Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2018.081

SHORT COMMUNICATION

Prevalence of Mycoplasmosis and Antibiotic Susceptibility of *Mycoplasma gallisepticum* in Commercial Chicken Flocks of Rawalpindi Division, Pakistan

H Khatoon[1], F Afzal[2], MF Tahir[2], M Hussain and SU Khan[1*]

[1]Department of Animal Sciences, Faculty of Biological Sciences, Quaid-I-Azam University Islamabad, Pakistan
[2]Disease Section, Poultry Research Institute, Rawalpindi, Pakistan; [3]Animal Health Section, Animal Sciences Institute, National Agricultural Research Center, Islamabad, Pakistan
*Corresponding author: saeedkhan@qau.edu.pk

ARTICLE HISTORY (18-164)

Received: April 28, 2018
Revised: July 05, 2018
Accepted: July 24, 2018
Published online: September 04, 2018

Key words:
Antibiotics
Chicken
Mycoplasma
Pakistan
PCR
Rawalpindi

ABSTRACT

Involvement of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in respiratory disease in commercial chicken showing respiratory signs was investigated using polymerase chain reaction (PCR). Out of the 124 suspected cases, 50 (40.32%) were found to be positive for MG and 17 (13.70%) were positive for MS. The MG isolates were recovered from birds and subjected to antibiotic sensitivity testing by the broth microdilution method. Tilmicosin with MIC_{90} and MIC_{50} values of 3.12 and 6.25 µg/ml were found to be the most effective drug, followed by tylosin, erythromycin and enrofloxacin, all of which gave MIC_{90} and MIC_{50} values of 6.25 and 12.5 µg/ml respectively. Oxytetracycline and chlortetracycline were found to be the least effective drugs and the MIC_{90} value for both these antibiotics was 100.0. The MIC values for various antibiotics recorded in this study were generally higher compared to those found in previous investigations.

To Cite This Article: Khatoon H, Afzal F, Tahir MF, Hussain M and Khan SU, 2018. Prevalence of mycoplasmosis and antibiotic susceptibility of *Mycoplasma gallisepticum* in commercial chicken flocks of Rawalpindi Division, Pakistan. Pak Vet J, 38(4): 446-448. http://dx.doi.org/10.29261/pakvetj/2018.081

INTRODUCTION

MG and MS are the most important mycoplasmal pathogens of chicken. MG causes chronic respiratory disease (CRD) in chicken and MS causes synovitis in chicken. The respiratory form caused by MS is generally subclinical, but the organism may be involved in air sac disease, which is a severe form of air sacculitis that results from infection with MG or MS combined with a respiratory virus or *Escherichia coli*.

Avian mycoplasmosis is widespread in Pakistan and seroprevalence rates of as high as 43.33% have been observed recently (Atta-ur-Rehman *et al.*, 2018). The gold standard for diagnosis of mycoplasmosis is isolation and identification of the causal organism. However, *Mycoplasmas* are slow growing organisms and it takes several days or even weeks for development of bacterial colonies. An early diagnosis of mycoplasmosis is critical in preventing the spread of infection. Detection of *Mycoplasma* DNA in infected birds by PCR is a quick method of diagnosis (Umar *et al.*, 2017). Antibiotics are used to treat MG infection and transmission, and to reduce egg production losses. MG can develop resistance against commonly used antibiotics such as macrolides, quinolones and tetracyclines (Gharaibeh and Al-Rashdan, 2011). It is, therefore, important that antibiotic sensitivity of MG involved in local disease outbreaks be monitored regularly. This helps in choosing the right antibiotics for treatment purposes.

The present research was aimed at studying the relative involvement of MG and MS in respiratory disease at broiler and layer chicken farms of the Rawalpindi Division. Another objective was to determine the susceptibility of the local MG isolates to commonly used antibiotics.

MATERIALS AND METHODS

Sample collection: From July 2015 to June 2016 samples (tracheal swabs and tissues from trachea, air sacs and lungs) from 124 chicken (62 broilers and 62 layers) showing signs of respiratory disease brought for postmortem examination at the Disease Section of the Poultry Research Institute, Rawalpindi. The birds were from different farms located at various places in the Rawalpindi Division. Each bird represented a single poultry farm.

