge (Sawata et al., 1982). Briefly, the sera were absorbed by 10% (v/v) glutaraldehyde (GA)-fixed chicken red blood cells for 2 h at room temperature. Then, the obtained supernatant at a final dilution of 1:5 was used for the HI test by two-fold serial dilution from 1:5 to 1:5120 (25 µl of each diluted serum). For this, 25 µl of the antigen containing 4 hemagglutinating units was added to each well and the mixture was shaken and incubated at room temperature for 30 min. Finally, 50 µl of 1% (v/v) GA-fixed chicken red blood cells was added and the mixture was shaken and incubated for 30 min before reading the HI titer. The highest dilution that completely inhibited hemagglutination was identified as the HI titer.

Statistical analysis: Serum antibody responses against *A. paragallinarum* detected by the developed I-ELISA for each serovar were compared among the groups by One-Way ANOVA test (SPSS for Windows).

Results

SDS-PAGE analysis: The three serovars of *A. paragallinarum*, A (221), B (0222) and C (Modesto), were cultured and identified by Gram's strain, biochemical and PCR tests. For the PCR results, a 500 bp amplicon was found for all three *A. paragallinarum* serovars. The bacterial suspensions prepared for the I-ELISA were checked for protein purity by SDS-PAGE and the three serovars exhibited a nearly identical protein profile, with three visible groups of bands divided at 60-100 kDa, 40-55 kDa and 25-38 kDa (Fig. 1).

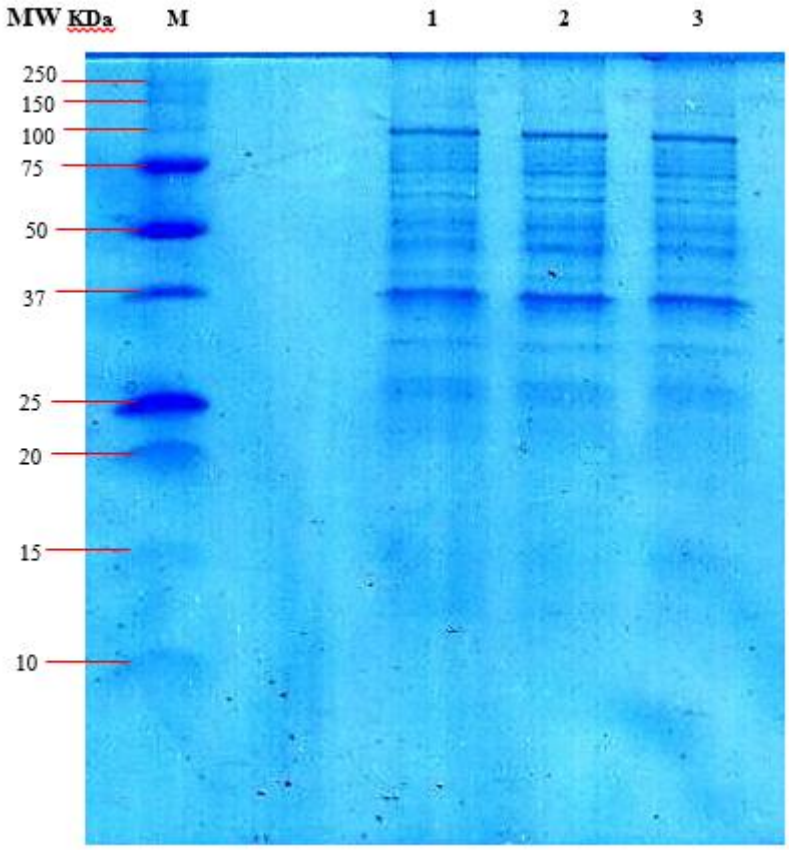


Figure 1 SDS-PAGE analysis of *A. paragallinarum* serovars. Lane 1: Serovar A (221), Lane 2: serovar B (0222), Lane 3: serovar C (Modesto).

Optimization of the developed I-ELISA: The optimal concentration of antigen, goat anti-chicken IgG conjugate and serum were 25 µg/ml, 1:1000 and 1:800, respectively, for serovar A (221) (Tables 1 and 2), and 25 µg/ml, 1:1000 and 1:400, respectively, for serovars B (0222) and C (Modesto) (data not shown). The largest difference in the OD value between the positive and negative serum samples was considered as the optimized condition.

