INT. J. BIOL. BIOTECH., 15 (1): 39-45, 2018.

DEVELOPMENT OF ELISA AND ITS COMPARISON WITH OTHER DIAGNOSTIC TESTS FOR AVIAN MYCOPLASMOSIS

Faiz Muhammad, Jehan Alam, Johar Hussain, Syed Khurram Fareed, Urooj Zafar, Shakeel Ahmed Khan and Aqeel Ahmad*

Department of Microbiology, University of Karachi, Karachi, Pakistan
*For Correspondence: aahmad57@gmail.com

ABSTRACT

Avian mycoplasmosis is caused by several pathogenic mycoplasmas species including *Mycoplamsa gallisepticum*, *M. synoviae, M. meleagrides* and *M. iowae*. *M. gallisepticum* (MG) and *M. synoviae* (MS) are responsible for considerable economic losses in the poultry industry, especially in Pakistan and other developing countries. Hence constant surveillance is required for effective control of avian mycoplasmosis in this region. In the present study serological (immunodiffusion, ELISA) and PCR tests were performed and their efficiency on healthy and experimentally and clinically infected chickens' sera was evaluated. Isolation of pathogen from infected birds is a gold standard for disease diagnosis. During the present study, mycoplasma was isolated from only less than 10% clinically positive samples and among these isolates 73.63% were identified as *M. gallisepticum* by using specie specific PCR technique. ELISA was found to be the most sensitive serological test compared to immunodiffusion for detection of anti-*M. gallisepticum* antibodies in the sera of infected birds; which gave 88% sensitivity with 92% specificity. Among the clinically infected bird samples, 72% samples were found positive for anti- *M. gallisepticum* antibodies. Interestingly, 28.71% of clinically healthy birds' sera also showed the presence of antibodies, indicating the extent of mycoplasma burden on the poultry farms of this region.

Key words: Avian Mycoplasmosis, *M. gallisepticum,* Diagnosis, ELISA

INTRODUCTION

Microbial infections are the constant threats and most important cause of economic loss in poultry industry world-wide, including Pakistan (Mustafa and Ali, 2005). The poultry industry in Pakistan has helped in poverty alleviation by providing jobs and low-cost good quality animal protein (Memon, 2013). However, poultry farming in Pakistan is a high-risk business due to maintenance cost and regular outbreaks of infectious diseases (Nauman *et al*., 2005). These outbreaks demand extensive work for development of early and cheap but reliable diagnostic tests for controlling infectious diseases.

Infectious diseases could be effectively controlled by good surveillance, which requires effective monitoring system for determining microbial prevalence in the poultry-farms. Diagnosis is generally based on detection of pathogens in clinical specimen or detection of antibodies in infected animals. Later method is easy to perform and helpful in monitoring the prevalence of pathogens. Since presence of antibodies, that are highly specific, indicate exposure to pathogens. So, detection of antibodies at an early stage can helpful in diagnosis of infectious disease and guide to take appropriate prophylactic measures to control outbreaks.

Mycoplasmosis, caused by *Mycoplasma* sp., is one of the major causes of economic losses in poultry. Mycoplasmas are important avian pathogens causing widespread diseases in chickens, resulting in decreased growth rate, weight, egg-production, and has higher mortality (Bradbury, 2000; Buim, *et al*., 2009). There are four commonly recognized avian mycoplasmas including *Mycoplasma gallisepticum*, *M. synoviae*, *M. melagridis* and *M. iowae*. But *M. gallisepticum* is considered to be the main cause of chronic respiratory disease in chicken which significantly affects commercial chicken worldwide (Buim *et al*., 2009; Yoder, 1991).

To control mycoplasma in poultry, it is necessary to carryout routine checkup at the poultry farms for the presence of *M. gallisepticum* and *M. synoviae*. Keeping the probable cause of mycoplasmosis, the present study was designed to perform diagnostic tests (culture and serology) for avian mycoplasmosis caused by *M. gallisepticum* that is prevalent in the poultry farms of Pakistan.

