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ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF ANTIBODY TO *HAEMOPHILUS PARAGALLINARUM*

M.Z. Zaini[1], M. Eguchi[2] and L.J. Tan[1]

[1]*ASEAN Poultry Disease Research and Training Center, Veterinary Research Institute, 31400 Ipoh, Perak, Malaysia.*
[2]*National Institute of Animal Health, Tsukuba, Ibaraki 305, Japan.*

SUMMARY

An enzyme-linked immunosorbent assay (ELISA) was developed and tested for its ability to detect antibody to *Haemophilus paragallinarum* infection in chickens. Six types of antigens were prepared and compared for the most suitable antigen on the criteria of specificity and sensitivity. Antigen, serum and enzyme conjugate concentration were standardised, and reproducibility was determined. The assay was then used to detect *H. paragallinarum* antibodies in experimentally infected chickens. Serum samples collected at 0, 12, 19, 25, 32 and 39 days post-infection were analysed by serum plate agglutination (SPA) test, haemagglutination-inhibition (HI) test and ELISA.

Results showed that *H. paragallinarum* antigen treated with Triton X-100 as antigen for ELISA was most specific, sensitive to homologous antisera, and the assay was reproducible. In experimentally infected specific-pathogen-free (SPF) chickens, the SPA detected antibody earlier than ELISA and HI test. Serum sample from 87.5% of chickens showing clinical signs of infectious coryza through natural infection with *H. paragallinarum* reacted in the ELISA, while 25% of chickens with unknown history reacted.

Keywords: ELISA, *Haemophilus paragallinarum*

INTRODUCTION

This present study was conducted to investigate the possibility of the various types of antigens used for the development of the ELISA and to test the applicability of ELISA for the detection of *H. paragallinarum* infection in chickens. Some modifications of the procedure by Yamamoto *et al.* (1992) for avian mycoplasmas were carried out to increase specificity and reduce the non-specific binding of chicken immunoglobulins. Antibody responses in a group of experimentally infected SPF chickens were also studied to provide comparative data on the sensitivity of the ELISA, HI and SPA tests.

MATERIALS AND METHODS

Preparation of ELISA coating antigen

Haemophilus paragallinarum strain 221 (Page's Type A) obtained from Dr. C. Kuniyasu, National Institute of Animal Health, Japan (designated as K), was grown in trypticase soy broth (BBL) containing 2% v/v of 25% fresh yeast extract. Following an 18-h incubation at 37°C, the organism was harvested by centrifugation at 4,000 *g* for 20 min at 4°C and washed three times with phosphate buffer saline (PBS), pH 7.0 Approximately 1 g (wet wt.) of the organism was suspended in PBS to two times the wet weight of the sediment. The concentration of the protein was measured by Lowry's method (Lowry *et al.*, 1957) with bovine serum albumin (BSA) as standard protein.

Six types of antigens were prepared from the suspension as follows:-

Phenol-water extract antigen

One gram (wet wt.) of the organism was suspended in distilled water to 20 times its original weight. To this suspension, an equal volume of 90% phenol was added and mixed. The mixture was heated at 65°C for 20 min on a magnetic stirrer. Following centrifugation at 2,000 *g* for 30 min at 4°C, the supernatant was collected. Phenol extraction was repeated once with the organism precipitated. The aqueous phase was collected, pooled before being centrifuged and dialysed against distilled water at 4°C overnight.

Heat-stable antigen

One gram (wet wt.) of the organism was suspended in PBS to make up to 250 mg/mL. The suspension was heated in a boiling water bath for 1 h prior to centrifugation at 10,000 *g* for 30 min at 4°C. The supernatant was collected.

Sonicated antigen

Cell suspension was disrupted for 1 h at 50% duty cycle using pulse sonicator (Sonifier 450, Branson, USA). Undisrupted cells and cellular debris were removed by centrifugation at 40,000 *g* for 30 min at 4°C.

Sodium dodecyl sulfate (SDS) antigen

To 9.09 mL of washed bacterial suspension (containing 1 mg/mL of protein) was added 0.91 mL of 1% SDS, giving a weight ratio of SDS/protein 1. The suspension was incubated at 37°C, for 90 min with continuous shaking and centrifuged at 2,000 *g* for 30 min at 10°C. The supernatant was used as antigen for ELISA.