PCR for detection of MG and MS: Genomic DNA was extracted from collected tissues and swabs using PureLink

Genomic DNA Extraction kit (Thermofisher Scientific, Cat. No. K1820-01) following manufacturer's protocol. PCR for detection was performed separately for MG and MS on extracted genomic DNA as described (OIE, 2008). For MG primers MG-14F 5' GAGCTAATCTGTAAAG TTGGTC '3 and MG-13R 5 'GCTTCCTTGCGGTTAG CAAC '3, and for MS primers MS–F 5'GAGAAGCAA AATAGTGATATCA '3 and MS–R 5'CAGTCGTCTCC GAAGTTAACAA '3 were used. The cycling conditions were 40 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, followed by 1 cycle (final extension) at 72°C for 5 min. Electrophoresis was performed on 1% agarose gels. Negative controls (containing water instead of genomic DNA) were included in the PCRs. PCR products from selected samples were purified and subjected to DNA sequencing for confirmation of species as MG or MS.

Isolation of MG: The tissue and swab samples that were found to be positive for MG through PCR were further processed for bacterial isolation. The samples were inoculated into PPLO broth (BD, Cat. No. 255420). Any growth obtained was then inoculated on to Frey's agar medium (BD, Cat. No. 241210). Colonies representative of MG (observed under a stereomicroscope) were sub-cultured in PPLO broth. Genomic DNA was extracted from the broth culture and the PCR, described above, was performed to verify the isolates as those of MG.

Antibiotic sensitivity testing: Isolates confirmed as MG were subjected to antibiotic sensitivity testing by the broth microdilution method. Each isolate was tested against six antibiotics i.e. tylosin, enrofloxacin, oxytetracycline, erythromycin, tilmicosin and chlortetracycline. Briefly each MG isolate was grown for 24 hours in Frey broth (BD, Cat. No. 212346). Then this culture was further diluted (1:5) in Frey broth and incubated at 36°C until the medium became orange. The culture was again diluted 1:10 in broth. Replicate doubling dilutions of each antibiotic were made in 50 µl volumes of broth in microtiter plates. Then 150 µl of broth (pH 7.8) culture containing known density (10^3 to 10^5 CFU/ml) of microorganisms was inoculated into each well. Controls containing microorganisms in broth without antibiotic were included in each case. Then microtiter plates were sealed and incubated at 36°C. The MIC was recorded when the color of controls (containing microorganisms only) just turned orange-yellow (pH 7.0). The MIC was noted as the lowest concentration (highest dilution) of an antibiotic that completely blocked color change in the medium containing microorganisms and that antibiotic. MIC_{50} and MIC_{90} values were calculated as the lowest concentrations that inhibited the growth of 50% or 90% of the isolates respectively as described (Hannan, 2000).

RESULTS AND DISCUSSION

DNA bands of expected sizes were observed both in case of MG (approximately 185 bp) and MS (approximately 211 bp) PCRs (Fig. 1). The DNA sequencing results of the PCR products and BLAST analysis revealed they were regions of 16S rRNA genes of MG and MS. Out of a total of 124 birds (62 broilers and 62 layers), 50 (40.32%) were found to be positive for MG and 17 (13.70%) were found to be positive for MS by the PCR. Out of the 62 broilers 27 (43.54%) and out of the 62 layers 23 (37.09%) were positive for MG. In case of MS, 10 (16.12%) out of 62 broilers and 7 (11.29%) out of 62 layers were found to be positive. Thus, the prevalence rates of both the MG and MS were higher in case of broiler chicken. The PCR positive cases either had MG or MS and there was no case of concomitant infection. MG was successfully isolated from all the 50 PCR positive cases. Fried egg-like colonies typical of MG appeared after 72 hours and the MG-specific PCR conducted on genomic DNA from broth culture confirmed the isolates were of MG.

