Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2021.024

SHORT COMMUNICATION

Mycoplasma gallisepticum Detection in Bangladesh Table Egg Laying Chicken Flocks

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ARTICLE HISTORY (20-574)

Received: November 07, 2020
Revised: January 18, 2021
Accepted: January 19, 2021
Published online: February 17, 2021

Key words:
Backyard poultry
Genotyping
Layer hens
Molecular
Mycoplasma

A B S T R A C T

There is limited information on strains of *Mycoplasma gallisepticum* (Mg) circulating in poultry flocks in Bangladesh. To address this, in a preliminary study, 14 table egg laying chicken flocks which presented with respiratory clinical signs were sampled (oropharyngeal swabs) for detection of the most common and economically important pathogens. DNA and RNA were extracted from the swabs, and subjected to PCR, sequencing and gene analysis. All 14 flocks sampled during the study were positive for Mg, with *mgc2*, *vlhA 3.04a*, *vlhA 3.05* and *mg03659* analyses demonstrating clusters of non-vaccine Mg strains circulating within table egg laying flocks in Bangladesh. The strains showed 93.88 to 97.85% identity to isolates of the neighbouring country, India. Testing by PCR, the same samples were negative for Newcastle Disease virus, infectious bronchitis virus, avian metapneumovirus, avian influenza H9N2 and *Mycoplasma synoviae*.

To Cite This Article: Ganapathy K, Ball C, Kabiraj CK, Nooruzzaman M, Chowdhury EH and Islam MR, 2021. *Mycoplasma gallisepticum* detection in Bangladesh table egg laying chicken flocks. Pak Vet J, 41(2): 306-308. http://dx.doi.org/10.29261/pakvetj/2021.024

INTRODUCTION

Poultry farming, including smallholding enterprises, continues to grow in developing countries, including Bangladesh. In 2015, it was reported that Bangladesh's poultry industry comprises of 30,000 commercial broiler and layer farms, which produces 0.2 million metric tons of poultry meat and 5,210 million table eggs per year (Ali *et al*., 2015). A recent report in 2017 described the total layer egg production in Bangladesh as 32 million eggs per day (Rahman *et al*., 2017), with production split evenly between commercial and smallholder/backyard poultry. Respiratory diseases can have serious ramifications on poultry health, production and welfare, which subsequently impacts the owner's income. Such diseases may also include drops in egg production in egg laying birds, and can be caused by many factors, including the infectious disease *Mycoplasma gallisepticum* (Mg) (Noormohammadi & Whithear, 2019). Information on Mg in Bangladesh's poultry flocks is currently limited, with recent studies focusing only on serology (Sikder *et al*., 2005; Hossain *et al*., 2007; Jalil and Islam, 2010; Ali *et al*., 2015). To date, there has been no characterization of infectious Mg strains through either conventional or molecular methods. We report the detection and molecular differentiation of *Mycoplasma gallisepticum* in Bangladesh's table egg laying chicken flocks.

MATERIALS AND METHODS

Ethical statement: The study was conducted according to the Bangladesh Agricultural University guidelines.

Sampling protocol: A total of 14 flocks of layer hens from farms located in Bhaluk upazilla of Mymensingh district and Sakhipur upazilla of Tangail district in Bangladesh were sampled. All flocks showed respiratory problems. Oropharyngeal swabs were taken from ten birds per flock and pooled together as a single sample. Pooled samples were inoculated onto Flinders Technology Association (FTA) cards and transported to the University of Liverpool for molecular testing.

Questionnaire information: At the point of sampling, questionnaire data was collected for each flock. Information gathered included management practices, purchasing and selling habits, production parameters, vaccination and treatment practices, and factors which farm owners considered important to their production levels.

Molecular detection and typing: A single, complete FTA circle from each flock was placed into 1,000 µl of TE buffer [(10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) (Thermo Fisher Scientific, Massachusetts, USA)] and incubated for 10–15 min. From the TE buffer, extraction of DNA was carried out for each flock sample using the DNEasy mini kit (Qiagen, UK), and subjected to PCR to detect *M. gallisepticum* (Mg) and *Mycoplasma synoviae* (Ms) (Moscoso *et al*., 2004). Positive gene amplicons (Mg – *mgc2*; Ms – *vlha*) were purified using exonuclease and shrimp alkaline phosphatase, and submitted to an external laboratory (Source BioScience, UK) for bi-directional Sanger sequencing. Chromatograms were analysed using Chromas PRO (http://technelysium.com.au/wp/chromaspro/) and MEGA X (Kumar *et al*., 2018), and subjected to BLAST (NCBI Database) to determine the strain identity and nucleotide similarity to previously deposited sequences. For samples positive for Mg (*mgc2* gene), three additional PCR assays were carried out on each sample targeting the *vlhA 3.04a* (187 bp), *vlhA 3.05* (158 bp) and *mg03659* (229 bp) genes as previously described (Ricketts *et al*., 2017). Samples showing a positive amplicon for all four genes were considered as a vaccine strain. Otherwise, samples were considered a field strain of Mg.