Table 1 The highest P/N values corresponding to optimal antigen serovar A (221) concentration and serum dilution (using 1:100 of conjugate dilution)

Dilution of positive and negative sera	Antigen dilution (µg/ml)					
	25	**12.5**	**6.25**	**3.125**	**1.56**	**0.78**
1:50	1.118	1.119	1.192	1.227	1.400	1.206
1:100	1.584	1.437	1.437	1.469	1.501	1.446
1:200	2.323	1.962	1.741	1.560	1.679	1.445
1:400	2.899*	2.293	1.916	1.605	1.805	1.465
1:800	2.898*	3.667*	2.597	1.786	1.560	1.549

*Optimal values **(**highest P/N ratio**)**

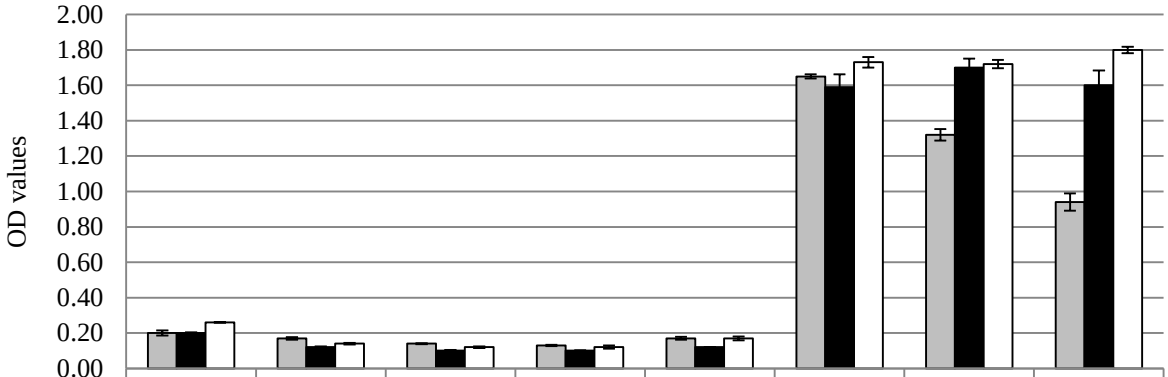
Validation of the I-ELISA method: Forty negative control sera were tested in duplicate for each of the developed I-ELISA coated serovar A, B and C antigens under the determined optimal conditions. The range and mean ± SD OD values were 0.111-0.343 and 0.184 ± 0.050 against serovar A, 0.174-0.423 and 0.274 ± 0.070 against serovar B, and 0.213-0.590 and 0.360 ± 0.106 against serovar C. The cut-off values of serovars A, B

and C, calculated by the mean OD value of the negative sera plus 3SD, were 0.334, 0.484 and 0.678, respectively. The specificity of the developed I-ELISA assay was evaluated by testing the reference positive control sera against IBDV, IBV, PM, MG and MS. The OD values for the IBDV, IBV, PM, MG and MS-positive samples ranged from 0.13 ± 0.004 to 0.20 ± 0.014 for serovar A, 0.10 ± 0.002 to 0.20 ± 0.004 for serovar B and 0.12 ± 0.005 to 0.26 ± 0.002 for serovar C. Thus, all the OD values from the other pathogens were less than the cut-off values for all three serovars, giving a high specificity for *A. paragallinarum* (Fig. 2). On the other hand, the OD values of the *A. paragallinarum*-positive samples tested with the different I-ELISA coated serovar antigens were all higher than the cut-off values (Fig. 2).

Table 2 The highest P/N values corresponding to optimal antigen serovar A (221) concentration and serum dilutions (using various dilution of conjugate in accordance with manufacture's recommendation)

Dilution of positive (P) and negative (N) sera	Antigen dilution (µg/ml)								
	Conjugate 1:500			Conjugate 1:1000			Conjugate 1:2000		
	25	12.5	6.25	25	12.5	6.25	25	12.5	6.25
P (1:400)	2.475	2.371	2.121	1.812	1.423	1.61	1.426	0.925	0.773
N (1:400)	0.293	0.292	0.278	0.188	0.163	0.183	0.137	0.143	0.157
P/N (1:400)	8.447	8.120	7.629	9.638	8.730	8.798	10.409	6.469	4.924
P (1:800)	2.514	2.280	1.893	2.012	1.298	1.169	1.395	0.925	0.596
N (1:800)	0.247	0.207	0.215	0.138	0.145	0.137	0.113	0.117	0.116
P/N (1:800)	10.178	11.014	8.805	14.58*	8.952	8.533	12.350	7.910	5.138

P = average OD values of positive serum samples against *A. paragallinarum* serovar A
N = average OD values of negative serum samples against *A. paragallinarum* serovar A
*Optimal values **(**highest P/N ratio**)**



	MG	MS	PM	IBV	IBD	serovar A	serovar B	serovar C
Plate A	0.20	0.17	0.14	0.13	0.17	1.65	1.32	0.94
Plate B	0.20	0.12	0.10	0.10	0.12	1.59	1.70	1.60
Plate C	0.26	0.14	0.12	0.12	0.17	1.73	1.72	1.80

Figure 2 Specificity of the developed I-ELISA coated *A. paragallinarum* serovar A, B and C antigens was tested with reference positive control serum against IBD, IB, PM, MG and MS, and *A. paragallinarum* serovars A, B and C. The antisera to the other pathogen species' antigens had OD values below the cut-off values, but the antisera to the different *A. paragallinarum* serovar antigens had OD values above the cut-off value.

