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Mycoplasma synoviae induced eggshell apex abnormalities in commercial layer chicken in Namakkal region of Tamil Nadu

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ABSTRACT

Commercial Babcock layer chicken, 60 to 70 week-old, in 3 flocks housed in Namakkal region of Tamil Nadu exhibited leg weakness and eggshell abnormalities mainly pronounced in apex region of the egg, suggestive of *Mycoplasma synoviae* infection. Choanal cleft and oropharyngeal swabs were collected from affected layer flocks in modified Frey's broth medium. Culture and polymerase chain reaction assay amplifying the conserved region of 16S rRNA gene was applied for the confirmation of *M. synoviae*. The isolates from culture were confirmed by specific amplification of 16S rRNA gene (207 base pair). The current report describe the first eggshell abnormality associated with *Mycoplasma synoviae* infection in India.

Key words: Eggshell apex abnormalities, Layer chicken, *Mycoplasma synoviae*

Mycoplasma synoviae (MS) is considered as the second most important mycoplasma affecting chickens (Kleven 2003). It causes respiratory disease and synovitis in chickens and turkeys. The scale of economic damage caused by MS in layers has been a controversial issue until recently when the possibility of natural or experimental MS infection in chickens causing eggshell apex abnormalities (EAA) was confirmed. In 2008, a new abnormality in the eggshell of chicken eggs was identified in the Netherlands (Feberwee *et al*. 2009b), followed by reports of the same abnormality in Italy (Catania *et al*. 2010), Germany (Ranck *et al*. 2010), and England (Strugnell *et al*. 2011). This egg abnormality is characterized by roughened shell surface, shell thinning, and increased translucency, which leads to an increase in the incidence of cracks and breaks. The abnormalities are confined to the top cone of the egg, up to approximately 2 cm from the apex, and frequently present a very clear demarcation zone. It was called as eggshell apex abnormalities (EAA). This condition has not been reported in India. The present communication deals with the report of MS associated with EAA and molecular confirmation of MS isolated in commercial layer chicken in Namakkal region of Tamil Nadu, India.

MATERIALS AND METHODS

Case history: Farms with sixty to seventy weeks old, three intensive commercial Babcock layer chicken flocks housed in Namakkal region of Tamil Nadu were investigated. A standard vaccination program for layers was followed in the farms. All the three investigated flocks were not vaccinated against *M. synoviae* and were naturally infected. In all the flocks, affected layers exhibited leg weakness, egg production drop up to 10–12% and eggshell abnormalities mainly pronounced in apex region of the egg. Rough patches of various sizes with different densities were found on the blunt end of the eggs. A clear demarcation zone separating the normal part of the shell from the affected portion of the eggs. Specks and cracks were also observed in the eggs with EAA. Breakage or crackling of eggs increased from 0.8 to 2% reaching peak along with increased mortality at 68–weeks old age.

Sample collection: Necropsy was conducted in dead birds. Oropharyngeal and Choanal cleft swabs from suspected live birds, and Tracheal swab, air sac, lungs and oviduct at necropsy were collected in modified Frey's broth medium.

Isolation of causative organism: Modified Frey's broth medium was used for isolation which contains frey's broth base 22.5 g/l, 10% swine serum, 5% yeast extract, dextrose 100 g/l, 10% thallium acetate and penicillin (1000 IU/ml) as mycotic and bacterial inhibitors, 0.0125% β-Nicotinamide adenine dinucleotide (NAD) as a necessary requirement of *M. synoviae* and 0.0125% cysteine hydrochloride as a reducing agent for NAD and 1% phenol red as indicator. pH of the broth medium was adjusted to 7.8 with 20% NaOH. Modified Frey's broth medium was

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sterilized using 0.45 filters.

After sampling, swabs placed in to 3 ml modified Frey's medium and transported to the laboratory, then agitated on a vortex mixer for 30 sec and then swab discarded. Broth medium was incubated under microaerophilic condition with 90% relative humidity at 37°C until the phenol red indicator changed from red to yellow (4–5 days).

Molecular confirmation

Nucleic acid extraction: The broth tube containing mycoplasma bacterial culture was centrifuged at 10,000 rpm for three min. The supernatant was discarded and pellet was used for extraction of nucleic acids. Pellet was washed with PBS, resuspended in 20 µl nuclease free water and boiled for 5–10 min and then kept on ice. The suspension was centrifuged at 1,500 rpm and supernatant was used as DNA template. The PCR was carried out in a 25 µl reaction.

Polymerase chain reaction (PCR): Polymerase Chain Reaction for detection of *Mycoplasma synoviae* was done by amplifying a 207 bp fragment corresponding to 16S rRNA gene sequence, as per the method of Lauerman *et al.* (1993) with minor modification. The following sequences were used: FP (MS1) 5'- GAAGCAAAATAGTGATATCA-3' and RP (MS2) 5'- GTCGTCTCCGAAGTTAACAA-3'.

The amplification reaction mixture contained 2 µl of template DNA, 1 µl of Primer FP MS1 (10 pmol/µl), 1 µl of Primer RP MS (10 pmol/µl) and 13 µl of master mix (Ampliqon III Taq 2× Master mix RED). DNA amplification was performed in the thermal cycler with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and 2 min at 72 °C, with a final extension cycle of 7 min at 72°C. The PCR product was kept at 4°C until processed. The amplified DNA product was visualized by electrophoresing 10 µl of the PCR product mixed with 2 µl tracking dye in a 1.5% agarose gel with ethidium bromide (0.5 µg/ml) and DNA bands were observed using transilluminator.