The results of antibiotic sensitivity of the MG isolates are shown in Table 1. The results showed tilmicosin was the most efficacious drug with a narrow MIC range (0.78-6.25 µg/ml). Its MIC_{90} and MIC_{50} values were 3.12 and 6.25 µg/ml respectively. Tylosin, erythromycin and enrofloxacin were next in effectiveness and had identical MIC range as well as MIC_{90} and MIC_{50} values. There was a wide range of MIC values both for oxytetracycline and chlortetracycline and both these antibiotics had the highest MIC_{90} and MIC_{50} values.

In the current study, we found 40.32 and 13.70% of the birds from various chicken farms of the Rawalpindi Division to be positive for MG and MS respectively. Both MG and MS were more prevalent in broilers compared to layers. Our results are in agreement with the findings of previous studies conducted in Pakistan. According to a province level study mycoplasmosis had one of the highest incidence among the poultry diseases in the Punjab province (Rehman *et al*., 2013). We have detected a high rate of involvement of MS in respiratory disease,

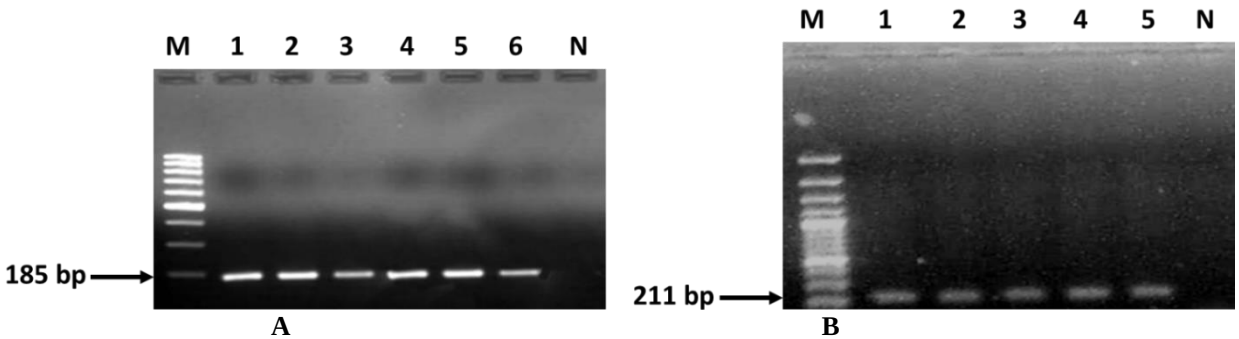

Fig. 1: Results of PCR for the detection of MG and MS in tissues of infected chicken. M: molecular size marker, N: negative control (in both A and B). A) 1-6: MG positive samples. B) 1-5: MS positive samples.

Table 1: Antibiotic sensitivity of MG isolates

Antibiotic	Number of MG isolates with MIC values (µg/ml)									Range	MIC_{50}	MIC_{90}
	0.78	1.56	3.12	6.25	12.5	25.0	50.0	100.0	200.0			
Tylosin	2	3	16	23	4	2	0	0	0	0.78-25.0	6.25	12.5
Erythromycin	2	3	16	23	4	2	0	0	0	0.78-25.0	6.25	12.5
Enrofloxacin	2	3	16	23	4	2	0	0	0	0.78-25.0	6.25	12.5
Chlortetracycline	0	0	3	7	11	14	9	5	1	3.12-200.0	25.0	100.0
Oxytetracycline	0	0	1	3	6	13	17	9	1	3.12-200.0	50.0	100.0
Tilmicosin	13	11	10	12	4	0	0	0	0	0.78-6.25	3.12	6.25

which is in line with results of the studies conducted in various parts of the world. In Jordan, chicken suffering from respiratory disease at 350 broiler farms were tested. The results showed higher number of these flocks were infected with MS (8.9%) compared to MG (6.6%) (Roussan *et al.,* 2015). Results of a large-scale study in Belgium, conducted on broiler chicken, turkeys and wild birds showed the prevalence rates of MS and MG in broilers were 12.9 and 2.7% respectively (Michiels *et al.,* 2016).