MATERIAL AND METHODS:

Isolation and identification of Mycoplasmas:

Sampling: Clinical samples of birds with observable symptoms like pale-comb, respiratory rales, listlessness, dehydration, emaciation, retarded growth and lameness, were obtained from diagnostic labs Micro laboratory , PRI (Poultry Research Institute, Karachi) and different farms located in Gaddap Town across the Northern bypass and Hub regions near Karachi.

Sample collection: The swab samples were directly inoculated in Frey's broth (Frey, *et al.*, 1968) at fields, while the tissue-samples were collected from the humanely euthanized birds in laboratory and processed before inoculation. Broth showing growth, were inoculated onto Frey's agar and mycoplasmas were identified on the basis of colonial morphology (Kleven, 1998) and confirm by PCR.

Polymerase Chain Reaction (PCR)

PCR based identification of *Mycoplasma* was performed by using species specific primers for *M. gallisepticum* and *M. synoviae* following method as described below.

DNA from mycoplasma isolates was extracted as described elsewhere (Garcia *et al*., 2005) with some modification. Briefly, A fully grown culture 48 hr old culture (10^8 colony forming unit (CFU) was harvested at 12000 rpm for 15 min., at 4^{o}C and the pellet was washed (x2) with 200 µl PBS and suspended in 25 µl PBS. Then heated to boil for 5 min., followed by incubation on crushed ice for 5 min., centrifuged at 12000 rpm for 3 min., and the supernatant containing DNA was collected and stored at -20^{o}C until PCR.

For *M. gllisepticum*, PCR reaction runs against *mgc 2* gene (F; 5' CGCAATTTGGTCCTAATCCCCAACA, R; 5' TAAACCCACCTCCAGCTTTATTTC). Cycles conditions were 93^{o}C for 3 min, and then followed with 35 cycles for 94 ºC for 30 sec, 58 ºC for 30 sec, and 72 ºC for 60 sec. Final product size is 236-302bp (Gracia *et al*., 2005).

For M. *synoviae* characterization 16S rRNA specific primer used (F; 5' GAGAAGCAAAATAGTGATA-TCA R; 5' CAGTCGTCTCCGAAGTTAACAA). The cycles comprise of 94ºC for 5 min, followed with 35 cycles of 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1min. final extension step carried out at 72ºC for 5 min. Final product size is 205 bp (Marois *et al*., 2000).

The amplification reactions were performed in a total volume 25 µl, containing 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.5 µM primers, and 0.5 unit of Taq polymerase (KAPA, Biosystem) and 1 µl template. After amplification, products were subjected to electrophoresis in 1.5% Agarose gel containing ethidium bromide (0.5µg/ml) at 100 Volt in TBE buffer.

Immunodiffusion

All sera samples were screened for the presence of antibodies against *M. gallisepticum* using Immunodiffusion method (Catty and Raykundalia 1989). Briefly, 4 ml melted agar prepared in barbitone/ buffer (0.07M, pH: 8.6) was evenly spread on a clean slide and allowed to solidify. Wells were prepared and 10 µl of serum samples were added to these wells surrounding the center well containing sonicated culture-extract of *M. gallisepticum*, as antigen, incubated at 4ºC in a moist chamber overnight. Gel was desalted, dried and fixed. The slides were stained with coomassie blue and then destained with solution containing methanol, acetic acid and water (5:10:85).

ELISA

All sera samples were screened for antibodies using ELISA as described elsewhere (Catty and Raykundalia, 1989). Briefly, 100 µl of 10 µg/ml of sonicated sample of *M. gallisepticum*, prepared in carbonate/bicarbonate buffer (0.05M), pH 9.6, was applied to 96 wells microtitre plate and incubated overnight at 4ºC. Unadsorbed antigen was removed by washing (x3) with saline containing 0.05% Tween-20. 100 µl of 1:100 diluted serum samples was added to the wells and incubated for one hour at 37ºC. Plates were washed (3x) with saline Tween-20 followed by addition of 100 µl of 1:1000 diluted Conjugate (Rabbit anti-chicken Ig.HRP- sigma) to each well and incubated at 37ºC for an hour. After washing, 100 µl of substrate (OPD) with H_2O_2 was added to each well and plates were incubated for 20-30min. The reaction was stopped by adding 20% H_2SO_4, and OD measured at 490nm in ELISA reader (BioRad Model 680XR).

