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Development of a Promising Agglutination Based Diagnostic Kit for Detection of *Mycoplasma gallisepticum* (MG) Infection in Chickens

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ABSTRACT

Background: *Mycoplasma gallisepticum* (MG) an important respiratory tract pathogen is responsible for morbidity in poultry flocks. It affects poultry birds of various age groups of layers as well as broiler, especially in younger ones. The diagnosis of such ailment is highly sought for adopting suitable control measures. In this regard, classical procedure of culture and isolation of mycoplasmas is cumbersome, tedious and time-taking. In an earlier study, it was found that serology was more sensitive than bacteriology in MG infection. The serum plate agglutination test was used in many of the earlier sero-prevalence studies. It is recommended to use the serology based detection methods for screening the poultry flocks rather than testing individual poultry birds. For field based rapid diagnosis, agglutination based serology methods are best comparison to nucleic acid detection methods, more so in remote areas.In view of the difficulties in the diagnosis of MG infection in field condition, the study was undertaken to develop a user-friendly agglutination kit, which can be used as screening test for an early diagnosis of MG infection in the poultry flocks.

Methods: Mycoplasma coloured antigen kit was developed and standardized using *Mycoplasma gallisepticum* F strain available at the Central University Laboratory. The prepared coloured antigen kit was stored at different temperatures such as 37°C, room temperature (22-28°C) and at 4-8°C at different period of time such as three months, six months and one year to know the shelf life of the kit.

Result: The sensitivity of the coloured antigen kit was found to be 92.25% and specificity as 100%. The kit was found promising in the field condition for an early diagnosis of the ailment among the poultry flocks.

Key words: Avian mycoplasmosis, Agglutination kit, Diagnostics, *Mycoplasma gallisepticum*.

INTRODUCTION

An effective control strategy relies on the rapid and accurate disease diagnosis followed by efficient therapeutic measures. Moreover, routine sero-surveillance is an indispensable part of epidemiological forecasting and preparedness against a particular ailment. With the increasing demand of rapidity in disease diagnosis at patient's bedside, the agglutination kits are promising diagnostic tools in field conditions.

Mycoplasma gallisepticum (MG) an important respiratory tract pathogen is responsible for morbidity in poultry flocks. It affects poultry birds of various age groups of layers as well as broiler birds (Manimaran *et al*., 2019), especially in younger ones. The diagnosis of such ailment is highly sought for adopting suitable control measures. In this regard, classical procedure of culture and isolation of mycoplasmas is cumbersome, tedious and time-taking. In an earlier study, it was found that serology was more sensitive than bacteriology in MG infection (Feberwee *et al*., 2005). The serum plate agglutination test was used in many of the earlier sero-prevalence studies (Elgnay and Azwai, 2013; Ali *et al*., 2015). It is recommended to use the serology based detection methods for screening the poultry flocks rather than testing individual poultry birds (OIE, 2018). However, in comparison to serology, molecular detection methods are having added advantages of having accuracy. Kahya *et al*., (2010) have suggested that nucleic acid detection methods can be adopted in sero-positive poultry flocks for diagnosis of MG infection. Similar strategy was opined by the Feberwee *et al*. (2020) regarding use of polymerase chain assay (PCR) based molecular diagnostic tools and Enzyme linked immune sorbent assay (ELISA) based diagnostics for confirmation of results of agglutination based diagnostics. However, these methods can only be adopted in sophisticated well-equipped laboratories. For field based rapid diagnosis, agglutination based serology methods are best comparison to nucleic acid detection methods, more so in remote areas.

In view of the difficulties in the diagnosis of MG infection in field condition, the study was undertaken to develop a

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user-friendly agglutination kit, which can be used as screening test for an early diagnosis of MG infection in the poultry flocks.

MATERIALS AND METHODS

Culture

Mycoplasma coloured antigen kit was developed and standardized using *Mycoplasma gallisepticum* F strain available at the Central University Laboratory during the period 2013 to 2016. *Mycoplasma gallisepticum* F strain was inoculated in 2 ml of PPLO broth and incubated at 37°C for 2-3 days. Then, the inoculum was transferred into 100 ml of medium and incubated at 37°C for 2 days. For bulk preparation of the antigen, sub-culturing was done in one litre of PPLO broth and incubated at 37°C for 2-3 days with intermittent shaking until the appearance of sharp yellow colour change in the medium indicating the acidic shift with optimum growth.

