



Synergistic pathological effect of *Mycoplasma gallisepticum* with *Ornithobacterium rhinotracheale* infection in layer chicken

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ABSTRACT

Synergistic pathological effect of *Mycoplasma gallisepticum* (MG) with *Ornithobacterium rhinotracheale* (ORT) infection in layer chicken was evaluated in 70 commercial layer chicken farms. *Ornithobacterium rhinotracheale* organism was confirmed by its growth characteristics on agar media and typical morphology on Gram's staining. Polymerase chain reaction technique was used for the confirmation of *Mycoplasma gallisepticum*. Other concurrent respiratory viral infections like Newcastle disease and infectious bronchitis were ruled out by hemagglutination and inhibition tests and bacterial diseases like colibacillosis, pasteurellosis and infectious coryza were ruled out by culture and staining techniques. The severity of clinical signs, gross pathology and histopathology increased in combined cases of MG and ORT than individual occurrence of these diseases. Zero and 7.8% mean mortality rate were observed in ORT and MG infections respectively, whereas 15% mean mortality rate was recorded in the combined cases. The synergistic increase in mortality rate of MG with ORT was clearly supported by the gross and histopathological alterations.

Key words: Chicken, *Mycoplasma gallisepticum*, *Ornithobacterium rhinotracheale*, Synergistic effect

Mycoplasma gallisepticum (MG) causing chronic respiratory disease (CRD) has a very small genome and evolved to this minimalist status by losing non-essential genes, including those involved in cell wall synthesis (Bradbury 2005). It affects all age groups of chicken, although very young birds are seldom affected (Bradbury and Levisohn 1996). The severity of the diseases exacerbate due to mixed infections with other respiratory pathogens (Murakami *et al.* 2002). Severe outbreaks with high morbidity and mortality observed in chicken were frequently due to concurrent infections (Kleven 1998). *Ornithobacterium rhinotracheale* (ORT) causing respiratory disease in broiler chickens is a gram-negative, pleomorphic, rod-shaped bacterium (Vandamme *et al.* 1994). The disease is common in broiler chicks at 3 - 6 week of age and in broiler breeders at 20 - 50 week of age (Allymetir 2006). Travers (1996) reported that dual infection with Newcastle disease virus and ORT in 28-day old broilers caused more severe respiratory lesions and higher mortality rates than in birds with only Newcastle disease and the pathogenicity of NDV was enhanced when it occurred with ORT infection. Concomitant ORT and *E. coli* infections in chicken broilers were reported by El-Sukhon *et al.* (2002).

When experimentally inoculated, ORT caused minimal pathologic lesions in chicken and turkey and the severity of lesions was enhanced when there was a concurrent

infection with respiratory viruses or bacteria (Back *et al.* 1997, Van Empel and Hafez 1999, Van Veen *et al.* 2000). Sivaseelan *et al.* (2013) reported synergistic pathological effect of *Mycoplasma gallisepticum* in layer chicken in association with other infectious agents like Newcastle disease and *E. coli*. This report deals with the synergistic pathological effect of *Mycoplasma gallisepticum* in layer chicken in association with ORT.

MATERIALS AND METHODS

Disease investigation: This study was conducted for 3 year, in which 70 commercial layer flocks (strength varied from 10,000 to 50,000 birds) with the history and symptoms of mycoplasmosis were investigated. Necropsy was carried out on recently died chicken carcasses and ailing birds. Five per cent of samples from each farm such as trachea, lungs, airsacs, spleen, proventriculus, intestine, ceecal tonsils and swabs of infraorbital sinus exudates, heart blood and liver were collected. These samples were utilized for the confirmation of viruses like Newcastle disease virus (ND) and infectious bronchitis virus (IBV) by haemagglutination (HA) and inhibition test (HI) and bacteria like *Escherichia coli* (colibacillosis, CB), *Pasteurella multocida* (fowl cholera, FC), *Avibacterium paragallinarum* (infectious coryza, IC) and *Ornithobacterium rhinotracheale* (ORT) by their growth characteristics on agar media and Gram's staining. Trachea and airsac pieces collected in Frey's medium were used in PCR for the confirmation of *Mycoplasma gallisepticum*.

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Diagnosis of *Mycoplasma gallisepticum*

Collection and culturing of samples: Pieces of trachea and airsacs from suspected birds were collected aseptically in Frey's medium and incubated at 37 °C for 5 –7 days.

