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Seropositivity, Involvement in Suspected Cases of Chronic Respiratory Diseases and Comparative Efficacy of Various Sero-Diagnostic Tests of *Mycoplasma Gallisepticum*

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ABSTRACT

Mycoplasma gallisepticum (MG) is considered as one of the most prevalent and major cause of Mycoplasmosis in poultry mainly isolated from cases of chronic respiratory diseases (CRD)— an economically devastating disease of poultry birds. Locally prevailed MG clinical isolates are not characterized and serodiagnosis based on exotic antigens may produce erroneous results that further complicate disease management. The current study was designed to investigate the occurrence rate of MG in suspected cases of CRD (Sus-CRD) and efficacy of available serological diagnostic tests that are routinely implied for the purpose of screening of MG in poultry birds in Pakistan. A total of 846 of poultry- and 22 of pheasantry-birds of Sus-CRD samples were subjected to different serological tests such as serum plate agglutination test (SPAT), ELISA, hemagglutination inhibition (HAI). Our results indicated that out of 846 sera samples, 29.71% (n=251) samples were positive with SPAT, 42.79 % (n=362) were positive with ELISA and 18.94% (n=160) with HAI against MG. Similarly, test results performed on samples obtained from Sus-CRD pheasantry birds indicated that 18.18% (n=4) were positive using SPAT, 27.27% (n=6) by ELISA and 4.55% (n=1) were positive by HAI. A total of 362 ELISA-positive poultry birds samples were processed for isolation and identification of MG by colonial morphology and specie specific PCR. Our results indicated that 152 (41.98%) samples were successfully cultured, of which, 119 isolates (78.2%) were identified as MG isolates. Our study concludes a higher frequency of MG causing CRD infection in poultry in Northern Pakistan and ELISA as sensitive test for screening.
KEYWORDS: Mycoplasmosis, Chronic Respiratory Diseases, Poultry, pheasantry birds

INTRODUCTION

Mycoplasmosis is one of the biggest threats to Poultry industry of Pakistan. Mycoplasmosis is caused by one of the four major species of *Mycoplasma* comprising of *M. gallisepticum* (MG), *M. synoviae* (MS), *M. meleagrides* (MM) and *M. iowae* (MI) [1]. Remarkably, MG is reported as the main cause of chronic respiratory disease (CRD) an economically devastating and long-lasting infection [2, 3 and 4]. As such in Pakistan, precise and an overall countrywide prevalence of MG infection is not known yet mainly due to lack of operational and regular vigilance and coordinated surveillance programmes. Nevertheless, there are reports on random infection as well outbreaks of MG in various parts of Pakistan [5]. Aerosol is one of the main routes for spread of MG infection, which is followed by local spread of the bacterium in the upper respiratory tract. The problem becomes more cumbersome with co-infection of other bacteria such as *E. coli,* Newcastle disease virus and infectious bronchitis virus *etc.* CRD infected flock severely goes down in production with apparent decline in weight gain, decrease in feed efficiency and egg production revealing huge economic losses to the industry.

The gross lesions of Mycoplasmosis include mild sinusitis, tracheitis and air sacculitis. Furthermore, eyelids of infected birds may become swollen with ocular discharge and drainage may be seen from nares. Macroscopic and microscopic lesions comprised of necrosed and swollen tracheal epithelium, loss of cilia and mucous layer that covered whole of trachea. Lungs show congestion, hemorrhages, focal necrosis and leukocytic infiltration particularly lymphocytes and polymorphs nucleated cells. The disease is vertically transmitted resulting poor hatchability and chick quality [6 and 7]. Poultry industry in Pakistan is a vibrant sector with a lion share in the national economy. Since 1984 of the first serological evidence of MG infection in Pakistan [8], a continuous higher incidence rate in different regions of Pakistan has resulted huge economic losses in poultry sector [9, 10 and 5].

