

## PREPARATION OF *MYCOPLASMA SYNOVIAE* ANTIGENS AND EVALUATION BY RAPID SLIDE AGGLUTINATION AND ENZYME LINKED IMMUNOSORBENT ASSAY

A. Rasool<sup>1</sup>, A. A. Anjum<sup>1</sup>, M. Rabbani<sup>1</sup>, M. Lateef<sup>2</sup>, M. Nawaz<sup>1</sup>, F. Akhtar<sup>3</sup>, A. Kanwal<sup>3</sup> and S. Sattar<sup>4</sup>

<sup>1</sup>Department of Microbiology, <sup>2</sup>Department of Parasitology, <sup>3</sup>University Diagnostic Laboratory, University of Veterinary and Animal Sciences, Lahore, <sup>4</sup>Veterinary Research Institute, Zargar Shaheed Road, Lahore, Pakistan.  
Corresponding author's email: aftab.anjum@uvas.edu.pk

### ABSTRACT

Poultry industry has high annual growth rate in Pakistan. Mycoplasmosis, separately or in combination with other diseases, cause major setbacks to poultry industry. Due to significant role of *Mycoplasmosis* in poultry industry, its control programme success depends on timely and accurate diagnosis of infected flocks. Poultry flocks are generally screened for Mycoplasma by Rapid Slide Agglutination (RSA) test and Enzyme Linked Immunosorbent Assay. Aim of the current project was to prepare and evaluate indigenous *Mycoplasma synoviae* RSA antigen and ELISA plate coating antigen. *M. Synoviae* isolates were characterized using 16s rRNA polymerase chain reaction. RSA antigen was prepared using Rose Bengal dye and compared with a commercially prepared RSA antigen. Incubation of ELISA plate, coated with indigenous antigen, at 4°C and 37°C had no significant difference (P=1.0000), but there was a significant difference (P value = 0.01) in the ELISA antibody titers of plates, coated with local antigen incubated at 37°C compared with pre coated plates available in imported kit. ELISA plate coating antigen and RSA antigen of *M. synoviae* prepared from local isolate is cheap and produced equally reliable results to their commercial companion.

**Keywords:** *Mycoplasma synoviae*, Rapid Slide Agglutination, Rose Bengal dye, Local isolate, ELISA

### INTRODUCTION

Poultry, being the second largest industry in Pakistan, has excellent annual growth rate (11.8%) (Anonymous, 2014-2015). It still faces many infectious diseases, of which respiratory diseases are responsible for major losses (Ali and Reynold, 2000). Mycoplasma, alone or in combination with other pathogens, is one of the major cause of respiratory tract infections and heavy economic losses in poultry industry (Ali and Reynold, 2000; Walker, 2004; Dufour-Gesbert *et al.*, 2006; Cobb, 2011; Ehtisham *et al.*, 2011). Different Mycoplasma serotypes (22) have been isolated from avian species throughout the world, of which *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are considered as highly pathogenic (Kleven, 2003; Yilmaz *et al.*, 2011).

*M. synoviae* infection generally occurs as a subclinical upper respiratory tract infection. It causes airsacculitis itself or in combination with *Mycoplasma gallisepticum* or with respiratory virus infections like Infectious bronchitis or Newcastle disease virus (Kleven and Ferguson-Noel, 2008; Catania *et al.*, 2016) or *Escherichia coli*. *M. synoviae* causes acute to chronic systemic infection (Senties-cue *et al.*, 2005), which results in synovitis and bursitis. Flocks infected with arthropathic strains of *M. synoviae* may suffer severe losses of growth retardation and culling of lame birds (Landman and Feberwee, 2001; Van Beek *et al.*, 2002).

Due to significant role of *M. synoviae* in poultry industry, farmers require an early detection and control of *M. synoviae*. To maximize the profits, infection free flocks should be maintained through good bio-security measures either by medication or vaccination. The success of control programs depends on accurate and timely diagnosis of infected flocks to prevent dissemination of infection.

For the Mycoplasma infection diagnosis, serology and antigen detection is done. For Serological screening methods, Rapid Serum Agglutination Test (RSA), Enzyme Linked Immunosorbent Assay (ELISA) and haemagglutination inhibition test (HI) are conducted and for antigen detection, culture techniques and polymerase chain reaction (PCR) are used (Kleven, 2008; Abdelwhab *et al.*, 2011; Yilmaz *et al.*, 2011).

