

Development of slide agglutination test for the rapid diagnosis of *Mycoplasma* infection in the chicken

M. Arefin, J. A. Begum, R. Parvin, M. M. Rahman, M. A. H. N. A. Khan and E. H. Chowdhury*

Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh,

Abstract

Mycoplasma gallisepticum is considered to be the most important pathogenic *Mycoplasma* sp. and occurs world-wide. For effective control of mycoplasmosis, early diagnosis is the cornerstone, but the cultural method is laborious, expensive and time-consuming, whereas agglutination test is easy to perform in field conditions and cheaper. *Mycoplasma gallisepticum* was isolated and two types of stained antigen produced. The antigen produced distinct tiny clumps with positive antisera of *Mycoplasma* spp. The slide agglutination system was sensitive, reliable and cheap. This method may be used to screen *Mycoplasma* infections in poultry farms. (Bangl. vet. 2011. Vol. 28, No. 2, 80 – 84)

Introduction

Mycoplasma is one of the major avian diseases in the emerging poultry industry in Bangladesh. Primarily, this is a disease of chickens and turkeys but it also infects many other domestic and wild birds (Jordan and Amin, 1980; Bradbury *et al.*, 1993). The disease is caused by four common pathogens: *Mycoplasma gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae* (Bradbury, 2001). *Mycoplasma synoviae* (MS) causes infectious synovitis or mild upper respiratory disease. *Mycoplasma gallisepticum* (MG) causes respiratory disease and is the most economically important avian mycoplasma (Ley and Yoder, 1997). Diagnostic laboratories are trying to reduce the time required for diagnosis of *Mycoplasma* infections. Usually it takes approximately 7 days to culture and identify *Mycoplasma*. This method is tedious and confers little guarantee of sensitivity and species specificity. Several serological tests have been used to detect *M. gallisepticum* antibodies (Jordan and Amin, 1980). The most commonly used serological tests are the rapid serum agglutination (RSA) test, haemagglutination inhibition (HI) test, agar gel immuno-diffusion (AGID) test, and enzyme-linked immunosorbent assay (ELISA) (Avakian *et al.*, 1988). Tube agglutination test for *Mycoplasma* disease was described by Thomas (1970), and microagglutination test by Harry and Yoder (1982). In Bangladesh, a commercially available Methylene-blue-stained *Mycoplasma gallisepticum* (MG) antigen is being used for RSA test to screen flocks. But this antigen, besides being less sensitive, is neither cheaper nor produced from a Bangladeshi isolate. It is difficult to read the result due to its strong colour background. The micro-agglutination (MA) system (microtest) has

*Corresponding author:- E-mail: emdad001@yahoo.com

been adapted for a wide variety of serological procedures due to its better sensitivity (Anonymous, 1970; Witlin, 1967). So far, no attempt has been undertaken to produce a stained antigen from a local *Mycoplasma* isolate in Bangladesh. Production of tetrazolium-stained antigen from a local isolate might be more sensitive and cheaper. The present study was designed to prepare *Mycoplasma*-coloured antigen, develop slide micro-agglutination and agglutination tests for the diagnosis of avian mycoplasmosis.

Materials and Methods

Production of seeds

Mycoplasma was maintained in Mycoplasma agar in the laboratory and the isolates were plated on Mycoplasma agar, and a fried-egg-like single colony was selected for each isolate (Fig. 1). This was transferred to Mycoplasma broth and incubated at 37°C for 48 hours and maintained by transferring from one agar plate to another for several months. Kanamycin was added to the medium to prevent the growth of Gram-negative bacteria, and horse serum was used for the growth of Mycoplasma organism. This culture was used for the production of stained antigens.