Early, quick and efficient diagnostic tools must be available to screen for MG infection. In a given set up, various serological procedures are generally applied for diagnosis of MG infected flocks. These include

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Serum Plate Agglutination test (SPAT), Hemagglutination (HA) test, Hemagglutination Inhibition (HAI) test and Enzyme Linked Immunosorbant Assay (ELISA) based principally on specificity of antigen antibody reaction. Notably, reports are not available on the antigenic characterization of the local MG isolates, and commercial antigens available in the market for SPAT test and MG-specific-monoclonal-antibody coated plates for ELISA may produce variable results due to possible slight variation in field isolates. A false positive or negative test thus could further complicate the issue that could extrapolate economic losses due to CRD. So far, local antigen prepared indigenously is not available in the market. Therefore, in order to obtain better outcomes, validation and comparison of different available serological tests in local conditions is crucial. Although PCR is highly sensitive and reliable diagnostic tools, but could be expensive and time consuming. Furthermore, procedures for MG bacterium isolation and culture are enormously tedious and expensive, and on top of that, clinical picture is not pathognominic in nature and misleading in the case of co-infection. Therefore, knowledge and skills about the most efficient, accurate and sensitive serological test is mandatory. Finally, literature regarding prevalence of MG infection in poultry birds in northern areas is also rare. Thus, the current study, on the one hands provides updated and comprehensive repot on the frequency of seropositivity and isolation rate of MG in poultry birds suspected of CRD in the Northern areas of Pakistan, while, on the other hands suggests an efficient serological test for MG infected birds.

MATERIALS AND METHODS

Ethical approval

The current study was approved from the ethical committee of the Hazara University, and all the reported work was carried out according to the local and national guidelines of animal ethics.

Sample collection

The current study was carried out during November 2009 and October 2011. Blood sample and swabs (from organs and nasal swabs) were collected from Hazara division Khyber Pakhtunkhwa Pakistan from different poultry farms and Pheasantries. A total of 846 blood samples were collected from CRD suspected poultry birds from 26 different poultry farms of Hazara Division Khyber Pakhtunkhwa. Of these 846 birds, a total of 226 tracheal/nasal swabs/organs samples were also collected immediately after the SPAT test were found preliminary positive. In parallel, all these 226 samples were also processed for isolation.

Serological tests

Serum samples were then subjected to various serological tests available for screening of MG infection. For serum plate agglutination test we used Nobilis® S-6 Adler Strain of MG antigen. Test procedures and interpretation of the results were followed according to the manufacturer's instructions. HAI test was performed as previously published [11]. Commercial ELISA kits (FLOCKSCREEN™ Mg ELISA Kit) were used for ELISA following the manufacturer's instruction. ELISA plate reader at 550nm was used to record the absorbance according to the instructions.

Bacterial isolation and specie specific PCR

Swab samples collected from tissue or nasal cavity were put in 5 ml tubes containing PPLO broth. These tubes were screwed, labeled, and transported under refrigeration (4°C) to veterinary research and disease diagnostic center, Abbotabad, Khyber Pakhtunkhwa. Modified Hay Flick media was used for MG isolation according to standard protocol as described by [12]. The broth and agar media were prepared as per directions of manufacturer. The examination of culture was carried out on regular basis to check the *Mycoplasma* growth by observing change in color and whirling movement inside the tubes. After 15 days, the tubes with no change in color were considered negative and were discarded from the CO2 incubator. The positive cultures *i.e.* showing color changes and turbidity were further processed and filtered through 0.45µ syringe filter. The filtrate was streaked onto solid agar medium incubated at 37°C in 5% CO2 incubator. The Agar plates were examined daily under stereomicroscope or light microscope at (10X) (Olympus) for about 5 days of incubation for observing typical *Mycoplasma* colonies. The isolated colonies were sub cultured up to four times to obtain possibly pure isolates. The identification of organism was made by colonial morphology of fried egg-like appearance, tiny, smooth and 0.1-1 mm in diameter with dense raised centers rooted in the medium were indicative of *Mycoplasma* species. Finally, PCR reaction was applied on chromosomal DNA obtained from purified colonies of mycoplasma as described earlier [13 and 14]. Chromosomal DNA from MG purified colonies and observation of amplified PCR product was performed as described elsewhere [15]. PCR reaction was performed in thermocycler (BioRAD T100). Primers were synthesized by InVitrogen. The expected amplified PCR product was resolved on 1% agarose gel and visualized on gel doc (BioRad, USA). PCR master mix was purchased from Thermoscientific. Furthermore, MG-specie specific PCR reaction was also carried out on clinical isolates as described below. Vaccinal strain of MG-TS11 (Merial Lyon, France) and genomic DNA of confirm clinical

