THE USE OF ELISA FOR THE DETECTION OF CHRONIC RESPIRATORY DISEASE IN CHICKENS

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ABSTRACT

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An enzyme linked-immunosorbent assay (ELISA) was developed for the detection of chronic respiratory disease (CRD) infection in chickens. Membrane antigens obtained from strain S6 of *Mycoplasma gallisepticum* (MG) were used for coating the wells of polystyrene microtitre trays. The membrane antigen and the conjugate were standardised before use. Control positive serum was serially diluted from 1:200 to 1:6400. Eighty control negative sera, taken from 2 week old commercial chickens, were used for evaluation of the ELISA negative cut-off level and 75 sera from chickens infected with MG were used as known positive sera. Field samples (750) were collected from commercial chickens in Bogor, Tangerang and Bandung, Indonesia. All sera were tested using a rapid serum agglutination (RSA) test and ELISA. The results showed that ELISA is more sensitive but the specificity is slightly less than RSA. Serologically, using ELISA, the prevalence of CRD in layers was higher than in broilers with 78% of laying chickens having MG antibody.

Key words: ELISA, detections, chronic respiratory disease, chickens

ABSTRAK

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Enzyme linked-immunosorbent assay (ELISA) dikembangkan untuk mendeteksi infeksi penyakit pernafasan menahun (PPM) pada ayam. Antigen membran dari galur S6 *Mycoplasma gallisepticum* (MG) dipergunakan untuk melapisi permukaan tiap sumuran mikrotiter polisterene. Sebelum digunakan untuk pengujian, antigen membrane dan conjugate dilakukan standardisasi lebih dahulu. Serum kontrol positif diencerkan dari 1/200 sampai 1/1600. Sebanyak 80 serum negatif kontrol yang diambil dari anak ayam komersial umur 2 minggu digunakan untuk mengevaluasi rataan nilai negatif ELISA dan 75 sampel sera diambil dari ayam yang diinfeksi dengan MG digunakan sebagai serum positif yang sudah diketahui dan sebanyak 750 sampel serum ayam dari peternakan ayam di Bogor, Tangerang dan Bandung digunakan untuk pengujian. Semua sera diuji dengan menggunakan uji serum aglutinasi cepat dan ELISA. Hasil dari pengujian ini memperlihatkan bahwa ELISA lebih sensitif daripada RSA tetapi sedikit lebih rendah spesifisitinya. Secara serologi dengan menggunakan ELISA ayam petelur memperlihatkan insiden CRD lebih tinggi daripada ayam potong dan sebanyak 78% dari ayam yang sedang bertelur mengandung antibodi MG.

Kata kunci : ELISA, deteksi, CRD, ayam

INTRODUCTION

Chronic respiratory disease (CRD) of poultry has been reported to occur worldwide (FAO-WHO-OIE, 1984; Anon, 1987), the primary cause of the disease being *Mycoplasma gallisepticum* (MG). Although the mortality due to CRD is low the morbidity is high (Jordan, 1979; Yoder, 1984) and the disease therefore causes significant financial losses in the poultry industries. Annual economic loss has been reported to be $millions (Carpenter *et al.*, 1979; Bickford, 1986; Gilchrist cited by Grimes, 1979). The economic losses are mainly due to decreased weight gain, decreased chicken price, decreased egg production, low hatchability and increased cost of medication (Jordan, 1979; Yoder, 1984; Soeripto, 1987, 1989).

There have been little investigations and reports of financial loss due to CRD in Asia, although preliminary findings have been reported by Naeem *et al.* (1980) and Soeripto (1989). In Indonesia, the presence of CRD has been confirmed using the rapid serum agglutination (RSA) test (Ronohardjo, 1974; Purnomo and Rahajeng, 1974; Purnomo *et al.*, 1986). RSA is used to detect the presence of immunoglobulin M (IgM) and the test is reported to be a sensitive test but non-specific and cross reactions against other mycoplasma strains, especially to *Mycoplasma synoviae* (MS), have been reported (Roberts, 1969, 1970; Vardaman and Yoder, 1971; Bradbury and Jordan, 1973; Glisson *et al.*, 1984).