Tween-20 antigen

The washed bacterial suspension was centrifuged and suspended in the initial volume of 0.025M Tris-buffer, pH 7.5 containing 1.45% of NaCl. An equal volume of 2% Tween-20 was added to the suspension. Following incubation for 30 min at 37°C, with continuous stirring, the mixture was centrifuged at 20,000 *g* for 30 min at 10°C.

Triton X-100 antigen

An equal volume of 1% Triton X-100 prepared in PBS was added to the washed bacterial suspension and incubated at 37°C for 90 min with continuous shaking. The mixture was centrifuged at 20,000 *g* for 30 min at 10°C and the supernatant was filtered through a 0.220 nm membrane filter (Whatman, England). Finally, the filtrate was dialysed against PBS with 0.2% sodium azide at 4°C for four days and at room temperature for three days.

The same procedures were repeated for *H. paragallinarum* (also Type A, designated as I) but provided by Dr. Y. Iritani of Shionogi and Co. Ltd., Japan. All antigens were stored at -80°C.

Source of sera

One hundred serum samples were collected from a group of SPF chickens at the ages of eight (44 sera) and 12 weeks (56 sera). Paired chicken sera were collected from 14 SPF chickens and 2 commercial chickens which were experimentally infected with *H. paragallinarum* strain 221 (I). The sera were collected prior to infection and at one week post-infection. Paired sera were also collected from SPF chickens vaccinated with a Japanese commercial vaccine prior to vaccination, and at three weeks post-vaccination. Eight serum samples were collected from chickens with clinical signs consistent with *H. paragallinarum* while 48 serum samples were collected randomly from commercial chickens with unknown disease history. Three antisera against *Leucocytozoon caulleryi*, were used as negative controls while three antisera against *Mycoplasma* against *M.synoviae*, by the courtesy of Dr. K. Yamamoto of National Institute of Animal Health, Japan were used as control sera.

Production of positive and negative reference sera

Hyperimmune sera *to H. paragallinarum* were produced and used to standardise the test. *Haemophilus paragallinarum* strain 221 (I) was cultured in chicken meat infusion broth for 18 h at 37°C. The organism was harvested by centrifugation at 8,000 rpm (rotor RPR 20-4, HIMAC, Japan) for 30 min at 4°C, and washed once with sterile PBS containing 0.01% methiolate at 12,000 rpm for 30 min at 4°C. Subsequently, the sediment was resuspended in the same buffer at turbidity No. 3 of a MacFarland nephelometer and dispensed into small volumes and kept at -20°C until use. This cell suspension served as antigen for immunisation.

Minimal-disease-free chickens were injected with the antigen once a week. Before exposure, the birds were bled and tested for antibodies to *H. paragallinarum* by the HI test (Yamaguchi *et al.*, 1989) to ensure that there had been no previous exposure to the organism. The volumes of the first to fourth injections were 0.3 mL, the fifth and the sixth were 0.4 mL while the seventh was 0.5 mL. All inoculations were administered intravenously. The chicken sera were collected when they had reached a high HI titre and then dispensed into small volumes and kept at -20°C. The hyperimmune sera served as positive control sera for the development of the test and as controls during testing of experimentally infected birds.

Negative sera were obtained from SPF birds approximately six weeks of age. The chickens were bled, and the serum samples were pooled for use as negative reference sera for the development of ELISA and as control sera for testing of experimentally infected birds. All sera were tested by HI test for *H. paragallinarum* to confirm whether they were free *from H. paragallinarum* infection. The sera were dispensed into vials and stored at -80°C.

ELISA procedure

The technique described by Yamamoto *et al.* (1992) for the ELISA was followed with some modifications. Linbro (Flow Laboratories, USA) flat bottomed polystyrene microplates were coated with five-fold dilutions of the various antigens prepared earlier, in 100 µL of carbonate-bicarbonate buffer, pH 9.6 and incubated overnight at 4°C. The plates were washed three times with washing buffer TW-TBS (Tris buffered saline, TBS with 0.05% Tween 20), blocked with 150 µL of SM-BSA-TW-TBS (TW-TBS containing 0.25% of skim milk and 1% of bovine serum albumin, BSA). Following a 30 min incubation (with shaking) at room temperature, the plates were rinsed with distilled water before being stored at -20°C.