To the best of our knowledge this is the first report from Pakistan on the antibiotic susceptibility of local MG isolates. In previous carried out in other countries tilmicosin has been found to be one of the most effective of antibiotics against MG. Very low MIC_{50} and MIC90 values of ≤ of 0.031 µg/ml and ≤ of 0.031 µg/ml for tilmicosin in MG isolates in Jordan were recorded (Gharaibeh and Al-Rashdan, 2011). In the present study the drugs found to be next in efficacy to tilmicosin were tylosin, erythromycin and enrofloxacin, which all had MIC_{50} and MIC_{90} values of 6.25 and 12.5 µg/ml respectively. These three drugs have shown good results against MG in previous studies. Wide range of MIC values against MG for erythromycin and low MIC values for tylosin and enrofloxacin have been observed in several previous investigations (Pakpinyo and Sasipreeyajan, 2007; Ghaleh Golab Behbahan *et al.,* 2008; Gharaibeh and Al-Rashdan, 2011). In our study, the least effective drugs against MG were found to be the tetracyclines. The MIC_{50} and MIC_{90} values were 25.0 and 100.0 µg/ml for chlortetracycline and 50.0 and 100.0 µg/ml for oxytetracycline, respectively. Increase in resistance overtime in MG against tetracyclines has been observed (Gharaibeh and Al-Rashdan, 2011). High MIC value of 25 µg/ml against MG for oxytetracycline have been observed in Iran and Taiwan respectively (Ghaleh Golab Behbahan *et al.,* 2008).

Conclusions: We have noted higher MIC ranges and MIC_{90} and MIC_{50} values as compared to those reported in previous studies, which is an alarming situation.

Acknowledgements: The authors are grateful to the Director Poultry Research Institute, Rawalpindi, Pakistan for providing facilities for this research work.

Authors contribution: SUK and FA conceived and designed the project. HK, MFT, SUK and MH performed the experiments. SUK wrote the article.

REFERENCES

Atta-ur-Rehman, Shah AH and Rahman SU, 2018. Seroprevalance of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in commercial broilers and backyard poultry in five districts of Khyber Pakhtunkhwa-Pakistan. Pak Vet J 38:149-52.

Ghaleh Golab Behbahan N, Asasi K, Afsharifar AR, *et al*., 2008. Susceptibilities of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* isolates to antimicrobial agents in vitro. Int J Poult Sci 7:1058-64.

Gharaibeh S and Al-Rashdan M, 2011. Change in antimicrobial susceptibility of *Mycoplasma gallisepticum* field isolates. Vet Microbiol 150:379-83.

Hannan PC, 2000. Guidelines and recommendations for antimicrobial minimum inhibitory concentration (MIC) testing against veterinary mycoplasma species. Vet Res 31:373-95.

Michiels T, Welby S, Vanrobaeys M, *et al*., 2016. Prevalence of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in commercial poultry, racing pigeons and wild birds in Belgium. Avian Pathol 45:244-52.

OIE, 2008. Avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*). In: OIE manual of diagnostic tests and vaccines for terrestrial animals. OIE, France. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.05_%20AVIAN_MYCO.pdf.

Pakpinyo S and Sasipreeyajan J, 2007. Molecular characterization and determination of antimicrobial resistance of *Mycoplasma gallisepticum* isolated from chickens. Vet Microbiol 125:59-65.

Rehman H, Fawad N, Abbas G, *et al*., 2013. Surveillance of poultry diseases in Punjab province, Pakistan; special reference to Newcastle Disease. Res J Vet Pract 1:1-4.

Roussan DA, Khawaldeh GY and Shaheen I, 2015. A survey of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* with avian influenza H9 subtype in meat-type chicken in Jordan between 2011-2015. Poult Sci 94:1499-503.

Umar S, Munir MT, Rehman ZU, *et al*., 2017. Mycoplasmosis in poultry: update on diagnosis and preventive measures. Worlds Poult Sci J 73:17-28.