M. gallisepticum fields isolate experimentally infected birds (no. 25) were used as Positive and Uninfected birds used as Negative control (data not shown here).

The serum samples obtained from diseased birds (from diagnostic laboratory and poultry farms) and healthy birds (from poultry farms & markets) were used to evaluate the performance of serological tests

RESULTS

Total 241 samples were culture for mycoplasmas collected from infected birds but only 22 samples (<10%) were found positive (Table 1). Furthermore, the most suitable collection site is choanal cleft, swabbing of choanal cleft was found easier and reproducible.

Typical mycoplasma colonies on Frey's medium were observed as smooth, 0.1-1 mm in diameter with dense and elevated centers (Figure-1).

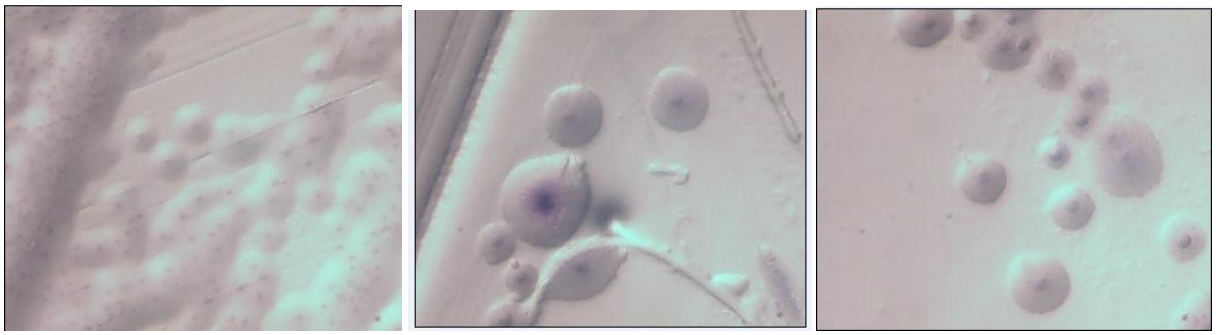

Fig.1. Fried Egg Colonies of *Mycoplasma* sp. (10x).

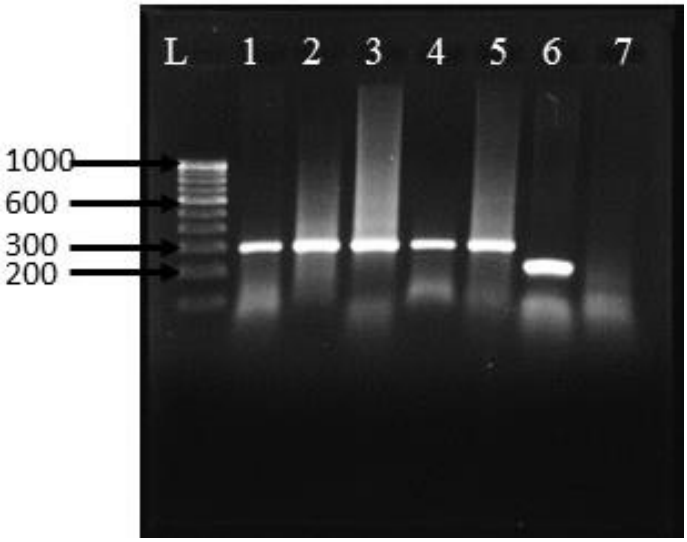

Fig. 2. Lane-1: Ladder (100bp), Lane 2: S_6 reference strain (ATCC 15203), Lane3-7 field isolates & Lane 8: *M. synoviae* WVU 1830 reference strain (ATCC 25204)

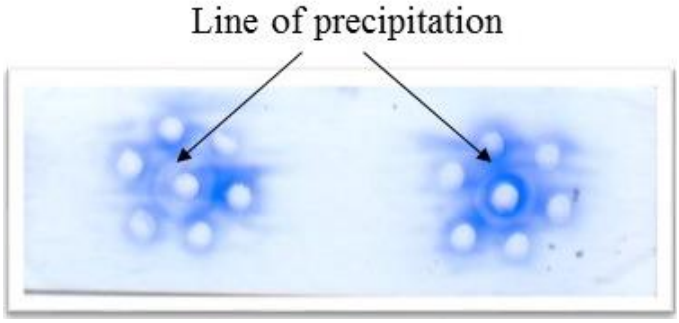

Fig. 3. Lines of precipitation showing presence of *Mycoplasma* antibodies by Immunodiffusion.