Five-fold dilutions of positive and negative

SM-BSA-TW-TBS and 100 μL of the respective dilutions were dispensed into each well. The plates were incubated as mentioned earlier, and washed three times. The wells were then filled with 100 μL of 1:10,000 dilution of horse-radish peroxidase conjugated anti-chicken IgG (ICN Immuno Biological). Following the conjugate incubation step, the plates were washed three times and 100 μL/well of freshly prepared substrate solution (30% H_2O_2, 100 μL; O-phenylene diamine dihydrochloride, OPD, 100 mg; citrate buffer pH 5.0, 100 mL) was added. The reaction was stopped by the addition of 50 μL of 3M H_2SO_4 following incubation at room temperature for 15 min. Absorbance or optical density(OD) was measured at 492 nm with a Titertek Multiskan (Flow Laboratories).

Investigation of non-specific reactions on negative sera

A number of treatments have been reported to reduce the non-specific binding with some negative sera investigated. In an attempt to reduce cross-reactions, different stages of the ELISA procedure therefore, were altered.

The effects of the composition of the serum diluting and conjugate diluting buffers were studied. Serum diluting buffer consisting of 3% and 5% (w/v) of BSA, gelatin and skim milk, 0.5% (protein w/v) crude chicken serum albumin and crude egg albumin were all prepared in TW-TBS, with TW-TBS as controls.

Conjugate diluting buffers, TW-TBS containing 1% and 6% BSA, gelatin and skim milk; 1% (protein w/v) crude chicken serum albumin and crude chicken egg albumin; 0.5% (w/v) skim milk with 0.2% (w/v) BSA were compared with TW-TBS.

To block any binding sites still present after the plates had been coated with the antigen, the wells were blocked with TW-BSA containing 1% or 3% (w/v) BSA; 0.25%, 0.5%, 1% or 3% purified chicken egg albumin and the absorbances were compared.

Preparation of crude chicken serum albumin and crude egg albumin

Anhydrous sodium sulphate (3.6 g) was added to 20 mL of SPF chicken serum and mixed. The mixture was left to stand for 30 min at room temperature prior to centrifugation at 6,000 rpm for 30 min at 4°C. The supernatant was dialysed for 3 days against PBS at room temperature and kept at -80°C until it was used. The crude egg albumin was prepared by separating the egg white (of a SPF chicken) from the yolk and purified by dialysing for 4 days; 1 day with normal saline and 3 days with PBS.

Storage of antigen coated plates

Freshly prepared antigen coated plates were rinsed with distilled water, placed in sealed plastic bags and stored at -20°C. At monthly intervals, a new plate was coated and kept as before. Following seven months of storage, the plates were taken out and ELISA was

Experimental inoculations of chickens

Six-week-old (n=30) SPF chickens were artificially infected with 0.1 mL of 10^8 cfu/mL of *H. paragallinarum* strain 221 I into the infraorbital sinus. They were bled on days 0, 12, 19, 25, 32 and 39 and the sera collected were tested by ELISA, HI and SPA tests for detection of antibody against *H. paragallinarum*.

RESULTS

Determination of suitable antigen

Seven antigens were compared by carrying out ELISA checkerboard titration test using serial diluted positive and negative control serum. Suitable antigen for ELISA was chosen in accordance with the following criteria: (1) The OD_{492nm} values of negative control serum were zero or as low as possible. (2) The OD_{492nm} values of negative control serum did not change accordingly to the change of concentration of antigen. (3) The ratio between OD_{492nm} of positive control serum and one of negative control serum (P/N ratio) is the biggest.

The results of the OD values of negative control sera showed high non-specific reaction when the SDS antigen and sonicated antigen were used as coating antigen, suggesting that the SDS antigen and the sonicated antigen were not suitable because of their high non-specific reactions. On the other hand, the results with negative and positive control sera showed that heat-stable antigen, Tween-20 antigen and phenol-water extract antigen showed almost no colour development with positive serum after the addition of substrate. These results suggested that the three antigens had little or no antigenic activities and they were not suitable as antigen for ELISA.

Comparing the ELISA absorbances among four antigens (SDS antigen, sonicated antigen, Triton X-100 antigen and whole cell antigen) using the same protein concentration (0.1 μg/mL to 12.5 μg/mL) to coat the plates, the P/N ratio is presented as in Table 1.

From the experiments carried out, Triton X-100 antigen was the most suitable among the seven antigens examined for the detection of antibodies by ELISA. Figure 1 shows the dose response curve at different concentrations of Triton X-100 antigen.