Statistical analysis: Statistical analysis was conducted in SPSS Statistics 25 (IBM, UK). Significance of differences between groups was analysed using univariate ANOVA, along with the homogeneity of variance test, to confirm statistical differences within the data set, followed by post hoc Bonferroni testing to compare between each group.

RESULTS AND DISCUSSION

Information regarding the circulating avian respiratory pathogens in Bangladesh is currently limited. A total of 14-layer flocks were sampled from two closely located geographical areas in central Bangladesh. All birds were ISA brown chickens, which were raised in a battery-caged system. The average flock size was 1,322±212 and the average age was 47.1±4.6 weeks old. The closest poultry farm was on average 464.29±57 meters away. Half of the farms disposed dead birds through refuse, with the other half burying the carcasses. All farms sourced their water from a bore well.

The most common reported vaccination (either live or inactivated) was for egg drop syndrome (n=14; 100%), with fowl cholera (Pasteurella) (n=12; 85.7%) and Salmonella (n=11; 78.6%) also included in the majority of flocks (Table 1). Infectious coryza was the least commonly administered vaccine. No mycoplasma vaccines were used in these flocks. Three of the 14 farms were practicing administration of anti-mycoplasma antibiotics (Tiamulin or Tylosin) for every 30 days throughout the laying period (Table 2). Flocks receiving anti-mycoplasma treatments showed production values much closer to the typical breed standard (average difference of 8±1.3) when compared to those flocks that did not receive any treatment (average difference of 22.8±4.7).

All 14 flocks were PCR positive for *M. gallisepticum*, and following Sanger sequencing of a 243 bp amplicon (*mgc2* gene), were identified as ts-11-like (accession numbers MT005810-MT005811 and MT005814-MT005825). Nucleotide similarity to the Mg vaccine strain known as ts-11 (accession number JQ770175) was high, ranging from 93.26 to 99.13%, although no flocks included in the current study were vaccinated against Mg (Figure 1). Previous studies have reported similar findings in the UK and USA, with high similarities of field Mg isolates to ts-11 being noted in commercial layer flocks (Ball *et al*., 2018; El Gazzar *et al*., 2011). Additional PCR gene (*vlhA 3.04a*, *vlhA 3.05* and *mg03659*) analyses showed that all 14 Mg samples were not related to the ts-11 vaccine strain. All three gene targets were amplified for the positive control (ts-11 vaccine) but not for the detected field strains.

Vertical transmission of ts-11 mutants from parent flocks previously vaccinated with ts-11 have been reported before (El Gazzar *et al*., 2011). In the current study, following the analysis of additional gene targets, all strains were determined as not belonging to the ts-11 vaccine strain. However, details of the Mg vaccination status of parent flocks from where the sampled layer hens originated is unknown. Based on the findings of El Gazzar *et al*. (2011) and high nucleotide similarities to ts-11, it is not possible to rule out potential vertical transmission or spread from other ts-11 vaccinated birds from neighbouring farms.

While no *mgc2* sequence data for previous Bangladesh samples was available, detected strains showed 93.88 to 97.85% identity to isolates from the neighbouring country of India (accession numbers: KY467403, KP300762 and KP279741). Previous work on Mg in Bangladesh has investigated seroconversion rates, and detection of the 16s RNA gene by PCR and RLFP (Sikder *et al*., 2005; Hossain *et al*., 2007; Sultana *et al*., 2008; Jalil and Islam, 2010; Ali *et al*., 2015). While Mg serology may have some value in diagnosing unvaccinated flocks, findings could be confusing in vaccinated flocks. Failure to detect and identify respiratory pathogens, including Mg, can have a substantial impact on the health, production and welfare of poultry, and the resulting excessive production losses may affect the livelihood of flock owners. Molecular diagnosis and further strain differentiation of Mg provides stronger evidence of infection (field or vaccine) and the prevalent strain types in Bangladesh, where Mg ts-11 vaccine is being used in some breeder and layer farms.