Preparation of mycoplasma-stained colour antigen

The local isolate of *Mycoplasma gallisepticum* was transferred to a conical flask containing 50 ml of Mycoplasma broth base, which was incubated for 11 days at 37°C, at which time each flask revealed dense growth. Fifty ml broth culture was divided into two conical flasks each containing 25 ml. Tetrazolium blue and neotetrazolium chloride were added aseptically, 0.5% to each culture. The flasks were agitated and incubated for 24 hours, then 0.5% phenol was added to each flask, which were incubated for a further 2 hours for inactivation. The stained broth suspension was filtered through sterile gauze and poured into eppendorf tubes. These were centrifuged at 14,000 rpm for 15 minutes. The supernatant fluid was decanted and the packed cells were suspended in 0.5% phenol saline. The suspension was vortexed vigorously with a few sterile glass beads and transferred to another eppendorf tube. A dense suspension of deeply stained cells resulted. The solution was kept at 4°C as neotetrazolium- and tetrazolium-stained antigen for future use. The total protein concentration of stained antigen was measured by the Folin Phenol method as described by Lowry *et al.* (1951) and the protein concentration was adjusted to 6.4 ng/ μ l with the help of BSA standard curve. The antigen was packaged for field use.

Collection of field samples

A total of 110 serum samples were collected from local markets and poultry farms into eppendorf tubes. The slide micro-agglutination test was performed with the new Mycoplasma antigen. For development of slide micro-agglutination test, 20 μ l of stained antigen and 20 μ l chicken sera were placed on a sterile glass slide by a micropipette and mixed thoroughly by stirring. The results were read within 1

minute. Serum and water were used as negative control. For proper mixing, the antigen was shaken before use. The stain that used for preparation of antigen was sensitive to light so it kept away from the light.



Fig. 1. White colour *Mycoplasma* colony showing a dense raised centre in colonies with typical fried egg appearance

Results and Discussion

Production of seeds

A single colony of *Mycoplasma gallisepticum* from Mycoplasma agar inoculated into Mycoplasma broth formed confluent growth that showed turbidity and sliminess (Fig. 2).

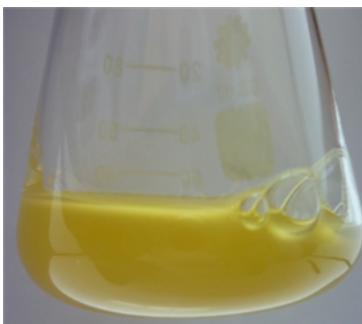


Fig. 2. Bulk culture of *Mycoplasma gallisepticum* in Mycoplasma broth showing turbidity and sliminess

Stained antigen preparation

The neotetrazolium-stained *Mycoplasma* antigen showed pink (Fig. 3), and the agglutination result was easily seen. The tetrazolium-stained Mycoplasma antigen showed light blue (Fig. 4) and the result was seen easily.

Slide micro-agglutination test

In positive case, granules (agglutinates) formed rapidly due to combination of homologous antigen and antibody, which was seen during rocking. In case of neotetrazolium-stained antigen the agglutinin was pink with purple background

(Fig. 5). For tetrazolium-stained antigen the agglutinin was deep blue with light blue background (Fig. 6) and less easily seen. Granules developed within one minute with tetrazolium stain and two minutes with neotetrazolium stain. Since the agglutinins were suspended on colourless serum, they were easily visible to the naked eye. In conventional agglutination test with other commercial antigens the cloudy reaction was not easy to detect. The specificity of the stained antigen was good: it did not react with the negative control serum or with water.



Fig. 3. *Mycoplasma* broth containing neotetrazolium stain

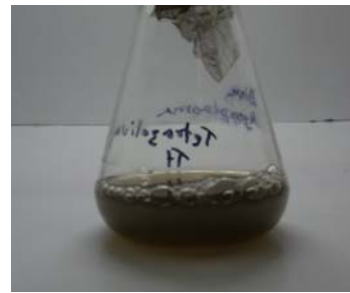


Fig. 4. *Mycoplasma* broth containing tetrazolium blue stain

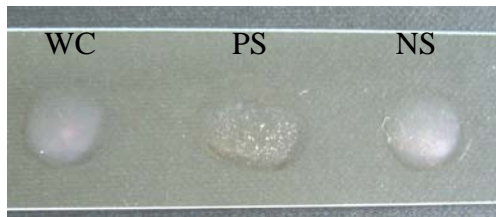


Fig. 5. Agglutination within two minutes with positive sera, negative sera and control with Neotetrazolium stained antigen