As RSA detects IgM, therefore, it can detect seroconversion earlier than HI or ELISA which detects IgG. RSA test is easy to perform in field because it is rapid and does not require any special equipment or technique (Butcher, 2007; Pakpinyo *et al.*, 2009). Serological screening for *M. synoviae* is often perplexed due to cross reactions and nonspecific reactants in serum of chicks, thus true picture of the flock status is revealed by ELISA which is used as back up test for screening of flock for mycoplasma infection. In Pakistan, RSA antigens and pre coated ELISA plates containing kits are imported from different technologically advanced countries. Therefore, this project was designed to prepare cheap, reliable and effective *M. synoviae* antigens for subsequent use in RSA and ELISA.

*Mycoplasma synoviae* was isolated from field samples collected from different farms at Abbotabad, Lahore, Jaranwala and Peshawar. All the samples were processed at University Diagnostic Laboratory, University of Veterinary and Animal Sciences, Lahore. Local isolate was confirmed as *M. synoviae* after molecular characterization using species-specific primer pairs targeting 16s ribosomal RNA gene (Anonymous, 2008; Ehtisham *et al.*, 2011; Perez *et al.*, 2011; Aliabad *et al.*, 2012; Lobova *et al.*, 2012). The local isolate after confirmation was maintained in freeze dried form to be used in the preparation of colored antigen and for ELISA plate coating antigen by the modification of the procedure of Arefinet *et al.* (2011) and Anonymous (2008).

#### Preparation of colored antigen from local isolate

**Preparation of 1% packed cell volume of *M. Synoviae*:** Lyophilized local isolate seed was reconstituted and transferred to Frey's broth (Frey *et al.*, 1968; Benicina *et al.*, 2001, 2005b; Lavric *et al.*, 2007). It was incubated for 72 hours at 37°C in 10% CO<sub>2</sub>. After incubation, there was dense growth in the cultured broth and the color of media was changed from red to yellow. The growth was pelleted by centrifugation at 14000 rpm for 20 minutes and supernatant was discarded. The pellet was washed with PBS thrice to remove the traces of media. One percent pack cell volume (PCV) was adjusted with the help of hopkin's tube. This 1% PCV of *M. synoviae* was used for the preparation of RSA antigen and ELISA plate coating antigen.

**Staining of 1 % PCV of *M. synoviae* by Rose Bengal Dye:** The 1% PCV antigen was again pelleted by centrifugation. The pellet was re-suspended in phenol saline (20 mL). Rose Bengal dye (1 %) was added in dense culture (0.5%) and culture was again incubated at 37°C in 10% CO<sub>2</sub> for 24 hours. Phenol (0.5%) was added in the stained culture, and the culture was incubated again for 2 hours for inactivation. The inactivated stained antigen was centrifuged at 14000 rpm for 15 minutes. The supernatant was discarded. The pellet was washed thrice in phenol saline to remove the residues of media and stain, in final suspension the pack cell volume (PCV) was adjusted 1% by Hopkin's centrifuge tube. Thiomerosal sodium was added (0.01%) as preservative. The stained antigen was homogenized thoroughly by vortexing.

On microscopic examination, the antigen was homogeneous suspension without any residue. It was free of contaminants. The pH was adjusted to 6.8. The stained antigen was then stored at 4°C for further use.

**Collection of chicken serum samples from field:** The whole blood (n=200) was collected from jugular vein of randomly selected birds at different farms from Abbotabad, Lahore, Jaranwala and Peshawar, with the help of sterilized 5cc syringes. The stationary slants were

prepared for complete separation of serum from blood. Then the separated serum was collected in clean properly-labeled microfuge tube of 1.5 ml capacity (Eppendorf, Germany).

**Evaluation of RSA colored antigen prepared from local isolate:** The rapid slide agglutination (RSA) antigen prepared from local isolate was evaluated by slide micro agglutination of field samples. *Mycoplasma synoviae* positive and negative hyper immune sera were used as control and results of locally prepared RSA were compared with commercial RSA antigen (Charles River, USA). Test was performed according to the Commercial RSA antigen manufacturer guideline (Charles River, USA). All the components of the test including test sera, colored antigen (commercial and prepared from local isolate) positive and negative controls and slides were pre warmed at room temperature. Antigen was homogenized by vortexing for few seconds. Serum samples (n=200) were screened by RSA test using local and commercial RSA colored antigen and results were noted by following the scoring methods of Anonymous (2008); Ley and Yoder (1997) and Papkinyo *et al.* (2007).