Detection of Mg was consistent, potentially promoted by all flocks being epidemiologically linked, either through similar rearing systems, close proximity to neighbouring farms, similar chick movements, or sharing of resources, amongst others. It is also of interest that Mg was detected in flocks both receiving and not receiving medication. Furthermore, flocks that had been receiving anti-mycoplasma drugs every 30 days were still Mg positive though the egg production was losses were minimal compared to those not having the drugs. In the future, it is advisable to culture, isolate and identify the Mg through a larger epidemiological study. A viable culture would allow for antimicrobial sensitivity testing. This will assist in the use of appropriate sensitive drugs which will be more efficient for control of infections, better production, and reduction of drug resistance development in flocks.

Based on this preliminary data, it appears that Mg will continue to challenge flock health and productivity in Bangladesh, therefore reducing the income of smallholders and other poultry producers. A thorough epidemiological study would provide scientific evidences on the importance and the role of Mg (and other pathogens) in Bangladesh's smallholder and commercial poultry flocks.

Table 1: Details of flock vaccination history at the time of sampling

Farm	Vaccine									
	Avian influenza	*Egg drop syndrome*	*Fowl Pox*	*Infectious bronchitis virus*	*Infectious bursal disease virus*	*Infectious Coryza*	*Marek's*	*NDV*	*Pasteurella*	*Salmonella*
B01	X	X	X	X	X	X	X	X	X	X
B02	X	X	X	X	X	X	X	X	X	X
B05		X	X	X	X	X	X	X	X	X
B06		X	X	X	X	X	X	X	X	X
S01		X	X	X	X		X	X		
S02	X	X							X	X
S03	X	X	X	X	X	X	X	X		
S04		X	X	X	X	X	X	X	X	
S05		X							X	X
S06	X	X							X	X
S07	X	X							X	X
S08		X							X	X
S09	X	X							X	X
S10		X							X	X
Total	7	14	7	7	7	6	7	7	12	11

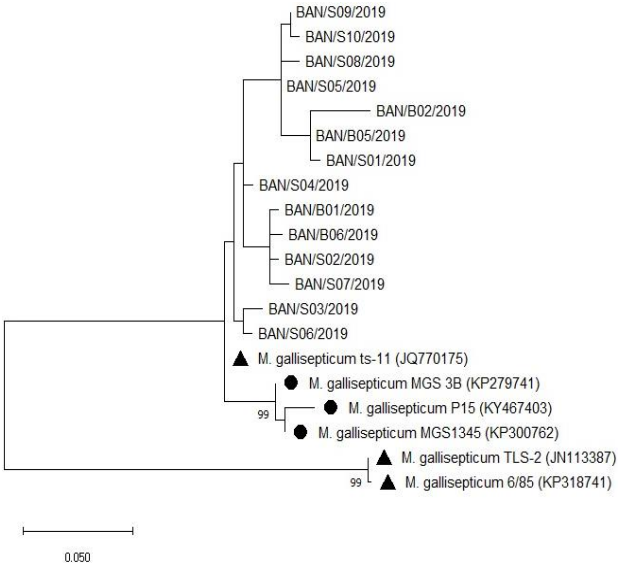

Fig. 1: Maximum likelihood phylogenetic analysis of *mgc2* nucleotide sequences (243bp) from commercial Bangladesh flocks (accession numbers MT005810-MT005811 and MT005814-MT005825). Reference *M. gallisepticum* strains are indicated with a black triangle, and neighbouring isolates from India are indicated with a black circle. Analysis was carried out with the Kimura-2 parameter model and gamma distribution. Bootstrap values below 70 are not displayed. Scale bar represents nucleotide changes per site.

Table 2: Comparison on actual against expected (standard) hen-housed egg production of chicken flocks in Bangladesh. [1]Extracted from the ISA Brown management guide (Hendrix Genetics, Netherlands)

Farm	Age	Production (%)			Anti-mycoplasma
#	(weeks)	*Actual*	*Breed standard*[1]	*Difference*	Treatment
B01	28	85	96	-11	Y*
B02	75	80	76	4	
B05	30	77	95	-18	
B06	80	80	73	7	
S01	52	81	88	-7	Y*
S02	26	75	96	-21	
S03	60	78	84	-6	Y*
S04	32	70	95	-25	
S05	40	65	92	-27	
S06	48	63	90	-27	
S07	32	60	95	-35	
S08	48	55	90	-35	
S09	48	55	90	-35	
S10	34	57	94	-39	

*Anti-mycoplasma drugs given every 30 days.

Acknowledgements: We would like to express our sincere thanks to Dr. Md. Mijanur Rahman, Upazilla Livestock Officer, Department of Livestock Services, Bangladesh for his help during sample collection. Laboratory analyses was supported by an ODA-University of Liverpool Seed Fund.

Authors contribution: KG and MRI conceived and designed the study. CB conducted laboratory analysis. CKK, MN and EHC conducted the field sampling. KG and CB wrote the paper. All authors critically revised and approved the final version.

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