The repeatability of the I-ELISA kits was assessed by determining the average %CV of the same and different batches of antigens. Most of the CV values were < 10% when calculated from the same and different batches of positive and negative sera (Table 3). There were only two %CV values from different batches of serovars B and C that were slightly higher than 10% (12.9% and 11.5%, respectively). The sensitivity and specificity of the I-ELISA coated with *A. paragallinarum* antigens were 100% and 36.7% for serovar A and 100% and 31.7% for serovars B and C, while the agreement rate between the I-ELISA and HI assay for serovars A, B and C were 59%, 53% and 59%, respectively.

Comparison of detected antibody response between I-ELISA and HI assay: The control sera from the immunized chickens at 2 weeks after the second vaccination had 100% positive rates in the I-ELISA test for all three coated serovars when tested for the homologous serovar, whereas the HI test was only 85% and 70% for serovars A and B, respectively (Table 4). However, antisera to each serovar were positive in the heterologous I-ELISA tests. For the negative control, one sample was positive (5% of positive rates) in both serovars B and C in the I-ELISA test, but was negative in the HI test. In addition, the serovar A I-ELISA test revealed significantly higher OD values against serovar A antiserum than against the other two heterologous serovars ($p < 0.05$). In contrast, the I-

ELISA results for B and C did not show any significant differences between the homologous and heterologous serovars (Table 5).

Detection of antibody response by HI test: The positive rate and HI antibody titers of the immunized sera with the commercial inactivated vaccines and the prepared *A. paragallinarum* bacterins against serovars A, B and C are shown in Fig. 3. The sera of the immunized commercial vaccine in Group 1 showed the highest positive rates and HI titer levels in all three serovars compared to the other groups at all times. At 2 weeks after the first vaccination, the positive sera in Group 1 were first detected (at 90%) in serovar A, while they were first detected in serovars B and C at 2 weeks after the second vaccination. Moreover, the HI test of all sera in Group 1 were positive for all three serovars at 2 weeks after the second vaccination and remained at 100% positive until 6 weeks after the second immunization for serovars A and C. All serum samples in the respectively prepared *A. paragallinarum* serovar bacterin groups were negative in the heterologous serovar for HI test. The sera in the prepared bacterins for serovars A, B and C (Groups 2, 3 and 4, respectively) had the highest average HI titer at 4 weeks after the second vaccination. The positive rates and HI titers of all serovars began to decline at 6 weeks after the second vaccination.

Table 3 Repeatability of I-ELISA coated *A. paragallinarum* serovar A, B and C antigens assessed by determining average CVs of the same and different batches. Positive samples (n = 3) and negative samples (n = 3).

I-ELISA		Range of CVs (n = 6)	Average of CVs
Same Batches	Serovar A	1.57-9.78%	5.45%
	Serovar B	1.11-9.16%	3.67%
	Serovar C	0.14-7.62%	2.85%
Different Batches	Serovar A	1.22-8.69%	4.10%
	Serovar B	3.87-12.92%	6.15%
	Serovar C	1.42-11.52%	6.28%

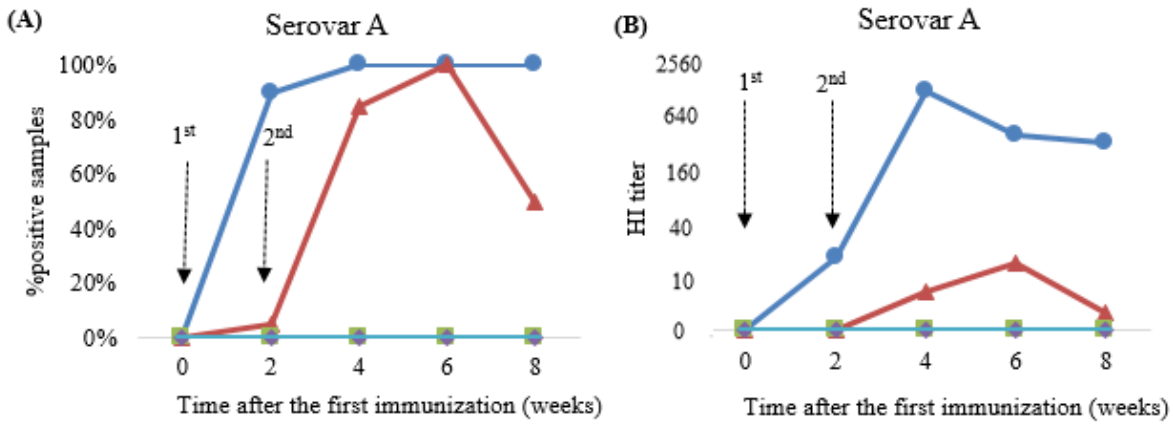
Table 4 Positive rate of samples from *A. paragallinarum* positive and negative serum control groups for I-ELISA and HI test (number of each group = 20 samples)