Table 1. Isolation of Mycoplasma from infected birds

No. of samples	Collected from body sites	Culture	% of positive sample
Oral swabs			
52	Trachea	2	3.846
105	Choanal cleft	13	12.38
At necropsy			
42	Trachea	6	14.285
27	Air sacs	0	0
15	Synovial fluid	1	6.66
Total =241		22	9.1286

Table 2. Mycoplasma antibodies in the sera by immunodiffusion.

Samples	Negative	Positive
Control negative (25)	25	00
Control Positive (25)	17	08
Clinically positive (25)	20	05
Clinically negative (25)	23	02

Table 3. Detection of antibodies against *M. gallisepticum* by ELISA in birds.

	<0.1	0.1 to 0.5	>0.5
Sample	Negative	Positive	Highly positive
Control negative (25)	23	2	0
Control Positive (25)	3	18	4
Clinically positive (25)	7	12	6
Clinically negative (491)	350	103	38

Table 4. Serological tests for the detection go antibodies against *M. gallisepticum.*

Test analysis	Immunodiffusion	ELISA
Sensitivity	32	88
Specificity	100	92
Positive predictive value	100	91.66
Negative predictive value	59.52	92

PCR Analysis

Out of 22 isolates only 16 were confirmed as *M. gallisepticum*, while the others could not be identified. No culture was found positive for *M. synoviae* during the present study. The remaining isolates may be of nonpathogenic mycoplasmas or may belong to some other group of microorganism.

Immunodiffusion

About one third control samples from infected bird positively reacted for the presence of antibodies by immunodiffusion (Figure-2); however, no antibody was detected in control negative samples. In clinically positive infected birds, only 20% samples were found positive for anti-*M. gallisepticum* antibodies (Table 2).

ELISA

Around 88% of the samples were positive in the positive-control subjects. While studying clinically positive samples, 72% samples were found positive for anti-*M. gallisepticum* antibodies. Remaining 28% samples were probably infected with some other agents. Interestingly 28.71% of clinically healthy birds' sera also showed antibodies against *M. gallisepticum* indicating a subclinical infection (Table 3).

ELISA was found to be most sensitive test for the detection of antibodies against *M. gallisepticum*. Sensitivity of ELISA was 88% with specificity of 92% in the present study. With control-positive and negative samples, the positive and negative predictive values were found to be 91.66% and 92%, respectively.

DISCUSSION

Poultry breeders and producers need to maintain their flocks free of pathogenic mycoplasmas, particularly, *M. gallisepticum*. Because the infection of poultry with either *M. gallisepticum* or *M. synoviae* is often subclinical, screening process should detect infection at an early stage.

In this study, we applied culture and serologic techniques for the diagnosis of avian mycoplasmosis. Both methods are useful but ELISA was found more economical and sensitive than either culture or immunodiffusion. The serological test should be sufficiently sensitive and specific to identify which particular *Mycoplasma* is causing the infection with minimum false positive results

Culture of any pathogenic organism is always a key for the detection and diagnosis of infection, having great value in epidemiological aspects as well as diagnosis. It provides information about the prevalence of organism and it's linked nearby forms. For clinical value, its provide information about the virulence potential and susceptibility to chemotherapeutic agent *etc* (Ley *et al*., 2003).

Overgrowth of contaminants is a major problem in isolation of mycoplasmas (Kleven, 1998). It was observed that choanal cleft swabs or minced tissue samples inoculated in Frey's broth produce color change after 12-24 hours, indicated the growth of contaminated organisms. However, the broth that showed color change or turbidity after 48 hours or more with less turbidity has higher chances of recovering the mycoplasmas. Furthermore, filtration of the suspension through 0.45µm before inoculation on Frey's agar media enhances recovery. Although the diagnosis of mycoplasmas on the basis of culture is ideal, but it has a limitation that it cannot be used for routine screening (Zain and Bradbury, 1996).