DNA extraction: Sample (1 ml) cultured in Frey's medium was centrifuged at 10,000 × g for 20 min twice and the pellet was washed with 70% ethanol. The pellet was resuspended with 50 µl of Tris EDTA buffer and boiled for 3 - 5 min to release the DNA. The extracted DNA was stored at – 20 °C until use.

Polymerase chain reaction

Primers: The following forward and reverse primers were used for the amplification of target sequence (530 bp) of *M. gallisepticum*.

Forward primer

5'- AAC ACC AGA GGC GAA GGC GAG G - 3'

Reverse primer

5' - ACG GAT TTG CAA CTG TTT GTA TTG G - 3'

The following mixture of materials was subjected to PCR in a thermal cycler as per Kiss *et al.* (1997).

Master Mix : 25 µl

(dNTPs, Taq polymerase and PCR buffer)

Forward primer : 1 µl

(40 picomols)

Reverse primer : 1 µl

(40 picomols)

DNA template : 2 µl

DNase free water to make up to 50 µl

The reaction consisted of initial denaturation for 5 min at 95°C followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec with final extension at 72 °C for 10 min. The PCR products were separated on 1.5% agarose gel in 1× TAE buffer containing ethidium bromide 50 µg / ml at 100 volts for 45 min to 1 h.

Diagnosis of *Ornithobacterium rhinotracheale*: Samples for trachea, lungs, air sacs, liver, and swabs of heart blood and infraorbital sinus exudate were utilised for the confirmation of the *Ornithobacterium rhinotracheale* by their growth characteristics on sheep blood agar media and Gram's staining. Growth on sheep blood agar with gentamicin discs was also performed to assess the gentamicin resisting nature of *O. Rhinotracheale* organism.

Diagnosis of other concurrent respiratory virus and bacteria: Tissue homogenate of pooled samples of trachea, lung, liver, spleen, proventriculus, intestine, ceecal tonsils and kidney were subjected to hemagglutination and inhibition tests to identify Newcastle disease and infectious bronchitis (Alexander 1988). The culture media (*Escherichia coli*, MacConkey and eosin methylene blue (EMB) agar; *Pasteurella multocida*, brain heart infusion (BHI) agar; *Avibacterium paragallinarum*, sheep blood agar and chocolate agar) were prepared as per instructions of the manufacturer and used for the isolation of below mentioned bacteria associated with respiratory diseases in chicken.

Samples from trachea, lungs, air sacs, liver, and swabs of heart blood and infraorbital sinus exudate were utilized for the confirmation of these bacteria by their growth characteristics on agar media and Gram's staining of organisms.

Pathology: After recording the gross lesions, a transverse section of tissue approximately 0.5 cm in thickness was taken from infraorbital sinus, trachea and lungs of birds. Air sacs were as such removed. Tissue pieces were fixed in 10% buffered neutral formalin and trimmed to a thickness of about 3 mm. The tissues were dehydrated, cleared and embedded in paraffin in a routine manual processing. Tissues were cut at 5 µm thicknesses, mounted on glass slides, stained with haematoxylin and eosin and covered with coverslips for histopathological examinations (Bancroft *et al.* 1996).

RESULTS AND DISCUSSION

Disease investigation: Occurrence of CRD and ORT out of 70 farms investigated is presented in the Table 1.

The mortality rate of 7.8 and 0% recorded in CRD and ORT individually was raised to 15%, when they combined indicated the synergistic effect of CRD with ORT, which is also supported by severe gross and histopathological lesions in combined cases than individual disease occurrence. Exacerbation of severity of CRD as the result of mixed infections with other respiratory pathogens was reported earlier (Kleven 1998, Murakami *et al.* 2002). This report might be the first report on synergistic effect of CRD with ORT.

Diagnosis of *Mycoplasma gallisepticum*: The MG positive samples produced 530 bp products corresponding to their 16S rRNA gene (Fig. 1), which confirmed the presence of *M. gallisepticum*. The results obtained are in concurrence with Kiss *et al.* (1997) and Singh *et al.* (2013) who used a primer pair complementary to 16S rRNA gene designated for the detection of MG.