Method	Serum control group (Serovar)				
	Coryza Oil-3®	A (221)	B (0222)	C (Modesto)	Negative
I-ELISA coated serovar A	100%	100%	100%	90%	0%
HI + serovar A antigen	100%	85%	0%	0%	0%
I-ELISA coated serovar B	100%	100%	100%	100%	5%
HI + serovar B antigen	100%	0%	70%	0%	0%
I-ELISA coated serovar C	100%	100%	100%	100%	5%
HI + serovar C antigen	100%	0%	0%	100%	0%

Table 5 Average OD values (mean ± SD) in homologous and heterologous serovar I-ELISA (number of each group = 20 samples)

Serum control group	Antigen coated on the plate		
	Serovar A	Serovar B	Serovar C
Serovar A (221)	1.193 ± 0.156 ^{a}	1.447 ± 0.158 ^{a}	1.501 ± 0.115 ^{a}
Serovar B (0222)	0.702 ± 0.230 ^{b}	1.615 ± 0.229 ^{ab}	1.566 ± 0.246 ^{a}
Serovar C (Modesto)	0.561 ± 0.204 ^{b}	1.299 ± 0.286 ^{ac}	1.580 ± 0.210 ^{a}
Negative	0.128 ± 0.016 ^{c}	0.289 ± 0.112 ^{d}	0.408 ± 0.153 ^{b}

Data are shown as mean ± SD, derived from independent repeats.
Different superscript letters within the same column indicate significant differences ($p < 0.05$).