The ELISA has been reported to be sufficiently sensitive to detect mycoplasma infections (Ansari *et al.*, 1982; Talkington *et al.*, 1985; Higgins and Whithear,

1986; Soeripto, 1987) and the assay can be made specific to either total immunoglobulin (IgM + IgG) or, more specifically, IgM or IgG. The aims of this study was to evaluate an ELISA for use in Indonesia which can be used to detect CRD infection in chickens.

MATERIALS AND METHODS

Mycoplasma isolates

Mycoplasma gallisepticum strain S6 obtained from the Institute of Medical and Veterinary Science, Adelaide, Australia was used for the ELISA antigen.

Mycoplasma media

Mycoplasma broth (MB) was made according to the method of Frey *et al.* (1968). It consisted of 10% (w/v) Mycoplasma broth base (Oxoid, UK), glucose (BDH Chemicals, UK), cysteine-HCl (BDH Chemicals, UK), thallous acetate (BDH Chemicals, UK), phenol red (Chroma stains, UK) and distilled water with the pH adjusted to 7.8. Mycoplasma agar (MA) was similar to MB with the addition of 1.5% (w/v) Noble agar (Difco Media, USA), and the deletion of glucose and phenol red. MB or MA media were autoclaved at 121°C for 15 minutes and then enriched with 15% (v/v) swine serum (inactivated at 56°C for 30 minutes), yeast extract (Difco, USA), DNA (Koch-Light, UK), actidione (UpJohn Ltd, USA) and silamox (Prafa Lab).

Test samples

Positive and negative control sera were taken from the control sera used for the RSA test. Eighty negative sera were obtained from 2-week old commercial chickens. At the age of 3 weeks, all of the chickens were infected with MG strain S6 via the intra-thoracic or intra-abdominal route. Two weeks after infection all the chickens were bled and 75 samples were used as positive sera. Seven hundred and fifty chicken sera were collected from commercial chicken farms in Bogor, Tangerang and Bandung, Indonesia. The breed and age of chickens were recorded.

Serology

The RSA was performed by adding 25 μL of serum to 25 μL of stained MG antigen (Balitvet) in a WHO tray and mixed thoroughly by tapping followed by 2 minutes on a rotary agglutinator. The agglutination reaction was recorded on a scale of 0 (no agglutination) to +3 (full agglutination).

ELISA

Antigen

Membrane antigen was produced from MG strain S6 grown in MB supplemented with 10% CO_2 in air at 37°C. After the broth culture changed from red to pink/yellow (16-24 hours) MG cells were harvested by ultracentrifugation at 30,000 *g* for 15 minutes at 4°C. The pellet was collected and resuspended in sterile 0.1 M phosphate buffered saline (0.8% NaCl) pH 7.4 (PBS) over three cycles. The cells were then resuspended in a small volume of PBS and sonicated for 60 seconds using a Soniprep 150. The protein content of the suspension was measured using a modified Lowry procedure (Hartree, 1972) on a Varian DMS 80 spectrophotometer and adjusted to 4.77 mg protein per mL in PBS.

Procedure

The ELISA was developed according to the method of (Plackett and Stewart, 1986). The MG S6 membrane antigen, diluted at 1:400 in 0.1 M carbonate/bicarbonate buffer pH 9.6, was added at 100 μL per well to Linbro microtitre plates (Flow Laboratories, USA). The plate was incubated at 4°C overnight, then washed at least six times in 0.1 M $NaH_2PO_4.H_2O$ pH 7.4 containing 0.05% tween 20. Test sera were diluted 1:200 in PBS containing 0.05% tween 20 (PBS-T) and 100 μL added to duplicate wells of the microtitre plate. The plate was incubated at room temperature for 1 hour with shaking, washed and then 100 μL of rabbit anti-chicken IgG-HRP conjugate, diluted 1:2500 in PBS-T with 0.2% casein, was added per well. The plate was incubated at room temperature for 1 hour with shaking, washed and then 100 μL per well of 1 mM ABTS/2.5 mM H_2O_2 chromogen/substrate was added. The plate was incubated at room temperature for 45 minutes with shaking and read in Titertek MCC ELISA platereader (Flow Laboratories, USA) at 415 nm. Reference positive serum, diluted 2-fold from 1:200 to 1:6400 and reference negative serum diluted 1:200 in PBS-T were included in each ELISA test. The control positive serum used for ELISA is the serum normally used as the positive reference serum for the RSA. The serum was assigned a value of 1000 ELISA units (EU).