Diagnosis of *Ornithobacterium rhinotracheale*: Non-haemolytic, grey to greyish white, opaque, convex, very small, circular colonies with a diameter of 1–2 mm and with butyrous odour were observed on sheep blood agar after 48 h of incubation at 37°C under anaerobic conditions. Growth on sheep blood agar with gentamicin was also noticed. Smears prepared from the colony revealed highly Pleomorphic Gram-Negative Rods designated as PGNR. The growth characteristics observed on sheep blood agar are similar to the reports of Zorman-Rojs *et al.* (2000) and Soriano *et al.* (2002).

Table 1. Occurrence and mortality of CRD and ORT in chicken

Disease	Occurrence out of 70 farms (%)	Average mortality
CRD	07 (10)	07.80
ORT	03 (04)	—
CRD + ORT	12 (17)	15.00

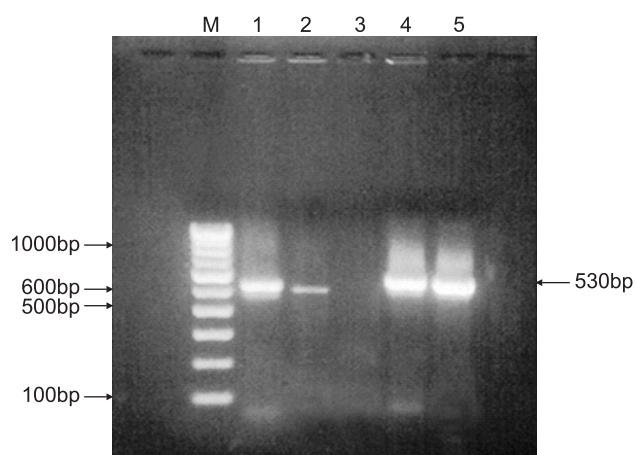


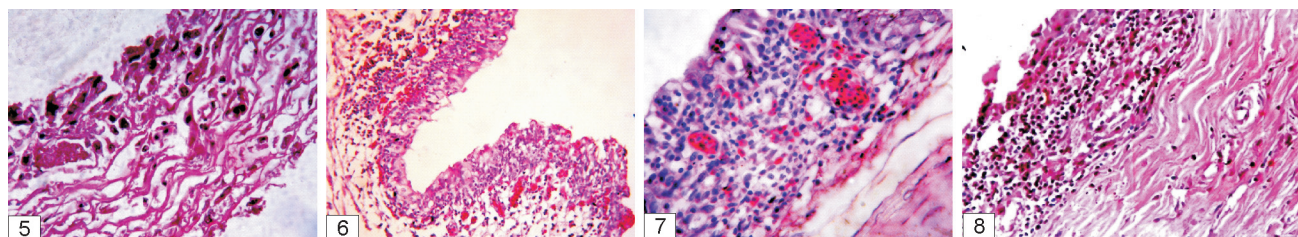
Fig. 1. *M. gallisepticum* - 530 bp PCR product on agarose gel electrophoresis.

catarrhal tracheitis, bronchitis, sinusitis and caseous airsacculitis were observed. Rarely, congestion and consolidation of the lungs were noticed. Gelatinous exudate in the infraorbital sinuses in one or both sides of the face was noticed in few cases. Airsac lesions varied from mild cloudiness to accumulation of caseous exudate in abdominal airsacs. These observations are in accordance with those of Nunoya *et al.* (1995) and Murakami *et al.* (2002).

Uncomplicated cases of ORT revealed mild sinusitis with crusts in the external nares, mild subcutaneous swelling of face, catarrhal rhinitis and tracheitis, airsacculitis and unilateral or bilateral pneumonic changes of the lungs. Foamy, whitish, yogurt like exudates with strands of fibrin were observed in the abdominal airsacs (Fig. 2). Unilateral pneumonia was more frequently noticed than bilateral type. In few cases, cut section of the lungs showed whitish miliary



Figs 2–4. **2.** ORT affected bird revealing foamy, white and yogurt like exudate in the air sac. **3.** CRD combined ORT affected bird showing severe edema in the facial subcutis. **4.** CRD combined ORT affected bird showing organized caseated material in the thoracic air sacs.