The swab samples are easy to collect from birds that permit to collect a vast number of samples with significant chances of recovery of avian mycoplasma. The choanal cleft (Palatine fissure) swabs were found reproducible than tracheal swabs.

The sensitivity of culture was considerably lower <10% in our study than other studies 18.5% (Mukhtar *et al*., 2012a); 25.5% (Osman *et al*., 2009); 57% (Muhammad *et al*., 2017), 62.9% (Heleili, *et al*., 2011). This study have been carried in hot weather while avian mycoplasmosis is more prevalent in winter season (Buim *et al*., 2009; Sun *et al*., 2014; Kahya *et al*., 2015, Muhammad *et al*., 2017). Kahya *et al.*, (2015) reported 33% of samples positive for MG in winter seasons while zero positivity in in summer season. Similarly 8.1% MS detected in winter season while 0% in summer seasons. Another reason could be due to indiscriminate prophylactic use of antibiotics by the farmers or as therapeutic measures (Feberwee, *et al*., 2005) or the number of organisms may be too low to be detected by culture technique.

Immunodiffusion and ELISA were carried out to detect antibody in the infected birds. In the control study, immunodiffusion was found to be a poor test, while ELISA was much better assay for detecting the anti-*M. gallisepticum* antibodies.

ELISA was superior in sensitivity than Immunodiffusion. But the latter is relatively cheaper and easier to perform. The low positivity of Immunodiffusion test may probably be due to too low titer of antibodies; and secondly, since only *M. gallisepticum* was used as antigen, birds may have had mycoplasmas infection other than *M. gallisepticum*. Nevertheless, clinically-negative samples also showed the presence of antibodies against *M. gallisepticum* in 8% of the specimen, an indication of possible subclinical infection of *M. gallisepticum* or probably due to an infection by some other agents exhibiting antigenic similarity to *M. gallisepticum* or because of some unknown reason.

The seroprevalence of *M. gallisepticum* was 72% and 28% in clinically infected and healthy birds respectively, detected by ELISA. Tossi *et al*., (2005) also detected 33% in single-age farm and in 77.8% multi-age farm in both eggs and sera. In broiler breeder farm 21.4% among which higher in female (56.2%) than male (43.79 %) (Seifi and Sheirzad, 2012); Feizi *et al.*, 2012). Kaboli *et al.*, (2013) also observed that seropositive sample by ELISA also were found positive by PCR. 52.33 % in backyard poultry (de Sa *et al*., 2015). However, all studies indicate a large burden of *M. gallisepticum* on poultry industry. The sensitivity of the test (88%) comparable with other study as 94% (Duffy *et al*., 1999).

PCR is one of the important tools for rapid diagnosis and detection. They offer high sensitivity and specificity (Marois *et al*., 2002; Nascimento *et al*., 1991). In this study we used PCR to identify *Mycoplasma* sp. using specific primer for *M. gallisepticum* and *M. synoviae*.

In developed countries, avian mycoplasmosis caused *M. gallisepticum* is greatly reduced by using effective vaccination program, but, there are complaints about *M. synoviae* (Buim *et al*., 2009). The present study aimed to develop methods to detect the presence of mycoplasmas infection in commercial poultry industry due to *M. gallisepticum* and *M. synoviae*. Currently there is no adequate serodiagnostic facility available for avian mycoplasmosis. ELISA proved to be highly sensitive and more specific compared to other tests. It could be combined with culture to confirm the infection.

Acknowledgements:

This work was conducted by the support of HEC under National Research Program for Universities. We are extremely thankful to all those who helped in this project, especially Dr. Muhammad Akram, Consultant, Micro Laboratory, Karachi, Dr. Aslam Jalali, Poultry Production & Research, Government of Sindh serum samples and clinical materials from infected birds.