**Preparation of enzyme linked immunosorbent assay (ELISA) plate coating antigen:** Adjusted one percent PCV was used for coating onto 96 well flat bottom micro titration plates. Coating buffer [Carbonate-Bicarbonate buffer (Na<sub>2</sub> CO 31.5gm, NaHCO<sub>2</sub>.93 g, distilled water, 1 liter, pH 9.6)] and blocking buffer, Skimmed milk (PBS 100 ml, Tween 20 .005ml, Skim milk 5 gm, pH 7.4) were prepared in the laboratory, while all other required reagents and chemicals were used from commercially available *M. synoviae* antibody test ELISA kit (ProFLOK *M. synoviae* antibody test kit, SYNBIOTIC, USA). Coating buffer (50 µL) was dispensed in 96 wells of flat bottom ELISA plates, then locally prepared *M. synoviae* antigen (50 µL) having 1% PCV was added in each well. Plates were covered with lid to prevent drying and evaporation of fluid during incubation. The plates were incubated at 37 °C for one hour after that one plate was remained at 37(Anonymous, 2008; Mehmood *et al.*, 2009) and the other was shifted to 4 °C (Rosenquist *et al.*, 1995; Kyvsgaard *et al.*, 1996) overnightly for further incubation.

On next day, the excessive fluid from the wells was discarded by inverting the plates and tapped to remove the excessive fluid. Blocking buffer (100 µL) was added in each well of plates and plates were again incubated at room temperature for 45 minutes. After that, the blocking buffer was discarded and plates washed thrice with washing buffer. Almost, all of washing buffer was removed by inverting and tapping the plates.

All the serum samples together with negative and positive controls available with test kit were diluted at 1:50 in an uncoated micro titration plate by adding test serum (6 µL) in dilution buffer (300 µL) available in Kit.

The plate was properly labeled and kept for 05 minute at room temperature to equilibrate the serum samples in dilution buffer.

Diluted test serum samples (50  $\mu$ L) were added in antigen coated plate whereas diluted reference negative and positive sera (50  $\mu$ L) were added in positive and negative wells. The loaded plate was incubated at 37°C for an hour. The plates were washed thrice with washing buffer (300  $\mu$ L/well), soaking for 03 minutes and then tapped away. After completion of wash procedure anti chicken IgG conjugated with horseradish peroxidase (Avalable in commercial Kit) was diluted at 1:100 dilution (50  $\mu$ L) was added in each well and the plates were incubated at room temperature for 30 minutes, then the solution was discarded by inverting and taping the plates, washing was again repeated thrice by the same procedure. Substrate solution (2,2'-AZINO-BIS(3-ETHYLBENZTHIAZOLINE-6- SULFONIC ACID – Hydrogen peroxide, 50  $\mu$ L) was dispensed in each well and plates were again incubated at room temperature for 15 minutes. Diluted (1:5) top solution (1 M H<sub>2</sub>SO<sub>4</sub>, 100  $\mu$ L) was added in each well .The optical density (OD) value of the test and control wells were evaluated at 405 nm at micro plate absorbance reader (BIO Rad, USA). The OD values were evaluated by the method described by Kreider *et al.* (1991a; 1991b). Same serum samples were also checked for the presence of Anti-MS Antibodies by using *M. synoviae* antibody test kit (PRO flok Synbiotics, USA) for comparison.

## RESULTS AND DISCUSSION

Poultry is the second largest industry with tremendous growth rate in Pakistan (Anonymous, 2015). In present study, locally prepared RSA colored antigen was prepared and evaluated for the detection of *M. synoviae*, from 200 serum samples by following the procedure of Anonymous (2008). Rose Bengal dye, used for the preparation of colored antigen, is an acidic dye and analogue to fluorescence dye. It stains the cells brightly which makes the agglutination reaction more visible in bright light (Alexander, 2006; Sabnis, 2010). Although serological screening for *M. synoviae* is often perplexed due to cross reactions and nonspecific reactants in serum of chicks, but it is also true that it is necessary to know the mycoplasma infection status of chicken flock as it helps to develop the strategies to combat infection (Haghighi-khoshkhoo *et al.*, 2011; Luciano *et al.*, 2011). Factors like, the vaccination of chicken flocks with inactivated oil emulsion or cell culture based viral vaccine in which serum is used as a part of culture media or presence of any contamination or freezing and thawing of samples before RSA test, similarly presence of infections of poorly immunogenic strain of Mycoplasma contribute in nonspecific reactants, Heat treatment at 56°C for 30 minutes and dilution to 1:4 and 1:8

(Feberwee *et al.*, 2005; Anonymous, 2008; Luciano *et al.*, 2011) prevent this non specificity.