Investigation of non-specific reactions of negative sera

Incorporation of 3% and 5% (w/v) BSA, gelatin and skim milk, 0.5% (protein w/v) crude chicken serum and egg albumin in TW-TBS diluent, failed to reduce the OD value of negative sera. However, when 3% BSA in TW-TBS was used as serum and conjugate diluent, the level of non-specific absorbance of a cross-reacting serum was significantly reduced without compromising specific reactivity (Figure 2 and 3).

Table 1. Positive/Negative[a] ratio of various antigens

Antigen	Serum dilution	Antigen concentration (mg/mL)			
		0.1	0.5	2.5	12.5
Whole cell	1/625	*	30	52	85
	1/125	*	53	38	62
	1/25	38	33	48	32
Sonicated	1/625	*	*	44	71
	1/125	38	42	79	86
	1/25	16	19	23	29
SDS	1/625	*	54	35	25
	1/125	16	105	59	63
	1/25	16	36	26	110
Triton X-100	1/625	*	*	74	79
	1/125	*	48	239	*
	1/25	5	20	110	322

a: P/N ratio = $\frac{\text{OD at 492nm of positive control serum}}{\text{OD at 492nm of negative control serum}}$

b: Concentration of antigen shown as protein concentration

*: At OD_{492nm} , the value of negative control is 0.00

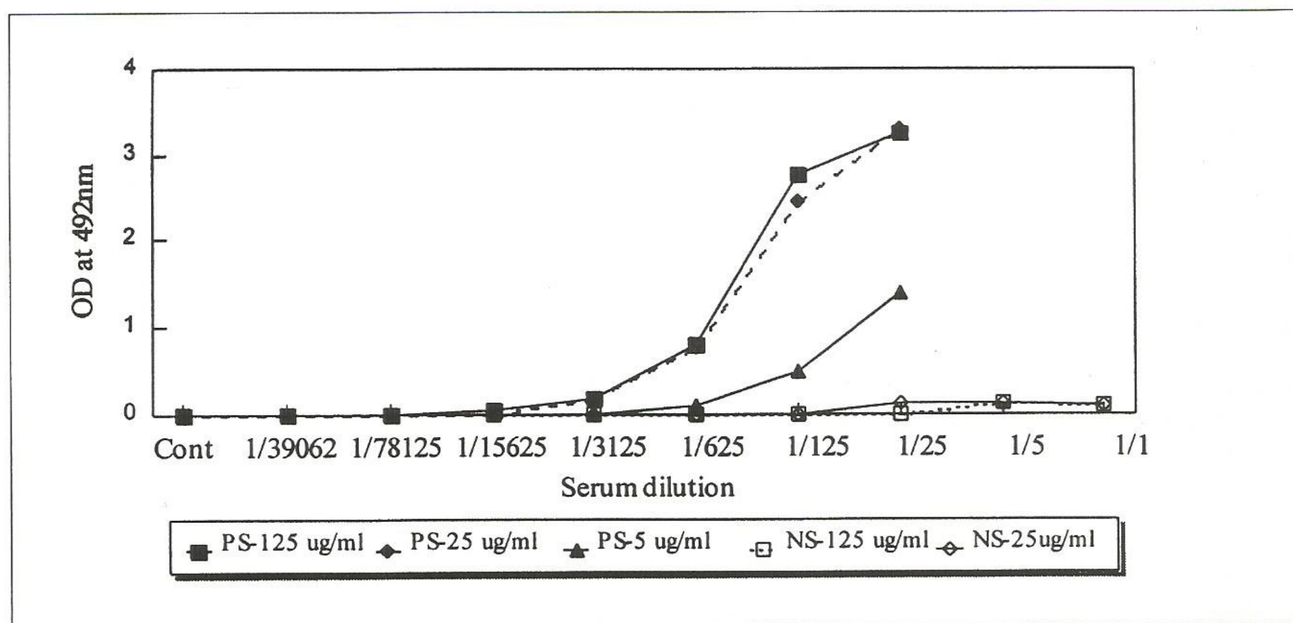


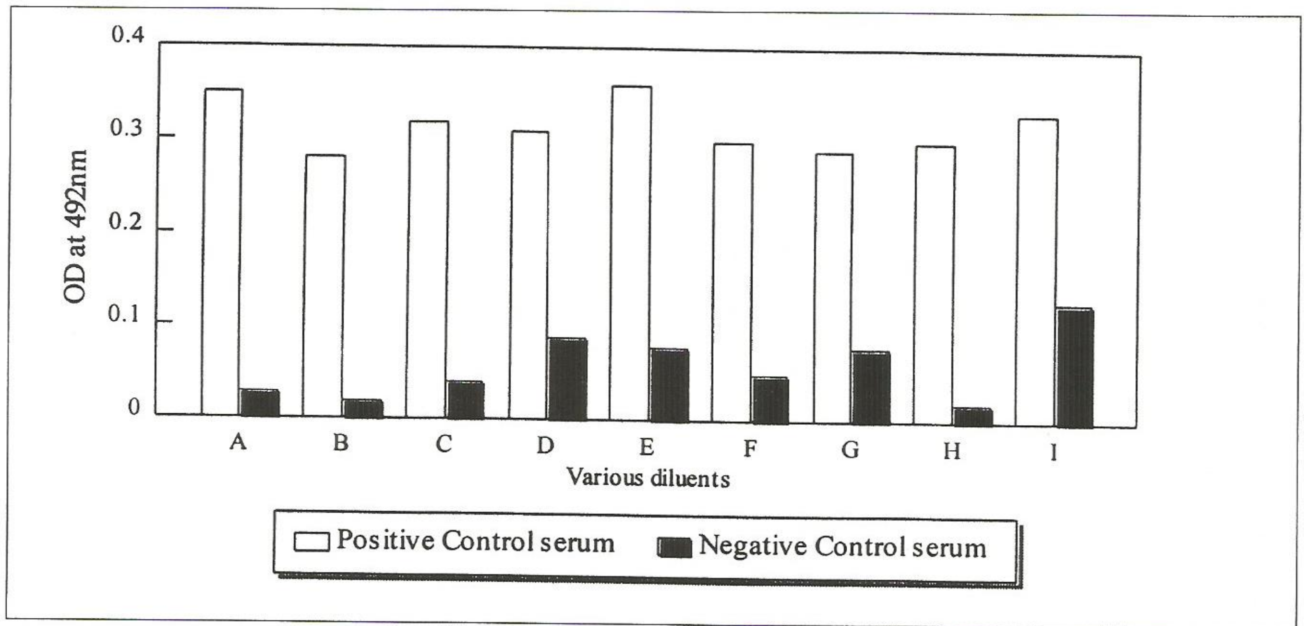
Figure 1. Dose response of Triton X-10 positive (PS) and negative (NS) control antisera

Determination of most suitable washing buffer

A higher background was seen when plates were washed with PBS and TBS than with TW-PBS or TW-TBS. The use of TW-TBS for washing improved the absorbance of homologous sera, due to an increase in the positive reaction rather than a reduction on non-specific reaction (Table 2).