REFERENCES

Avakian, A.P., S.H. Kleven, and J.R. Glisson (1988). Evaluation of the specificity and sensitivity of two commercial enzyme-linked immunosorbent assay kits, the serum plate agglutination test, and the hemagglutination inhibition test for antibodies formed in response to *Mycoplasma gallisepticum*. *Avian Disease*, 32 (2): 262 – 272.

Bradbury, J..M. (2001). Avian mycoplasmas; In: *Poultry Diseases* (F. Jordan, M. Pattison, D. Alexander, T. Faragher, W.B. Saunders, (eds.) 5th Ed: London, UK, p:178-193.

Buim, M.R., E. Mettifogo, J. Timenetsky, S.H. Kleven, and A.J.P. Ferreira (2009). Epidemiological survey on *Mycoplasma gallisepticum* and *M. synoviae* by multiplex PCR in commercial poultry. *Pesquisa Veterinaria Brasileira*, 29(7):552-556.

Catty, D. and C. Raykundalia (1989). *ELISA and related enzyme immunoassay in Antibodies: A practical approach*. Vol. II. IRL Press Oxford and Washington DC, p 97-154.

De Sa, S.G., J.W.P. Junior, S.M. de Vilela, E.P.B.X. Moraes, P.P.F. Albuquerque, D.R.A. Ferreira and R.A. Mota (2015). Occurrence and risk factors assessment associated with Mycoplasma gallisepticum infection in chickens in the semi-arid region of Pernambuco, Brazil. *Pesquisa Veterinaria Brasileira* 35 (6); 531-535.

Duffy M.F., K.G. Whithear, A.H. Noormohammadi, P.F. Markham, M. Cotton, J. Leydon J, and G.F. Browning (1999). Indirect Enzyme-Linked immunosorbent assay for detection of immunoglobulin G reactive with a recombinant protein expressed from the Gene encoding the 116-kilodalton protein of *Mycoplasma pneumonia, Journal of Clinical Microbiology*, 37 (4) 1024-1029.

Feberwee A, D.R. Mekkes. J.J. De Wit, G.E. Harman and A. Pijpers (2005). Comparison of culture, PCR, and differential serologic tests for detection of *Mycoplasma gallisepticum* and *M. synoviae* infections. *Avian Dis.*, 49: 260-268.

Feizi A., P. Bijanzad, M. Khakpour, H. Nikpiran, K. Kaboli, A.R. Jeyrani-Moggadam (2012). Seroprevalence of *Mycoplasma gallisepticum* infection in Iranian north-west broiler breeder farms, *Annals of Biological Research*, 4(4): 109-111.

Frey M.L., R.P. Hanson, and D.P. Anderson (1968). A medium for the isolation of avian mycoplasmas. *American Journal of Veterinary Research*. 29:2163.

Garcia M., N. Ikuta, S. Levisohn and S.H. Kleven (2005). Evaluation and comparison of various PCR methods for detection of *Mycoplasma gallisepticum* infection in chickens. *Avian Disease*, 48:125-132.

Heleili, N., B. Mamache, and A. Chelihi (2011). Incidence of avian mycopasmosis in the region of Batna, Eastern Algeria. *Veterinary World*, 4(3): 101-105.

Hossain, K.M.M., M.D.T. Hossain, I. Yamato (2010). Seroprevalence of *Salmonella* and *Mycoplasma gallisetpicum* infection in chickens in Rajshahi and surrounding districts of Bangledesh. *International Journal of Biology*, 2: 74-80.

Kaboli, K., P. Bijanzad, A.R.J. Moggadam, M. Shahbazi, H. Hosseini (2013). Evaluation of *M. gallisepticum* Infection diagnosis in rural poultry by 16S rRNA PCR methods. *European Journal of Zoological Research*, 2 (4):63-66.

Kahya, S., Yilmaz, O., Eyiogr, A., Temelli, S., Carli, and K.T. 2015. Detection of *Mycolasma gallisepticum* and *M. synoviae* by real-time PCR and *Mycoplasma gallisepticum*-antibody detection by an ELISA in chicken breeder flocks. Kafkas Univ. Vet. Fak. Derg. 21(3), 361-366

Kleven, S. H. (1998) *Mycoplasmosis*; In: *A Laboratory Manual for the Isolation and Identification of Avian Pathogens* (D.E.Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson, and M.W. Reed, eds) 4th ed. Kennett Square: American Association of Avian Pathologists p 74 – 80.