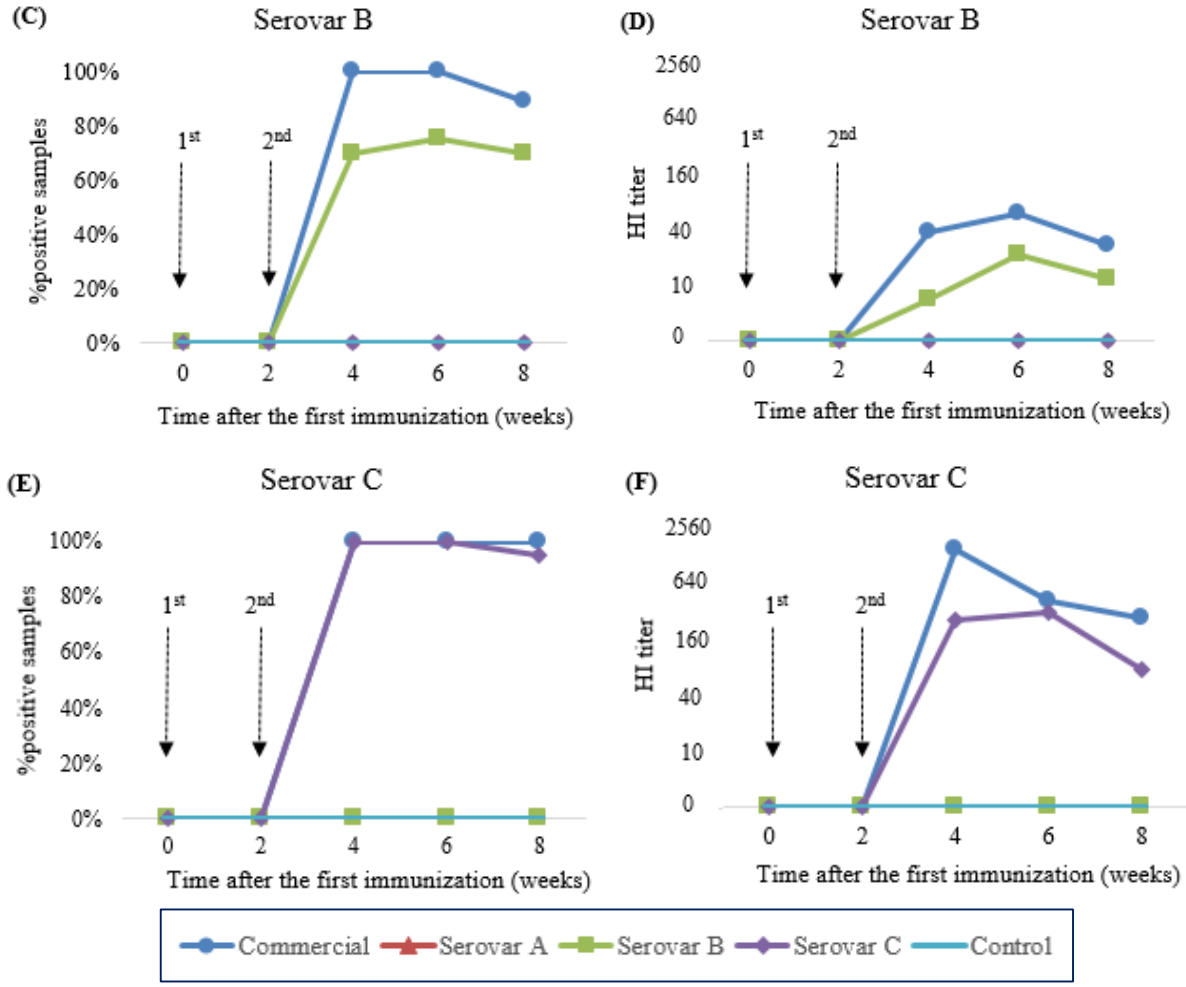


Figure 3 (a, c, e) proportion of positive samples (%) and (b, d, f) HI titer examined with HI test of the *A. paragallinarum* serovars (a, b) A, (c, d) B and (e, f) C. Positive and negative control serum samples were divided into five groups of commercial vaccine 1 (Group 1), prepared bacterin serovar A (Group 2), prepared bacterin serovar B (Group 3), prepared bacterin serovar C (Group 4) and negative control (Group 5).
Remark: 1st = the first immunization, 2nd = the second immunization

Discussion

Serological tests for infectious coryza based upon the HI test and blocking ELISA have been performed for many years (Iritani et al., 1977; Yamaguchi et al., 1988; Zhang et al., 1999). However, these methods still have limitations of being a complicated method with uncertain interpretation and unavailable commercial monoclonal antibodies. The limitations of the HI test and blocking ELISA assay imply that they are not routinely used for antibody detection after vaccination or infection. Therefore, using whole *A. paragallinarum* bacterial cells of each respective serovar to separately coat the I-ELISA plates to detect the antibody response was performed in this study, because I-ELISA is user-friendly, saves time and reduces the required labor due to being compatible with computerized analysis. Although I-ELISA has already been developed with good efficacy using a subunit of the serovar-specific regions of HMTp210, an outer membrane protein (Sakamoto et al., 2012), whole bacterial cells may provide a more comprehensive antigenicity than this subunit. For this study, whole *A. paragallinarum* bacterial cells of each of the three serovars showed a similar protein pattern to each other and to that previously reported (Amal et al., 2012), and so these bacterial antigen preparations were likely to be suitable for I-ELISA plate coating (no contamination).

From the higher cut-off values for serovars B and C, the I-ELISA showed that the reaction with serovars B and C had more variation than with serovar A, which is possibly due to nonspecific immune-reactivity. No antigenic cross-reactivity of the antisera against other chicken pathogens (IBDV, IBV, PM, MG and MS) was detected, whereas cross-reactivity of each antisera to the other two *A. paragallinarum* serovar antigens was seen in all three cases. Thus, these I-ELISA tests are likely to be of use in the detection of antibody response against *A. paragallinarum* but not in the discrimination between serovars. The average CVs of the same and different antigen batches were mostly < 10% and so of a good repeatability. The efficacy of the I-ELISA showed 100% sensitivity in all coating serovars, but the specificity of all serovars of I-ELISA was low (approximately 30%) because of the high cross-reactivity among the three *A. paragallinarum* serovars. A reason for the high cross-reactivity is possibly the similar protein antigens among whole bacterial cells of the three *A. paragallinarum* serovars

which showed a nearly identical protein profile. This is in contrast to previous studies of monoclonal antibody based blocking ELISA that reported serovar specificity (Zhang et al., 1999; Sun et al., 2007). The validation tests indicated that the new I-ELISA was highly sensitive to detect an antibody response against *A. paragallinarum* from immunized chickens by vaccination, compared to *A. paragallinarum*-free chickens. However, the serovar A I-ELISA showed slightly higher specificity (36.7%) than the other two serovars (31.7%), and gave significantly higher OD values against serovar A than against the other two heterologous serovars ($p < 0.05$). It is interesting that the serovar A I-ELISA could distinguish the serovar A antibody response from the other two serovars (B and C). Perhaps the serovar A antigen had a more specific-immunoreactivity than serovars B and C.