RESULTS AND DISCUSSION

Eggshell apex abnormalities in table eggs, leading to a significant decrease in eggshell strength, are described here for the first time in India. The eggshell pathology was associated with an increased incidence of soft-shelled eggs and egg breakage, as well as a general decrease in egg production.

In this report, affected flock showed 10–12% egg production drop, which is in line with the work of Santos *et al.* (2014). After observation of the eggs produced by the affected flocks, detected abnormalities were compatible with those previously reported by Feberwee *et al*. (2009). Eggshell apex abnormalities in MS infected layers are shown in Fig. 1. Egg apex abnormalities incidence of 2.5% was noticed in investigated farms whereas previous studies reported EAA incidence of 1.3% in an intensive layer farm in Italy (Catania *et al*. 2010), 25% in layers housed on the floor in the Netherlands (Feberwee *et al*. 2009b)

While changes of eggshell quality may be attributed to non-infectious factors (Roberts 2004), an experimental analysis was conducted to confirm the existence of infection. Factors such as sudden changes in egg production or concentration of specific lesions on the apex of the eggs strongly suggest the possibility that infectious agents have altered egg quality.

Fig. 1. Egg apex abnormalities in MS infected layers.

On necropsy examination, affected birds revealed mild cloudiness of abdominal air sac, inflammation of oviduct. Other organs showed no visible gross lesions. Previous studies revealed that pathologic lesions may be absent in the oviduct despite the presence of eggshell abnormalities, especially in cases of primary MS infections (Feberwee *et al.* 2009a).

It is not clear, how *M. synoviae* affects the normal eggshell calcification process and why the defect is confined to a distinct zone at the apex of the egg. *M. synoviae* may affect the composition and concentration of eggshell matrix proteins in the uterine fluid, which are needed for the regulation of the growth of calcite during eggshell calcification (Hinke *et al.* 2003).

M. synoviae may also affect ciliary motility in the oviduct, which could lead to changes in the uterine fluid content affecting the deposition of calcium carbonate crystals (Dominquez-Vera *et al*. 2000). Preferential colonization of a specific area of the uterus might also offer an explanation for the localization of the eggshell defects. Although eggshell abnormalities could be directly correlated with *M. synoviae* infection, the mechanism of pathogenicity remains unclear and further investigations are recommended to clarify this issue.

Infection was confirmed by isolating MS from choanal cleft, oropharyngeal swab and trachea in Frey's mycoplasma broth as previously described by Catania *et al.* (2010). Samples for which the Mycoplasma broth colour changed (yellow) were subjected to PCR for amplification of the conserved region of 16S rRNA gene. The isolates from culture were confirmed by specific amplification of 16S rRNA gene (207 base pair) (Fig. 2). *M. synoviae* was isolated

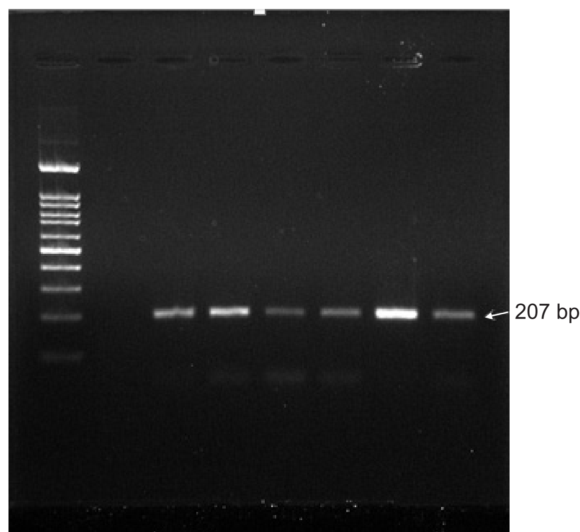

Fig. 2. PCR electrophoresis analysis in 1% agarose gel. Marker (Bio Basic Inc. DNA Marker-H1 (100 bp-1000 bp). PCR assay amplifying the *M. synoviae* species specific conserved gene region of 16S rRNA (207 base pair).

from all the investigated flocks that produced eggs with EAA.

Provisional diagnosis and monitoring of *M. synoviae* infection is usually made using serological assays, while definitive diagnosis is made by isolation and identification of the organism. However, the latter is generally expensive and time consuming (requiring 1–2 weeks to complete). Recently, PCR has been employed for identification of suspected cultures or for rapid detection of *M. synoviae* directly from clinical samples (Maricarmen *et al.* 2004). In this study, broth culture followed by PCR was used for the diagnosis of *M. synoviae*. The primary advantage of PCR is that it is a rapid and sensitive method of direct detection of organisms, compared with isolation techniques, which are time consuming and may be problematic.

The presence of abnormal eggshells in commercial layer flocks affected with MS, as confirmed by PCR and culturing with reduced eggshell strength and thickness, indicates the possibility of the presence of EAA in Namakkal region of Tamil Nadu, India. Further studies are required to fully understand the correlation between MS oviduct infection and EAA. The eggshell apex abnormality reported here and the concomitant egg production losses that results from *M. synoviae* infection, further highlight the economic significance of *M. synoviae* in commercial layers. Further studies with larger samples to ascertain the occurrence of this disease in industrial layer flocks in India are essential for better implementation of control measures.

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