Inactivation

The above culture was inactivated by formalin (0.2% final concentration) and incubated at 37°C for 2 days. Then, the whole culture was kept at room temperature for 3 days for cell maturation. To detect complete inactivation, the inactivated culture was inoculated into PPLO broth and PPLO agar plate and incubated at 37°C for one week for checking the presence of mycoplasma growth, if any.

Extraction and standardization of native membrane antigen

The inactivated culture was centrifuged at 9000 rpm for 20 min in refrigerated centrifuge. The supernatant was discarded and pellet was washed thrice with PBS (pH 7.2) and resuspended in PBS (pH7.2) used as native membrane antigen. The native membrane antigen was prepared at different concentration of mycoplasma cells (1-10%).

Preparation and standardization of Rose Bengal dye

1% Rose Bengal dye was prepared from the stock rose Bengal dye (HiMedia). This 1% dye solution was further diluted as 1:1000 to 1:10000 in PBS. Each dilution was mixed with equal volume of native membrane antigen (3% mycoplasma cells) and vortexed at time period (one minute to ten minutes) in shaker incubator at 37°C.

Working procedure of coloured antigen kit

A volume of 25 µl of suspected serum was placed on to a clean glass slide. Similarly, 25 µl of prepared coloured antigen of *Mycoplasma gallisepticum* was placed nearby the serum. Using a stirring rod antigen and serum mixed to a circular area of approximately 1.5 cm diameter. The glass plate was rocked gently for 2 minutes. *Mycoplasma gallisepticum* positive serum procured from Poultry diagnostic and research Centre (PDRC), Venkateshwara Hatcheries Pvt. Ltd., Pune was taken as positive control where as serum from specific pathogen free (SPF) birds was taken as negative control in the study.

Shelf life of coloured antigen kit

The prepared coloured antigen kit was stored at different temperatures such as 37°C, room temperature (22-28°C) and at 4-8°C at different period of time such as three months, six months and one year to know the shelf life of the kit.

Applicability

The coloured antigen kit was tested for its field applicability using the sera samples collected from the suspected poultry flocks. A total of 540 blood samples were collected at random along with the tracheal/nasal swabs from the birds of different age groups ranging from grower to adult stage and suspected of MG infection. The samples were collected from various poultry farms of broilers and layers consisting of various commercial chickens, *viz*., Lohmann, Babcock, Bovans and Vencobbs reared in deep litter/cage system of housing in and around areas of Namakkal, Salem and Dharmapuri districts of Tamil Nadu, India. The sera samples were prepared by centrifuging the collected blood samples at 1000×g for 10 minutes at 4°C temperature and preserved at -20°C temperature until further use. All tracheal/nasal swab samples were tested for molecular detection of the MG genome through Polymerase Chain Reaction (PCR) as per the protocol described (Manimaran *et al*., 2019). Similarly, all sera samples were tested by the coloured antigen kit and results were recorded. All the sera samples collected were tested in the same time to avoid any variation of the flocculation due to storage among the sera samples.

RESULTS AND DISCUSSION

On inoculation of inactivated culture, there was no growth observed both in PPLO broth and agar plate which ensured a complete inactivation of the mycoplasma culture prior to preparation of native membrane antigen. The native membrane antigen prepared with 3% of mycoplasma cells was found to be optimum during standardization for the test. The coloured antigen prepared with 1:4000 dilution of 1% rose bengal dye with native membrane antigen (3% mycoplasma cells) vortexed for two minutes was found to produce visible agglutination. Flocculation of the antigen within two minutes was considered as agglutination. The presence of agglutination was considered as positive for MG infection. Absence of agglutination was considered as negative for MG infection (Fig 1). The coloured antigen kit stored at 4-8°C produced a clear visible agglutination (Fig 2) at all the test periods *i.e.* three months, six months and one year. A very similar slide micro-agglutination kit was developed by Arefin *et al*. (2011) using tetrazolium dye for diagnosis of *Mycoplasma* spp. infection in poultry flocks in field condition.