Comparison between the I-ELISA method and the HI test in terms of sensitivity and specificity was not performed in this study because the HI test is not the best comparable method since HI negative results do not always equate to no antibody response. For example, chickens inoculated with the ACΔ5-1 fusion peptide had no detectable antibodies in the HI test but were protected from infectious coryza challenge (Sakamoto et al., 2012; Sakamoto et al., 2013). Indeed, protective immunity was not only induced by the hemagglutinin protein (HA), but also by the outer membrane protein (Noro et al., 2007; Noro et al., 2008). These reasons account for the agreement rate for the I-ELISA and HI assay of around 60%. Comparison between the I-ELISA and HI assay from serum samples at 2 weeks after the second immunization with the prepared bacterins revealed that the HI test showed a higher serovar specific antibody response than the whole cell I-ELISA due to genetic differences in the HA gene between serovars A and C (Amal et al., 2012). In addition, other explanations have included (1) post-translational modifications of the HA protein, (2) variations in the expression or sequence of other proteins such as the steric hindrance of HA and (3) multiple HA (Kume et al., 1983).

There have been few studies of the HI antibody response against serovar B. The present study examined the HI titer response pattern against three serovars of *A. paragallinarum*. The sera in Group 1 showed a similar pattern to that reported before (Yamaguchi et al., 1988) in which the serovar A-HI titer was rapidly detected at 2 weeks after the first vaccination. The serovar C-HI titer in Group 1 was detected later than the serovar A-HI titer, in agreement with a previous report (Sun et al., 2007) in which the positive serovar C titer was first detected at 3 weeks post-challenge. The serovar B-HI titer pattern in Group 1 was detected at 2 weeks, peaked at 4 weeks after the second immunization, and then declined, similar to that previously reported (Yamaguchi et al., 1991) in which the HI titer level of serovar B was lower than those of serovars A and C at all times. The HI titers in the prepared bacterins (Groups 2, 3 and 4) showed a serovar specific positive rate of more than 70% and were higher than the 1:5 HI titer at 2 weeks after the second vaccination. From the findings, it can be expected that these prepared bacterins were effective at inducing protection against infectious coryza.

In conclusion, the developed I-ELISAs were unable to clearly differentiate antibody raising among the three serovars of *A. paragallinarum*. However, they could be used to distinguish antibody raising from vaccinated birds compared to *A. paragallinarum*-free healthy chickens. They will be of use in the monitoring of any immune response against infectious coryza vaccine to ensure vaccine efficacy and accuracy of administration. Overall, the development of this I-ELISA assay will be a model for improving ELISA test kit to measure antibody against infectious coryza, aiming at disease control in Thailand. Further studies are required to find the cut-off values for determining the protective antibody response or a specific subunit protein for coating the ELISA plates to eliminate the non-specific effects among different serovars in the whole bacterial cell ELISA.

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References

Abbas G, Khan SH, Hassan M, Mahmood S, Naz S and Gilani SS. 2015. Incidence of poultry diseases in different seasons in Khushab district, Pakistan. J Adv Vet Anim Res. 2(2): 141-145.

Amal M, Soliman YA and Shafey SM. 2012. Molecular characterization of *Avibacterium paragallinarum* strains used in evaluation of coryza vaccine in Egypt. J Am Sci. 8(3): 253-263.

Blackall PJ. 1999. Infectious coryza: overview of the disease and new diagnostic options. Clin Microbiol Rev. 12(4): 627-632.

Blackall PJ and Hinz K-H. 2008. Infectious coryza and related diseases In: Poultry Diseases 6th ed M Pattison, PF McMullin, JM Bradbury and DJ Alexander (eds). Edinburgh: W.B. Saunders. p. 155-159.

Byarugaba DK, Minga UM, Gwakisa PS, Katunguka-Rwakishaya E, Bisgaard M and Olsen JE. 2007. Virulence characterization of *Avibacterium paragallinarum* isolates from Uganda. Avian Pathol. 36(1): 35-42.

Chen X, Miflin JK, Zhang P and Blackall PJ. 1996. Development and application of DNA probes and PCR tests for *Haemophilus paragallinarum*. Avian Dis. 40(21): 398-407.

Chukiatsiri K and Chansiripornchai N. 2007. Case report: An outbreak of infectious coryza in a layer farm. J Thai Vet Med Assoc. 58(3): 98-107.

Chukiatsiri K, Chansiripornchai N and Chotinun S. 2010. An outbreak of *Avibacterium paragallinarum* serovar B in a Thai layer farm. Thai J. Vet. Med. 40(4): 441-444.