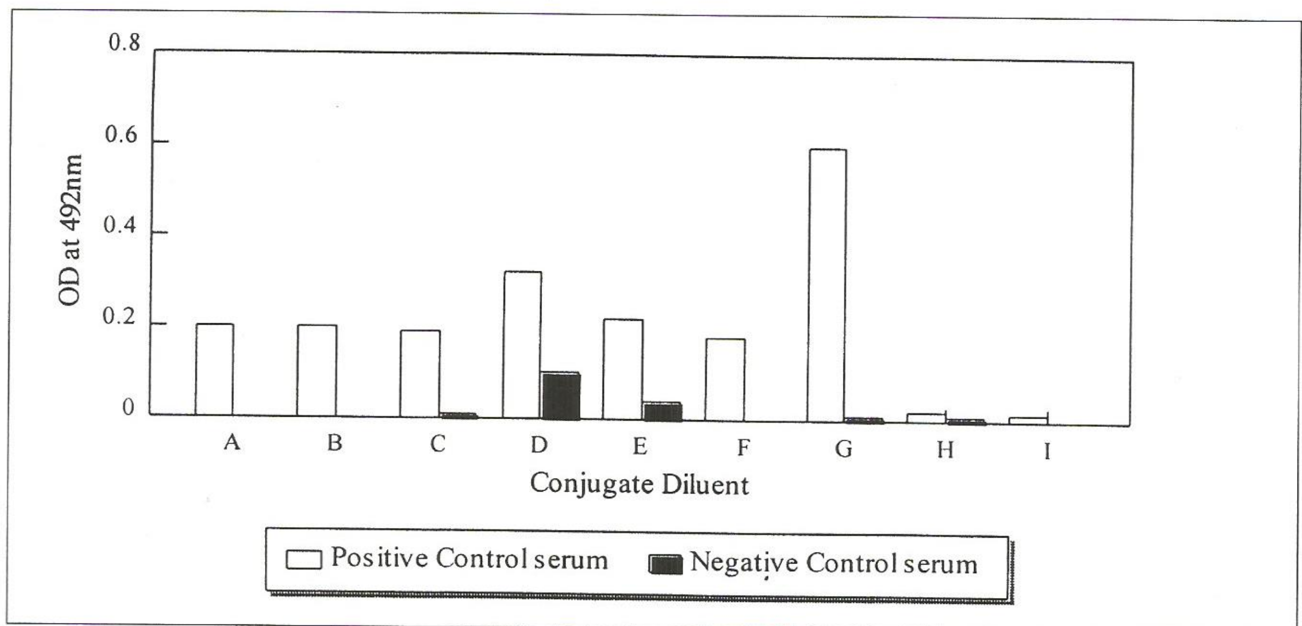
Reproducibility of the ELISA

Between three batches of antigen treated with Triton X-100 and between two strains of *H. paragallinarum* (K and I), the results of their absorbances using positive and negative control sera were consistent, indicating high reproducibility.



A: Tw-TBS-0.25%SM-1% BSA
B: 3.0% BSA
C: 0.5% BSA
D: 3.0% Gelatin
E: 0.5% Gelatin
F: 3.0% Skim milk
G: 0.5% Skim milk
H: 0.5% Chicken serum albumin
I: 0.5% Chicken egg albumin

Figure 2. Effect of serum diluent on absorbance



A: Tw-TBS-0.25%SM-1% BSA
B: 3.0% BSA
C: 0.5% BSA
D: 3.0% Gelatin
E: 0.5% Gelatin
F: 3.0% Skim milk
G: 0.5% Skim milk
H: 0.5% Chicken serum albumin
I: 0.5% Chicken egg albumin

Figure 3. Effect of conjugate dilution buffer on absorbance

Table 2. Effect of washing and diluting solutions on OD readings in *H.paragallinarum* ELISA

Buffer	OD at 492nm	
	No Serum	Hpg positive serum
PBS	0.065 ± 0.016	0.540 ± 0.008
PBST	0.005 ± 0.001	0.573 ± 0.003
TBS	0.104 ± 0.042	0.506 ± 0.037
TBST	0.062 ± 0.004	0.824 ± 0.002

Storage of antigen coated plates

Results are shown in Table 3. Titres were maintained for at least seven months when *H. paragallinarum* coated plates were stored at -20°C. The P/N ratio for serum diluted to 100 times were found to be constant for at least seven months of storage.

Determination of positive/negative cut-off value

The distribution of OD_{492nm4}values with sera obtained from SPF chicken is shown in Figure 4. The cut-off point was selected as the upper limit of negative reaction. This value was determined as an OD of 0.07.

Titres in chicken sera

Serum samples collected from 14 commercial and 2 SPF chickens pre and one-week post infection with *H. paragallinarum* showed an increased in OD value in two of the commercial chickens and both sera of the SPF chickens. Sera from six chickens which were vaccinated with a Japanese commercial vaccine, however, failed to react although there was an increase in OD value at three weeks post-vaccination.

Table 4 shows ELISA results for eight serum samples obtained from *H. paragallinarum*-infected chickens showing signs of coryza in the field and 48 serum samples collected randomly from chickens in farms with unknown disease history. Seven of the former group (87.5%) were positive and 12 of the latter were positive (25%). However, it is not clear whether the 25% positive reaction was due to infection to *H. paragallinarum* or vaccination.

When antisera against *Leucocytozoon caulleryi*, *M. gallisepticum* and *M.synoviae* were tested with the ELISA, all of the antisera failed to react.