In the present study, out of 540 tracheal/nasal swabs subjected for molecular detection, 131 samples were found positive through PCR. Similarly, out of the corresponding 540 sera samples, 91 samples were found strong positive whereas 29 samples were found weak positive producing

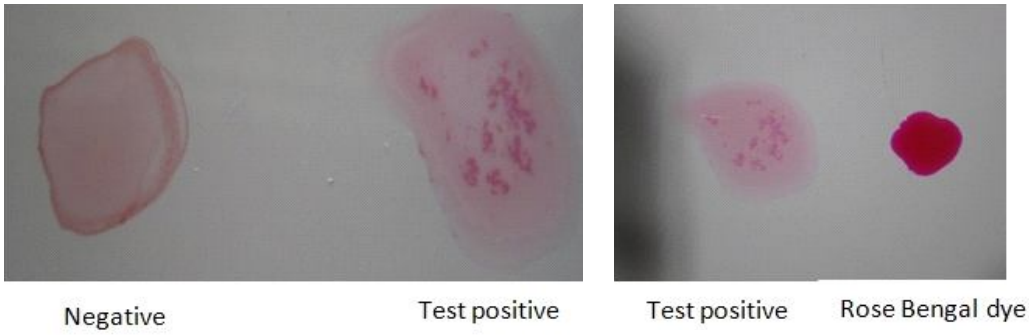

Fig 1: Agglutination with the MG agglutination kit. Presence of flocculation was inferred as positive result whereas absence of flocculation was indicated as negative for MG infection.

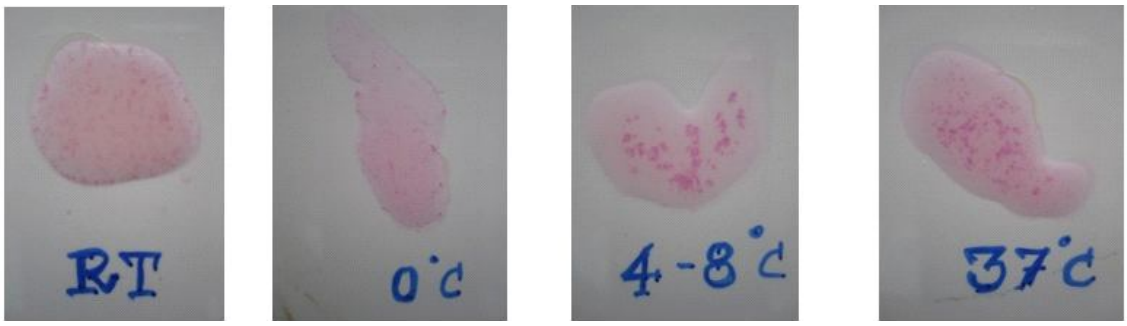

Fig 2: A clear visible agglutination produced by the coloured antigen kit stored at 4-8°C temperature.

the agglutination using the coloured antigen kit. There were 11 sera samples with absence of agglutination but found positive for the corresponding tracheal/nasal swabs through PCR. The sera samples found positive through PCR but absence of any flocculation were considered as false negative reactors (n=11) and these 11 sera samples showing such false negative result were repeated thrice to conclude the reactivity and similar results were obtained. None of the agglutination test positive sera samples were found negative through PCR while testing the corresponding tracheal/nasal swabs. Such samples were considered as false positive reactors (n=0). In an earlier study, it was suggested that false-positive reactors may result in such serologic tests (Feberwee *et al*., 2005). The nucleic acid based diagnostics are always preferred than the serology based diagnostics because of higher sensitive and specificity. In accordance, the results of the present study with certain level of false negative reactors but absence of false positive reactors indicated the superiority of nucleic acid based diagnostics than serology based diagnostic tools. Limitations of such serology based rapid plate agglutination based diagnostics were already reported (Feberwee *et al*., 2005). However, the agglutination based kits are always preferred in the field conditions where proper laboratory facilities are not there. Furthermore, such agglutination based diagnostics are useful for an ease and rapid diagnosis of the infection in field condition. Moreover, such agglutination kits can be used most suitably for surveillance of infection in flocks on large scale rather than individual testing. In a recent study it was suggested that screening of the flocks suspected of avian mycoplasmas can be undertaken with serological tests like rapid plate agglutination tests followed by confirmation through molecular based diagnostics (Feberwee *et al*., 2020). Hence, the present agglutination kit reported in the study can be used for flock screening followed by molecular confirmation because of occasional presence of non-MG infection in the flocks may affect the results as suggested in earlier report by Feberwee *et al*., (2020). The sensitivity of the coloured antigen kit was found to be 92.25% and specificity as 100%. In one of the earlier studies with experimental infection of MG, sensitivity of the PCR method was found higher as compared to bacterial culture and serology (Asgharzade *et al*., 2013).

In conclusion, the agglutination kit developed was found suitable for early diagnosis of the MG infection among the poultry flocks in the field condition. However, a large number of sera samples from diverse climatic zone may add more value to it and a more evident diagnostic picture can be anticipated. This may facilitate for an efficient sero-surveillance study against the MG infection among the poultry flocks.

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