Results of *M. synoviae* local RSA antigen revealed that out of 200 serum samples, 139 (69.5%) samples were positive and 47(23.5%) were negative. After the heat treatment and dilution, out of 200 serum samples, 105 (52.50%) were positive, 34 (17%) were probable and 61 (30.50%) were negative (Table 01) as shown in fig. 1. Sero-positivity of *M. synoviae* in poultry flocks has been determined previously in Pakistan (Ehtisham *et al.*, 2011; Saddique *et al.*, 2012) and other parts of world (Fiorentin *et al.*, 2003; Feberwee *et al.*, 2005; Haghighi-khoshkhoo *et al.*, 2011; Luciano *et al.*, 2011; Seifi and Shirzad, 2012, Feizi *et al.*, 2013) by using RSA colored antigen. Results of *M. synoviae* commercial RSA antigen showed that 140 (70%) samples were positive and 60(30%) were negative while after heat treatment and dilution of serum samples, 103 (51.50%) were positive, 38 (19%) probable and 59 (29.50%) were negative. From Fisher's exact test it can be resulted that there was a non-significant difference ( $p=1.0000$ ) in detection rate of *M. synoviae* by local and commercial RSA antigen. Non-significant differences ( $P>0.05$ ) in detection of *M. synoviae* by commercial and locally prepared RSA antigen shows that locally prepared RSA antigen, in this study, can be used for rapid screening of *M. synoviae* in poultry flocks. It will mitigate the imports cost and save national capital. Serum samples were heated and diluted to increase the specificity of *M. synoviae* detection by RSA. Similar results have also been reported in different studies (Arefin *et al.*, 2012).

ELISA is another technique used for the detection of *M. synoviae* (Saadia *et al.*, 2014). It is more specific, sensitive, and reproducible tool as compared to RSA testing for diagnosis of mycoplasma infection. Different studies have reported the comparison of RSA and ELISA for the detection of *M. synoviae* (Abdelmoumen and Roy 1995; Luciano *et al.*, 2011; Seifi and Shirzad 2012). Different studies, from different parts of world, have reported the preparation of *M. synoviae* coated ELISA and compared with commercially available ELISA kits (Rosenquist *et al.*, 1995; Yilmaz *et al.*, 2011). Similarly, there are different studies from Pakistan which report the development of ELISA from local antigens of different microbes. To best of our knowledge, this is first report of preparation and evaluation of ELISA plate coated with local isolates of *M. synoviae* from Pakistan. Two different incubation temperatures 37°C and 4°C were used for coating of plates with locally prepared antigen of *M. synoviae*. Fisher's exact revealed that there was a non-significant difference ( $p=1.0000$ ) between ELISA plates incubated at 37°C and 4°C (Table 02) indicated in fig.2. It indicates that any temperature can be used for incubation of the antigen coated plates. Antibody titers against *M. synoviae* detected by ELISA developed in study and commercially available ELISA are given in

table (3). Local antigen coated ELISA plates showed significantly higher positive samples (89.13%, 41/47) as compared to commercial ELISA pre-coated plates (80.43 %, 37/47) (P < 0.05). This difference can be explained by the fact that whole cell was used for coating of ELISA

plate instead antigenic portion of cell. It is concluded that RSA test colored antigen and ELISA plate coating antigen prepared from local isolate of *M. synoviae* are equally good for screening of flocks as compared to commercial kits.

**Table 01: Comparative efficacy of locally prepared and imported antigens for detection of antibodies against *Mycoplasma synoviae* by RSA**