isolate (a generous gift from University Diagnostic Lab, University of Veterinary and Animal Sciences) were used as positive control during PCR.

RESULTS AND DISCUSSION

Seropositivity against MG of sus-CRD poultry and pheasantry birds

Our results indicated that out of 846 sera samples, 362 (42.79%) were found positive by ELISA, 251 (29.71%) samples were declared positive with SPAT and 160 (18.94%) were found positive using HAI suggesting that ELISA was the most sensitive and accurate assay for screening and diagnostic assays (Table 1). Similarly, analysis on the serum samples of pheasantry birds indicated that out of 22 samples 6 were positive by using ELISA, 4 were positive by using SPAT and one sample was declared as positive through HAI (Table 1). Our results indicated an overall 42.79% seropositivity rate determined by ELISA suggesting a significantly higher prevalence rate of 10% as reported earlier from Punjab Pakistan [9]. In agreement with our findings, Mukhtar *et al*., reported 49.1% seropositivity in Faisalabad, Punjab, Pakistan [5]. Our studies show a lower seropositivity frequency of broiler in all three districts of Hazara division as compared to layers and breeders. This is possibly due to the fact that broiler chicken are kept for shorter period of time before they are marketed thus with minimum period of exposure. Most of the mycoplasma infection is acquired and droplet infection is a common and most crucial route of infection. Interestingly, almost half of the seropositive samples obtained from CRD suspected cases were found positive for MG in this study indicating an active infection. We assume that the infection rate could be even higher than this as MG is highly fastidious and difficult to culture.

Table-1 Seropositivity of MG using different serological tests

Tests applied	Poultry birds		Pheasantry	
	Total number of samples analyzed	Positive + (%)	Total number of samples analyzed	Positive + (%)
SPAT*	846	251 (29.71)	22	4(18.18)
HAI	846	160 (18.94)	22	1(4.55)
ELISA	846	362 (42.79)	22	6(27.27)

*only samples with three positives (+++) and clear-cut positive were taken as positive

Comparative seropositivity of three Districts

A total of 63 blood samples (as well as swabs) were taken from poultry breeder with 40 of them exhibited vaccination history against MG. Interestingly; results indicated that not all serum samples of vaccinated breeders were found positive against MG. Thus, the overall seropositivity (excluding vaccinated birds) was found 42.3% (322/760) by ELISA. The highest seropositivity was shown by layers followed by broiler (Table-2). Hazara Division of Khyber Pakhtunkhwa is comprised of three main districts, and layer birds of District Mansehra were found relatively exposed higher (69.04) as compared to District Abbotabad (63.88) and Haripur (35.71). Serological testing is quite common for flock surveillance against MG, but these are often perplexing and facade intricacy in elucidation due to false positive and false negative results. The multifariousness is essential to be addressed to plan the relevant use of antibiotics in flocks, vaccination schedules and designing bio-security strategies. Several commercial ready to use flock screening test kits with variable sensitivity and specificity have been manufactured and are being used by poultry producers to screen the flock for constant surveillance. Serum Plate Agglutination test also known as rapid slide plate agglutination test has been an established test with rapid results and preferred due to its simplicity and high sensitivity [16]. In line with our findings, Kempf et al., worked on comparison of serological tests including Rapid Slide Agglutination (RSA) Test, HAI and commercially available ELISA test kits [17]. They evaluated the sensitivity and specificity of all these serological tests on sera isolated from specific pathogen free (SPF) chickens infected with MG. In contrary to our findings, their results confirmed that the sensitivity of RSA was superior to ELISA and HAI tests in the capability to identify antibodies formed in early reaction to MG infection [17]. Other studies reported that serum plate agglutination test and ELISA assay could detect antibodies against MG-infections in 69.9% (320/458) and 58.3% (267/458) of the chicken samples, respectively [7]. Similarly, our results corroborate with those reported by Ewing et al., who compared ELISA with HI. They concluded that ELISA is superior to HI in moderate infected flocks but no remarkable difference was found where flocks showed low MG infection prevalence [18]. Altogether, this study indicates that MG is involved in the development of CRD infection screening of which can preferably be carried out with MG specific ELISA.