RESULTS

Titration of the MG membrane antigen indicated that a 1:400 dilution was optimal for the assay (data not presented). Each well of the microtitre plate therefore contained 1.2 μg protein of MG membrane antigen. The mean EU of 80 negative sera was 49 with a standard deviation (SD) of 19 EU. The negative cut-off level, calculated as the mean + 2SD, was therefore 87 EU.

A comparison of the RSA and ELISA using a range of positive and negative sera is presented in Table 1. All sera from non-infected birds gave negative reactions with the RSA. However, four of the RSA negative sera gave positive reactions (≥ 87EU) in the ELISA. Of the infected sera, 41 were positive in the RSA and 46 were positive in the ELISA. From these results the sensitivity of the RSA was calculated to be 55% and the ELISA to be 61%. The specificity of the RSA was calculated to be 100% and the ELISA to be 95%.

A total of 140 serum samples were collected from broiler birds, 171 sera from layer birds less than 20 weeks of age and 436 from layer birds greater than 20 weeks of age. The incidence of CRD as measured by ELISA was 36% for broiler birds, 65% for pullets less than 20 weeks of age and 78% for layer birds greater than 20 weeks of age (Table 2).

Table 1. Comparison of the RSA and ELISA

Disease Status	RSA				ELISA			
	+ve	-ve	Sensi-tivity	Speci-ficity	+ve	-ve	Sensi-tivity	Speci-ficity
Non-infected	0	80			4	76		
			55%	100%			61%	95%
Infected	41	34			46	29		
Total	41	114			50	105		

Table 2. Testing of field samples for CRD by RSA and ELISA

Chicken Type	RSA			ELISA		
	+ve	-ve	% +ve	+ve	-ve	% +ve
Broiler	53	87	38	51	89	36
Layer - <20 weeks	102	69	60	112	59	65
Layer - >20 weeks	260	176	60	342	94	78
Total	415	332	-	505	242	-

As shown in Table 3 one hundred and eighteen sera that were negative in the RSA were found to be positive in the ELISA. Conversely 29 sera that were positive in the RSA were found to be negative when tested by ELISA (Table 4).

Table 3. Distribution of RSA negative, ELISA positive samples

ELISA titre (EU)	Broiler	Layer	
		<20 weeks	>20 weeks
87-250	2	-	84
251-500	-	3	10
≥ 501	2	9	8
Total	4	12	102

Table 4. Distribution of ELISA negative, RSA positive samples

RSA score	Broiler	Layer	
		<20 weeks	>20 weeks
1+	6	21	4
2+	-	-	7
3+	-	-	-
Total	6	2	21

DISCUSSION

This study is in agreement with others (Ansari *et al.*, 1982; Talkington *et al.*, 1985; Higgins and Whithear, 1986; Socripto, 1987) who have reported that the ELISA is more sensitive than the RSA in detecting MG-specific antibody in chickens. This study indicated, however, that the specificity of the ELISA was less than that of the RSA. The specificity of the ELISA would probably be improved by testing samples from flocks where there was a better history of the MG status and vaccination history. Chronic respiratory disease (CRD) has been reported to spread worldwide (FAO-WHO-OIE, 1984; Anon, 1987).

Using the ELISA, the incidence of CRD in layers was higher than for broiler birds. In the case of many of the layer birds they did not show clinical symptoms, indicating that many cases of CRD occurred subclinically.

A number of sera were negative in the RSA and positive in the ELISA, and some positive in the RSA and negative in the ELISA. As the RSA mainly detects IgM and the ELISA was specific for chicken IgG in ELISA the discrepancies in the test result may be because of the initial development of IgM and then the secondary development of an IgG antibody response. The RSA may therefore be expected to detect a higher number of recently infected animals and not be as efficient at detecting chronically infected animals. The IgM level would be expected to have decreased and the IgG antibody level to have increased in the latter group. It is recommended that routine testing for CRD should be carried out by ELISA and RSA.

The ELISA was found to be a sensitive and reproducible method for the detection of CRD of chickens. Therefore, it would be suitable for evaluating CRD infection and is recommended as a routine assay for monitoring the infection in the field.

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