Ley, D.H. (2003). *Mycoplasma gallisepticum* infection. In: *Diseases of poultry* (Y.M. Saif, H.J. Barnes, A.M. Fadly, D.L. Glisson, L.R. Mcdougald, and D.E. Swayne eds) 11th ed: Iowa State University Press: Ames, Iowa, 722-744.

Marois, C.F., F. Dufour-Gesbert and I. Kempf (2002). Polymerase chain reaction for detection of *Mycoplasma gallisepticum* in environmental samples. *Avian Pathology,* 31, 163-168.

Memon, N.A. (2013). Poultry: Pakistan's fastest growing industry. Pak. Food J. 24-27.

Muhammad, F., J. Hussain, S.K. Fareed, T.A. Khan, S.A. Khan, and A. Ahmad (2017). Diagnosis of avian mycoplasmas: A comparison between PCR and culture technique. *Archive of Razi Institute*. DOI. 10.2292/ari.2017.108217.1085. In press.

Mukhtar, M., M.M. Awais, M.I. Anwar and Z. Hussain (2012a). Pathogenicity and effect of exogenous antibodies on the viability of *Mycoplasma gallisepticum* in chicken embryos. *Pakistan Journal of Life Social Science,*10(1): 13-17.

Mukhtar, M., M.M. Awais, M.I. Anwar, Z. Hussain, N. Bhatti and S. Ali (2012b). Seroprevalence of *Mycoplasma gallisepticum* among commercial layers in Faisal Abad, Pakistan. *Journal of Basic and Applied Sciences,*8:183-186.

Mustafa, M.Y. and S.S. Ali (2005). Prevalence of infectious diseases in local and fayoumi breeds of rural poultry*(gallus domesticus), Punjab University Journal of Zoology* 20 (2), 177-180.

Nascimento, E. R., R.Yamamoto, K.R. Herrick and C. Tait (1991). Polymerase chain reaction for detection of *Mycoplasma gallisepticum*. *Avian Disease*, 37:203-211.

Nouman, M., M.A. Zahoor, H.A. Khan and M. Siddique (2005). Serologic status of Newcastle disease in broilers and layers in Faisalabad and surrounding districts. *Pakistan Veterinary Journal*, 25(2): 55-58.

Osman, K.M., M.M. Aly, Z.M.S. Amin and B.S. Hassan (2009). *Mycoplasma gallisepticum*: an emerging challenge to poultry industry in Egypt. *Scientific and Technical Review-International office of Epizootics*, 28: 1015-1023.

Seifi, S. and M.R. Shirzad (2012). Seroprevalence and Risk Factors of *Mycoplasma gallisepticum* Infection in Iranian Broiler Breeder Farms. *International Journal Animal and Veterinary Advance*, 41(1) 45-48.

Sun, Y., L. Lin, Z. Chen, L. Wu, Z.L. Xiong and X.M. Li (2014). Serology study of *Mycoplasma gallisepticum* in broiler chickens in Chon Qing. *J. Anim. Vet. Adv.*, 13, 5-8.

Tosi, G., L. Roberto, T. Marco, C. Roberto (2005). Evaluation of an egg yolk enzyme-linked immunosorbent assay antibody test and its use to assess the prevalence of *Mycoplasma gallisetpticum* infection in laying hens in Italy. *Italian Journal of Animal science*, 4: 272-275.

Yoder, H. W. Jr. (1991). *Mycoplasma gallisepticum* infection. In: *Diseases of Poultry* (B.W. Calnek, H.J. Barnes, C.W. Beard, W.M. Reid and H.W. Yode Jr. eds). Iowa State University Press, Ames Iowa. pp: 198-212.

Zain, Z.M. and J.M. Bradbury (1996). Optimising the condition for isolation of *Mycoplasma gallisepticum* collected on applicator swabs. *Journal of Veterinary Microbiology*, 49: 45-57.

(*Accepted for publication December 2017*)