Chukiatsiri K, Sasipreeyajan J, Blackall PJ, Yuwatanichsampan S and Chansiripornchai N. 2012. Serovar identification, antimicrobial

sensitivity, and virulence of *Avibacterium paragallinarum* isolated from chickens in Thailand. Avian Dis. 56(2): 359-364.

Chukiatsiri K, Sasipreeyajan J, Neramitmansuk W and Chansiripornchai N. 2009. Efficacy of autogenous killed vaccine of *Avibacterium paragallinarum*. Avian Dis. 53(3): 382-386.

Iritani Y, Sugimori G and Katagiri K. 1977. Serologic response to *Haemophilus gallinarum* in artificially infected and vaccinated chickens. Avian Dis. 21(1): 1-8.

Kume K, Sawata A and Nakai T. 1983. Serologic and immunologic studies on three types of hemagglutinin of *Haemophilus paragallinarum* serotype 1 organisms. Jap J Vet Sci. 45(6): 783-792.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. nature. 227(5259): 680-685.

Miao D, Zhang P, Gong Y, Yamaguchi T, Iritani Y and Blackall PJ. 2000. The development and application of a blocking ELISA kit for the diagnosis of infectious coryza. Avian Pathol. 29(3): 219-225.

Morales-Erasto V, Falconi-Agapito F, Luna-Galaz GA, Saravia LE, Montalvan-Avalos A, Soriano-Vargas EE and Fernandez-Diaz M. 2016. Coinfection of *Avibacterium paragallinarum* and *Ornithobacterium rhinotracheale* in Chickens from Peru. Avian Dis. 60(1): 75-78.

Noro T, Oishi E, Kaneshige T, Yaguchi K, Amimoto K and Shimizu M. 2008. Identification and characterization of haemagglutinin epitopes of *Avibacterium paragallinarum* serovar C. Vet Microbiol. 131(3): 406-413.

Noro T, Yaguchi K, Amimoto K and Oishi E. 2007. Identification and expression of a gene encoding an epitope that induces hemagglutination inhibition antibody to *Avibacterium paragallinarum* serovar A. Avian Dis. 51(1): 84-89.

Sakamoto R, Baba S, Ushijima T, Kino Y, Honda T, Mizokami H and Sakaguchi M. 2013. Development of a recombinant vaccine against infectious coryza in chickens. Res Vet Sci. 94(3): 504-509.

Sakamoto R, Sakai AT, Ushijima T, Imamura T, Kino Y, Honda T and Sakaguchi M. 2012. Development of an enzyme-linked immunosorbent assay for the measurement of antibodies against infectious coryza vaccine. Avian Dis. 56(1): 65-72.

Sawata A, Kume K and Nakase Y. 1982. Hemagglutinin of *Haemophilus paragallinarum* serotype 2 organisms: occurrence and immunologic properties of hemagglutinin. Am J Vet Res. 43(7): 1311-1314.

Shen Y, Cheng A, Wang M, Chen S, Jia R, Zhu D, Liu M, Sun K, Yang Q and Chen X. 2015. Development of an indirect ELISA method based on the VP3 protein of duck hepatitis A virus type 1 (DHAV-1) for dual detection of DHAV-1 and DHAV-3 antibodies. J Virol Methods. 225: 30-34.

Sun H, Miao D, Zhang P, Gong Y and Blackall PJ. 2007. A comparison of a blocking ELISA and a haemagglutination inhibition assay for the detection of antibodies to *Avibacterium* (*Haemophilus*) *paragallinarum* in sera from artificially infected chickens. Biologicals. 35(4): 317-320.

Yamaguchi T, Blackall PJ, Takigami S, Iritani Y and Hayashi Y. 1991. Immunogenicity of *Haemophilus paragallinarum* Serovar B Strains. Avian Dis. 35(4): 965-968.

Yamaguchi T, Iritani Y and Hayashi Y. 1988. Serological response of chickens either vaccinated or artificially infected with *Haemophilus paragallinarum*. Avian Dis. 32(2): 308-312.

Zhang P, Blackall PJ, Yamaguchi T and Iritani Y. 1999. A monoclonal antibody-blocking enzyme-linked immunosorbent assay for the detection of serovar-specific antibodies to *Haemophilus paragallinarum*. Avian Dis. 43(1): 75-82.