Table 3. Stability of antigen coated plates after storage at -20°C

Antiserum dilution	Dilution	Months of storage (OD_{492nm})						
		1	2	3	4	5	6	7
Positive	x100	0.84	1.23	0.84	1.28	1.29	1.23	1.61
Negative	x100	0.02	0.03	0.02	0.03	0.03	0.03	0.04
P/N	x100	42.0	41.0	42.0	42.6	43.0	41.0	40.3

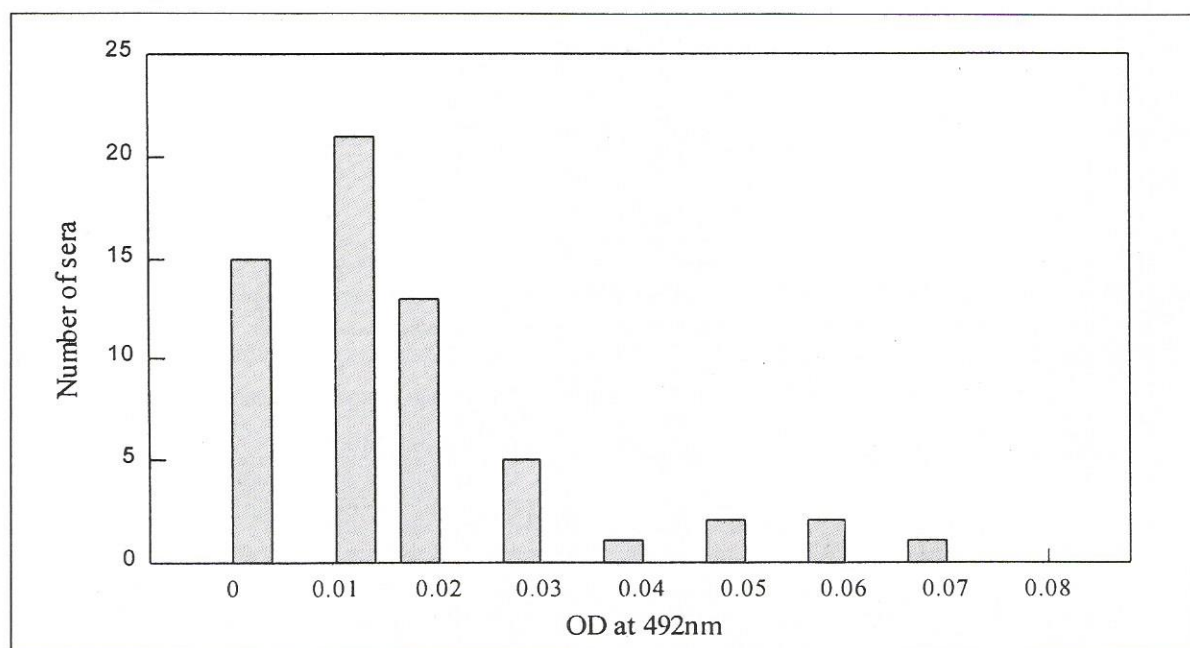


Figure 4. Distribution of ELISA titres of SPF sera

Table 4. ELISA titres of sera from *H. paragallinarum* naturally infected chickens and chickens of unknown history.

	ELISA titre (OD_{492nm})	
	≤ 0.07	≥ 0.07
H. paragallinarum-isolated chickens	1	7
Chickens with unknown disease history	36	12

Development of antibodies in experimentally infected chickens

Low serum antibody titres were detected by ELISA 12 days post-infection and they rose to a peak at day 19 but later declined. The SPA test, however, detected antibodies at day 12 and titres persisted till the end of the experiment, while the HI test could only detect antibodies at a very low titre, three weeks after infection. Table 5 compares the ability of the ELISA, HI test and the SPA test to detect antibodies following the inoculation of chicken with *H. paragallinarum* strain 221 through the lower eyelid.

Table 5. Percentage of positive SPA test, ELISA and HI test reactions of serum samples from artificially infected birds

Days post inoculation	Positive reactions (%)		
	SPA	ELISA	HI
0	0	0	0
12	100	0	0
19	100	100	33.3
25	100	100	100
32	100	66.7	100
39	100	66.7	100

DISCUSSION

One major objective of the present work was an attempt to develop and standardise the ELISA, as well as to minimise non-specific reactivity, while at the same time maintaining the maximum possible sensitivity. Many ELISA require a highly purified antigen for the initial coating of the wells to avoid cross-reactions with closely related antigens (Malcolm, 1980). The present study showed the suitability of Triton X-100 antigen for coating the plates. Triton X-100 disrupts the bacterial cells releasing somatic as well as membrane antigen into the solution. However, Iritani (1979) found that Triton X-100 dissolved the