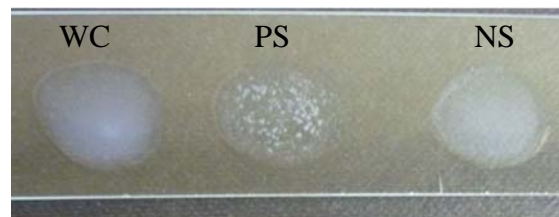


Fig. 6. Agglutination within one minute with positive sera (PS), negative sera (NS) and control (WC) with Tetrazolium stained antigen

Validation results with field samples

Among the 110 serum samples tested, 9% were positive, 82% negative and 9% suspect. The indigenous birds showed the highest prevalence at 50%. Broilers showed 15.9% positive. Growing birds showed no positive case (Table 1).

Table 1. Screening of field sera using new *Mycoplasma* stained antigen

Birds	Number of samples	Age in weeks	Agglutination result					
			Positive		Suspect		Negative	
Indigenous bird	6	28-32	3	50.0%	0	0%	3	50.0%
Broiler	44	4.5-5.0	7	15.9%	0	0%	37	84.1%
Growing bird (East shed)	20	52.1-78.2	0	0%	2	10.0%	18	90.0%
Growing bird (West shed)	40	52.1-78.2	0	0%	8	20.0%	32	80.0%

Conclusions

A tetrazolium-stained *Mycoplasma* antigen from a local isolate was successfully developed. [With the elapse of time, the stained antigen may react with the non-specific antibody present in the serum. Therefore, results within one minute were indicated suspecting cases of mycoplasmosis. After testing specificity with other closely related organism and sensitivity with some commercial antigen, a test kit may be prepared.

References

- Anonymous 1970: *A selected bibliography of micro methods in microbiology with special emphasis on microtiter techniques*. Cooke Engineering Co., Alexandria, Va, USA.
- Avakian AP, Kleven SH, Glisson JR 1988: Evaluation of the specificity and sensitivity of two commercial ELISA kits, the SPAT, and the HI test for antibodies formed in response to *Mycoplasma gallisepticum*. *Avian Diseases* **32** 262-272.
- Bradbury JM, Abdul Wahab, Yavari CA, Dipiellet JP, Bove JM 1993: *Mycoplasma imitates* SP. Nov. is related to *Mycoplasma gallisepticum* and found in birds. *Int. Journal of Systemic Bacteriology* **43** 721-728.
- Bradbury JM 2001. *Avian mycoplasmas*. In: *Poultry Diseases*, 5th edn, Jordan F, Pattison M, Alexander D. & Faragher T, eds. WB Saunders, London, UK pp. 178-193.
- Harry W, Yoder JR 1982: Microagglutination test for detection of antibodies to *Mycoplasma gallisepticum* and *M. synoviae* in avian sera. *Avian Diseases* **26** 606-611.
- Jordan FTW, Amin MM 1980: A survey of Mycoplasma infections in domestic poultry. *Research in Veterinary Science* **28** 96-100.
- Ley DH, Yoder HW 1997: *Mycoplasma gallisepticum* infection. In: *Calnek BW* (eds.). *Diseases of poultry*. 10th edn. Iowa State University Press, Ames, Iowa, USA pp. 194-207.
- Lowry OH., Rosbrough NJ., Farr AL., and Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193** 265-275.
- Thomas H, Harry W, Yoder JR 1970: Preparation of *Mycoplasma synoviae* antigen for the tube agglutination test. *Avian Diseases* **15** 462-466.
- Witlin B 1967: Detection of antibodies by microtitration techniques. *Mycopathol. Applied Microbiology* **33** 41-257.