บทคัดย่อ

การพัฒนาชุดทดสอบอีไลซ่าในการตรวจสอบแอนติบอดีต่อเชื้อ เอวิแบคทีเรียม พารากัลลินารุม

พัณณ์ชิตา หงษ์ประเสริฐกุล[1] วิษณุ วรรณแสวง[2] นิวัตร จันทร์ศิริพรชัย[1*]

การศึกษานี้ได้พัฒนาชุดทดสอบอีไลซ่าชนิดอินไดเร็ก (indirect ELISA, I-ELISA) สำหรับการตรวจวัดแอนติบอดีต่อเชื้อ *A. paragallinarum* ซีโรวาร์ A, B และ C ซึ่งแยกเพลททดสอบสำหรับแต่ละซีโรวาร์ แบ่งกลุ่มไก่ไข่เพศเมียสายพันธุ์ Babcock 308 จำนวน 100 ตัว แบบสุ่มออกเป็น 5 กลุ่ม ๆ ละ 20 ตัว ดังนี้ กลุ่มที่ 1 เป็นกลุ่มควบคุมบวกที่ได้รับวัคซีนเชื้อตายเชิงการค้าชนิดรวม 3 ซีโรวาร์ กลุ่มที่ 2, 3 และ 4 เป็นกลุ่มควบคุมบวกที่ได้รับวัคซีนเชื้อตายที่เตรียมเองจากเชื้อซีโรวาร์ A, B และ C ตามลำดับ ส่วนกลุ่ม 5 เป็นกลุ่มควบคุมลบ การคำนวณค่าความไวรับและความจำเพาะของชุดทดสอบ I-ELISA ที่พัฒนาขึ้นใหม่พิจารณาจากตัวอย่างซีรัมควบคุมผลบวกและซีรัมควบคุมผลลบที่ได้จากไก่กลุ่มควบคุมที่ 1-5 โดยเก็บตัวอย่างที่ 2 สัปดาห์หลังการทำวัคซีนครั้งที่สอง วิเคราะห์ค่า cut-off ของชุดทดสอบ I-ELISA ของแต่ละซีโรวาร์จากตัวอย่างซีรัมควบคุมผลลบที่เก็บก่อนการทำวัคซีนจำนวน 40 ตัวอย่าง ภายใต้สภาวะที่เหมาะสมค่า cut-off ของซีโรวาร์ A, B และ C ซึ่งคำนวณจากค่าเฉลี่ยการดูดกลืนแสง (OD) ของตัวอย่างซีรัมควบคุมผลลบทั้งหมดบวกสามเท่าของค่าเบี่ยงเบนมาตรฐาน เท่ากับ 0.334, 0.484 และ 0.678 ตามลำดับ ชุดทดสอบ I-ELISA ที่พัฒนาขึ้นทั้ง 3 ซีโรวาร์มีประสิทธิภาพในด้านความไวรับเท่ากับ 100% แต่มีความจำเพาะต่ำ คือ เท่ากับ 30% เนื่องจากมีการตอบสนองข้ามซีโรวาร์ ในขณะที่ความสอดคล้องระหว่างชุดทดสอบ I-ELISA และ HI อยู่ที่ประมาณ 60% อย่างไรก็ตาม ชุดทดสอบ I-ELISA สำหรับซีโรวาร์ A มีการตอบสนองต่อแอนติบอดีของตัวอย่างซีรัมควบคุมผลบวกซีโรวาร์ A มากกว่าอีก 2 ซีโรวาร์อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ในทางตรงข้าม ชุดทดสอบ I-ELISA สำหรับซีโรวาร์ B และ C ไม่มีความแตกต่างกันในด้านการตอบสนองต่อแอนติบอดีของตัวอย่างซีรัมควบคุมผลบวกซีโรวาร์ A, B และ C ผลการทดลองนี้แสดงให้เห็นว่า ชุดทดสอบ I-ELISA ที่พัฒนาขึ้นใหม่สามารถเป็นวิธีทางเลือกของการทดสอบทางซีรัมวิทยาเพื่อใช้แยกความแตกต่างระหว่างฝูงไก่สุขภาพดีที่ไม่เคยสัมผัสเชื้อ *A. paragallinarum* และฝูงไก่ที่เคยได้รับวัคซีน แต่ไม่สามารถแยกความแตกต่างของแอนติบอดีของซีโรวาร์ทั้งสามของ *A. paragallinarum*

คำสำคัญ: แอนติบอดี *เอวิแบคทีเรียม พารากัลลินารุม* ไก่ ชุดทดสอบอีไลซ่าชนิดอินไดเร็ก หวัดหน้าบวม

[1]หน่วยปฏิบัติการวิจัยสุขภาพสัตว์ปีก ภาควิชาอายุรศาสตร์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพมหานคร 10300

[2]ภาควิชาพยาธิวิทยา คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเทคโนโลยีมหานคร กรุงเทพมหานคร 10530

****ผู้รับผิดชอบบทความ** E-mail: cniwat@chula.ac.th*