All ELISA systems are prone to problems associated with anomalous reactions, mostly due to increase in the non-specific colour production or backgrounds. BSA at concentrations ranging from 0.1% to 2% is widely used as solid phase blocker (Offit *et al.*, 1984) while Tween 20, a non-ionic detergent is used as a liquid phase blocking reagent. Tw-TBS containing 3% BSA was chosen as the conjugate diluent and as serum diluent, not only to simplify the ELISA procedure but also to reduce non-specificity. The inclusion of 0.05% Tween 20 in the TBS as washing buffer, therefore, increased the absorbance of specific reaction which simultaneously reduced non-specific binding.

In this work, the threshold value for ELISA was set at an OD reading which was higher than that obtained with 100% of the negative sera tested. This automatically compares the sera to the normal population of negative control sera. Absorbance method is used as the sera can be tested at a single dilution and no data processing is necessary.

Reproducibility of the assay was maintained by the use of positive and negative sera on each plate. Results showed that both strains of *H. paragallinarum* (K and I) as well as the three batches of the strains tested were similar in their specificity, as well as sensitivity, which suggest that there was no antigenic differences between strains and between batches.

The shelf-life of reagent and antigen coated plates is important if ELISA is to have practical application. Conjugate was stored up to 1 year without loss of activity. *Haemophilus paragallinarum* coated plates retained activity for at least 7 months at -20°C. A longer period of storage at different temperatures warrants further investigation.

The SPA test was superior to the ELISA and the HI test in their ability to detect antibody production in response to infection. This is because the SPA test is more sensitive in detecting IgM than IgG (Kuniyasu, 1969), thus confirming the test to be the most suitable method for early diagnosis of infectious coryza (Iritani *et al.*, 1977). SPA agglutinin can be detected for 10-14 days post-infection and may persist up to 1 year, however, problems of autoagglutination have been encountered (Yamamoto, 1980). The present study shows that IgG production, measured by ELISA and HI test were delayed for up to three weeks following infection. Between the HI test and the ELISA, the latter is superior because it could detect antibodies in a higher percentage of serum samples than did the HI test.

In conclusion, results of this study suggest that the ELISA is suitable for large-scale testing of flocks for antibodies to *H. paragallinarum*. It is rapid and convenient for screening large numbers of sera. However, this test will not differentiate between titres due to infection or exposure to *H. paragallinarum* or field strains of *H. paragallinarum* nor between vaccination or post-infection antibody titres, but would

the management and control of *H. paragallinarum* infection. Since the number of samples tested to date are small, further studies to verify the sensitivity and specificity of the assay are therefore essential.

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RINGKASAN

ASSAI IMUNOERAP TERANGKAI ENZIM UNTUK PENGESANAN ANTIBODI TERHADAP HAEMOPHILUS PARAGALLINARUM

Satu assai imunoerap terangkai enzim (ELISA) telah dikembang dan diuji keupayaannya mengesan antibodi terhadap jangkitan Haemophilus paragallinarum *dalam ayam. Enam tip antigen telah disedia dan dibanding untuk menentukan antigen paling sesuai mengikut kriteria kekhususan dan kepekaan. Kepekatan antigen, serum dan konjugat enzim telah distandardkan dan kehasilan semulanya ditentukan. Assai ini kemudian diguna untuk mengesan antibodi* H. paragallinarum *dalam ayam terjangkit ujikaji. Sampel serum yang dikumpul pada 0, 12, 19, 25, 32 dan 39 hari pascajangkitan dianalisis melalui ujian pengaglutinatan plat serum (SPA), ujian penghemaglutinatan-perencatan (HI) dan ELISA.*

Hasil kajian menunjukkan antigen H. paragalinarum *teperlaku Triton X-100 sebagai antigen untuk ELISA adalah paling khusus, peka terhadap antiserum homologus, dan assai ini mudah dihasil semula. Dalam ayam bebas patogen khusus (SPF) terjangkit ujikaji, SPA dapat mengesan antibodi lebih awal daripada ELISA ataupun ujian HI. Sampel serum daripada 87.5% ayam yang menunjukkan petanda klinikal koriza berjangkit melalui jangkitan* H. paragalllinarum *bertindak balas dalam ELISA, sambil hanya 25% daripada ayam yang tidak diketahui riwayatnya bertindak balas.*