Table-2 Comparative seropositivity of three Districts using different serological techniques

%	Serology	Broiler (%)	Breeder (%)	Layer/Golden (%)
Mansehra	SPAT positive	25.97	41.26	45.23
	HAI positive	14.59	28.57	35.71
	ELISA positive	34.51	58.73	69.04
Abbotabad	SPAT positive	30.15	52.38	38.88
	HAI positive	17.17	28.57	25.00
	ELISA positive	43.12	66.66	63.88
Haripur	SPAT positive	20.16	25.00	21.42
	HAI positive	9.24	25.00	14.28
	ELISA positive	34.45	37.50	35.71

Incidence of MG in Sus-CRD cases

Nasal swab samples or tissue samples were collected from a total of 362 ELISA positive poultry birds and were directly subjected to MG isolation. Typical purified and suspected colonies of MG were then confirmed by specie specific PCR and results were presented in Table-3. Our results indicated that of 346 samples obtained from ELISA positive poultry birds, 152 (43.3%) could be positively cultured suggesting active infection stage. Of these 152 cultured samples, 119 (78.2%) were identified as MG isolates. Interestingly, most of the clinical isolates (>90%) obtained from poultry breeder were confirmed as MG species suggesting higher infection rate (Table-3). CRD infection caused by MG is economically devastating due to its prolonged nature resulting severe decline in production; and recurrent infection can actually make a farmer out of business. Poultry industry in Pakistan is a huge and vibrant economic sector providing huge employment. CRD infection is a huge challenge for the industry and proper diagnosis of the disease is crucial for control and management. Different serodiagnostic tools such as SPAT, ELSIA and HAI *etc*. are widely used for screening of MG infection in poultry. So far, to the best of our knowledge, literature regarding antigenic characterization of MG field isolates in Pakistan is not available, and therefore, diagnostic test based on the exotic antigen may produce erroneous results. Hence, validation of different diagnostic test is necessary in order to improve diagnosis. In the current study, we have compared three different diagnostic procedures, SPAT, ELISA and HAI, generally used for screening and diagnosis of MG infection in poultry in Pakistan. Further, we also report on the overall seropositivity and isolation rate of MG from CRD suspected birds.

Table-3 Isolation rate (n=346) and identification of MG in sus-CRD birds

	Culture isolates							
	Broiler	PCR + (%)	Breeder	PCR + (%)	Layer	PCR + (%)	Total Cultures	PCR + (%)
Mansehra	28	21 (75)	13	12 (92.30)	31	23 (74.19)	72	56 (77.78)
Abbotabad	31	24 (77.42)	9	7 (77.77)	17	14 (82.35)	57	45 (78.94)
Haripur	14	11 (78.57)	2	2(100)	7	5 (71.42)	23	18 (78.26)
Total	73	56 (76.71)	24	21 (87.5)	55	42 (76.36)	152	119 (78.28)

Conclusion

A total of 846 sera samples of poultry were tested, of which 42.79 % were positive with ELISA, 29.71% samples were positive with SPAT, and 18.94% with HAI suggesting that ELISA is significantly sensitive for screening of MG infection. Almost 50% samples from the ELISA-positive birds could be cultured for MG suggesting active infection. These results have strong implications in the epidemiology, management control of the disease, and proper medication and eradication of MG associated 0infection such as CRD.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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