Figs 5–8. **5.** CRD affected bird showing epithelial necrosis, and submucosal stray infiltration of lymphocytes and macrophages in the air sac. H & E. $\times 400$. **6.** CRD combined ORT affected bird showing severe congestion and lymphocytic infiltration of submucosal area in the sinus. H & E. $\times 100$. **7.** CRD combined ORT affected bird showing severe congestion and lymphocytic infiltration of submucosal area in the trachea. H & E. $\times 1000$. **8.** CRD combined ORT affected bird showing epithelial necrosis, subepithelial massive infiltration of mononuclear cells, and thickened connective tissue in air sacs. H & E. $\times 400$.

Growth on sheep blood agar with gentamicin indicated the gentamicin resisting nature of *O. rhinotracheale*, which is in concurrence with Hafez *et al.* (1993). Smears prepared from the colony revealed highly pleomorphic rods. Van Empel and Hafez (1999), and Gopalakrishnamurthy *et al.* (2008) also reported similar findings.

Diagnosis of other concurrent respiratory virus and bacteria: Concurrent infection of Newcastle disease, infectious bronchitis, colibacillosis, pasteurellosis and infectious coryza were ruled out in the farms under report by using standard diagnostic technique. These diseases were ruled out to ascertain that the observed lesions are pertaining to either CRD or ORT or combination of these two.

Gross pathology: In uncomplicated cases of CRD, mild

nodules ranging from 2–3 mm in diameter. The findings are in agreement with the findings of Van Empel and Hafez (1999). Airsacculitis was mostly observed in abdominal airsacs than thoracic ones. On the contrary Joubert *et al.* (1999) reported more involvement of thoracic airsacs. Cloudy airsacs without foamy exudate noticed in the affected farms might be the early lesions when compared to the whitish yoghurt like exudate commonly observed in ORT affected birds later.

In combined cases of CRD with ORT, keratoconjunctivitis and marked edema in the facial subcutis and eyelids were observed (Fig. 3). Bilateral thoracic (Fig. 4) as well as abdominal airsacculitis with caseous exudate were also observed in the combined cases. This might be

the reason for increased mortality rate in the combined cases owing to the fact that occlusion of airsacs will cause asphyxiation due to failure of bellowing activity that regulates respiration in birds.

Histopathology: In uncomplicated cases of CRD, thickening of the tracheal mucous membrane due to hyperactivity of the mucous glands was noticed. Lungs showed interstitial pneumonia characterised by septal thickening due to the deposition of fibrin and infiltration of lymphocytes. Organised exudate composing of RBCs, lymphocytes and fibrin occluding the parabronchial lumen was also noticed. Airsacs revealed epithelial necrosis, and subepithelial stray infiltration of lymphocytes and macrophages (Fig. 5). The microscopic lesions observed in uncomplicated cases of CRD concurred with the earlier reports (Nunoya *et al.* 1995, Gaunson *et al.* 2000). Less severity of airsac lesions of epithelial necrosis, and subepithelial stray infiltration of lymphocytes and macrophages revealed that MG needs ORT to induce severe changes.

In ORT affected cases, focal deciliation and necrosis of surface epithelial cells of trachea were observed. Fibrinopurulent pneumonia characterised by collection of fibrin admixed with macrophages and heterophils within the lumen of air capillaries and interstitial septa, and parabronchi were observed. The microscopic changes observed coincided well with the earlier findings (Odor *et al.* 1997, Gopalakrishnamurthy *et al.* 2008).

In combined infection of CRD with ORT, sinus (Fig. 6) and trachea (Fig. 7) revealed severe congestion and lymphocytic infiltration in the mucosal and submucosal region. Lung showed severe aircapillary haemorrhage and parabronchial epithelial necrosis and sloughing. Secondary bronchi showed epithelial hyperplasia and accumulation of fibrin and heterophils mixed fibrinopurulent exudate. Epithelial necrosis and sloughing, subepithelial massive infiltration of mononuclear cells, and thickened connective tissue were noticed in airsacs (Fig. 8). This indicated the complimentary action of ORT with CRD could cause loss of bellowing activity of affected airsacs to bring about asphyxiation and death.

It is concluded that the synergistic effect of CRD with ORT could cause more mortality in chicken, which was clearly supported by gross and histopathological changes. Though ORT infection is not causing any mortality in chicken, it is boosting the death rate caused by CRD.

A survey of Hassan *et al.* (2014) indicated the wide spread prevalence of MG in all kinds of chicken, which warrants a suitable control strategy. This current report infers that during the outbreaks of CRD, treatment and control strategies should not only include CRD but also ORT.

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