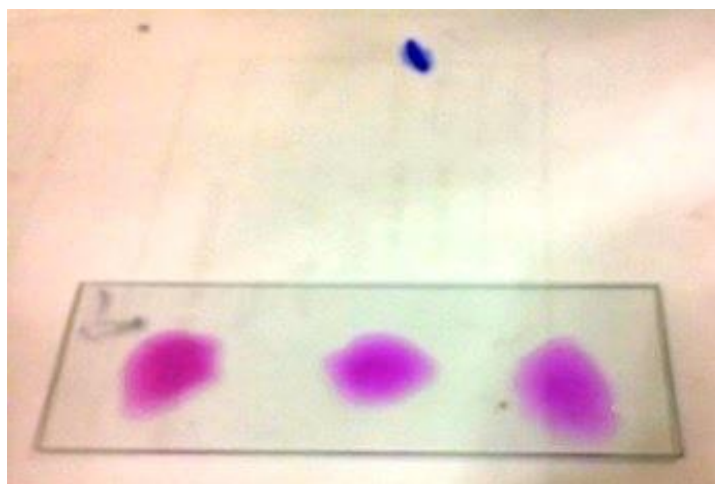
Antigen	No. of samples tested	RSA PRE-TREATMENT		RSA POST-TREATMENT (Heat and dilution)			P value
		Positive	Negative	No. of positive samples	No. of probable Samples	No. of negative Sample	
Local	200	139 (69.5%)	47 (23.5%)	105 (52.5%)	34 (17%)	61 (30.5%)	1.00
Imported	200	140 (70%)	60 (30%)	103 (51.5%)	38 (19%)	59 (29.5%)	

**Table 02: Comparative efficacy of *Mycoplasma synoviae* local isolate coated plates (37 and 4°C) and pre-coated (imported) by Enzyme linked Immunosorbent assay**

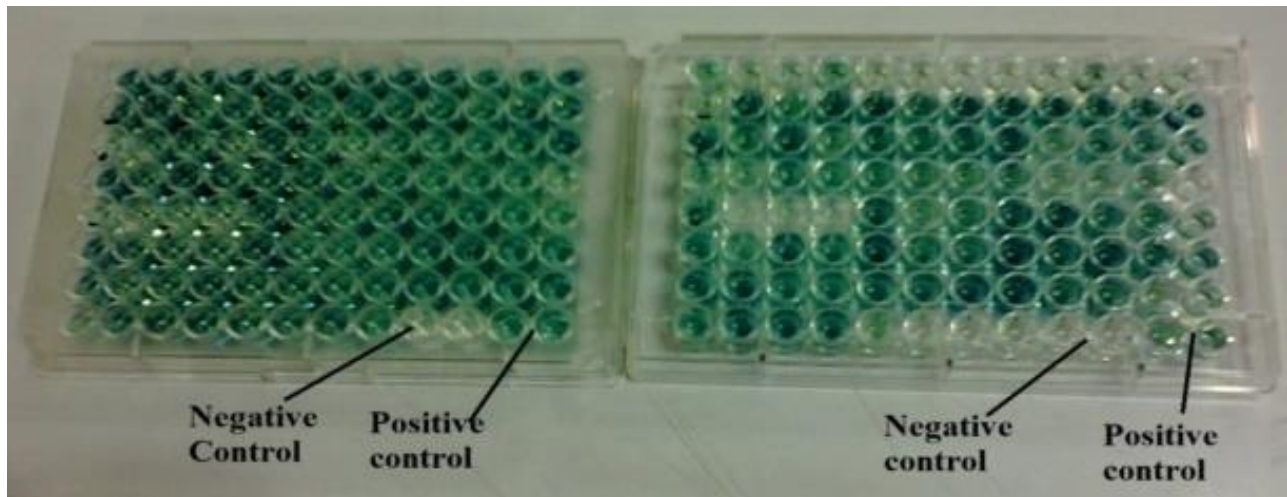
Antigen used	Incubation temperature	No. of samples tested	No. of positive samples	No. of probable samples	No. of negative samples	P valve
Local	37°C	47	41	04	02	P
Local	4°C	47	41	03	03	1.0000
Imported	37°C	47	37	01	09	

**Table 03: Comparative efficacy of *Mycoplasma synoviae* local isolate coated plates (37°C) and pre coated (imported) by Enzyme linked Immunosorbent assay.**

Antigen used	No. of samples tested	No. of positive samples	No. of probable samples	No. of negative samples	P value
Local	92	82 (89.13%)	08 (8.69 %)	02 (2.17 %)	0.0177
Imported	92	74 (80.43 %)	07 (7.60%)	11 (11.95 %)	



**Figure 1: RSA comparison of local and commercial colored antigen**



Enzyme linked Immunosorbent assay using *Mycoplasma synoviae* local isolate coated plate and pre coated (imported) Left: ELISA plate coated with local isolate and Right: Pre coated ELISA plate

**Figure 2: Comparison of locally prepared *Mycoplasma synoviae* antigens with commercially available by Rapid slide agglutination test and enzyme linked immune sorbent assay.**

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