Experimental Investigation on Ornithobacterium rhinotracheale and Enterococcus faecalis Co-Infection in Chickens

Peng Zhao¹, Guojiang Wu², Qiang Zhang¹, Jun Chu¹, Chen Xie¹, Yifei Wang¹, Yanxin Deng¹, Yuxin Hao¹ and Cheng He¹*

¹Key Lab of Animal Epidemiology and Zoonosis, Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, 100193 Beijing, China; ²College of Life Sciences, Agricultural University of Hebei, Baoding 071000, China
*Corresponding author: hecheng@cau.edu.cn

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ABSTRACT
A severe hemorrhagic pneumonia with respiratory distress prevalent has been observed in broilers with approximately 80% morbidity and 50% mortality. In our initial studies, Ornithobacterium rhinotracheale (ORT), Enterococcus faecalis (E. faecalis) and Streptococcus zooepidemicus were isolated from the diseased birds. However, the respective contribution of these organisms in the pathogenesis of hemorrhagic pneumonia is unclear. The objective of this study was to evaluate the role of ORT-E. faecalis co-infection in hemorrhagic pneumonia in chickens. Biochemical assays and 16S rRNA-based PCR were used to identify 5 E. faecalis isolates. Subsequently, forty-eight 21-day-old SPF chickens were divided randomly into six groups, 8 birds in each group. Chickens were co-infected intraperitoneally with ORT and E. faecalis isolates simultaneously, or inoculated with ORT first, then with E. faecalis three days later, and vice versa. Control groups consisted of chickens inoculated with ORT alone or E. faecalis alone. Post inoculation, the mortality was 87.5, 62.5, 37.6, 62.5 and 50% in birds co-infected with ORT and E. faecalis, infected with E. faecalis first followed by ORT, with ORT first followed by E. faecalis, and mono-infected with ORT and E. faecalis, respectively. Moreover, serum specimens from 60 out of 194 randomly selected chickens (30.9%) were positive for antibodies against E. faecalis. Our co-infection studies suggest that E. faecalis infection is able to trigger hemorrhagic pneumonia with a rapid mortality, while ORT infection was able to prolong pathological lesions. Therefore, the occurrence of ORT and E. faecalis co-infection may be associated with the outbreak of chicken hemorrhagic pneumonia.


INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is a Gram-negative bacterium associated with respiratory diseases in many avian species, causing significant economic losses in the poultry industry due to growth retardation and the discarding of infected carcasses as unacceptable for human consumption. It has been isolated from many bird species, such as: pheasant, pigeon, rook, duck, ostrich, goose, guinea fowl, turkey, chicken, red-legged partridge and falcon (Canal et al., 2005; Chansiripornchai et al., 2007; Van Empel et al., 1996). ORT affects the respiratory tract causing severe respiratory signs, depression, reduction in feed uptake and growth rate. It can be a primary or secondary etiological agent with avian pneumovirus (APV), Newcastle disease virus (NDV), influenza virus and Escherichia coli (Van Empel and Hafez, 1999; Thachil et al., 2009). Outbreaks of respiratory disease associated with ORT have been reported all over the world, including China, Germany, England, Slovenia, Mexico, Peru, Jordan, Egypt, Canada, Brazil, Belgium, Israel, USA, France, the Netherlands, Hungary, Korea, Japan, Taiwan, Turkey, and South Africa (El-Sukhon et al., 2002; Erganis et al., 2002; Canal et al., 2005; Misirlioglu et al., 2006; Chansiripornchai et al., 2007; Asadpour et al., 2008; Hafez and Vandamme, 2011). Recent reports disclosed that co-
infection with ORT and H9N2 or Streptococcus zooepidemicus contributed to outbreak of chicken bronchial embolization as well as broiler airsacculitis (Pan et al., 2012a; Pan et al., 2012b).

Enterococci are members of the normal microbiota of the gastrointestinal and urogenital tracts of humans and animals (Pan et al., 2012a; El-Ashy et al., 2013). In chickens, Enterococcus faecalis and Enterococcus faecium were found to constitute the dominant bacterial flora of the intestinal tract in 1-day-old chicks, while Enterococcus cecorum was found to be dominant in birds older than 12 weeks. In addition, these organisms have the potential to cause clinical infections (Devriese et al., 1991). In poultry, E. faecalis has been isolated from septicemia with valvular endocarditis (Chadfield et al., 2004), growth depression and amyloid arthropathy (Landman et al., 1994), pulmonary hypertension syndrome (Tankson et al., 2001). E. faecalis also occurs as a potential pathogen in other animals, and is the most common Enterococcus sp. associated with infections in humans (Malani et al., 2002). Very little is known about the pathogenesis and epidemiology of infections in avian species due to the infection occurring in all age groups. However, the most serious infections have previously been associated with the late embryos mortalities and high death in very young birds due to the egg contamination (Wages, 2003). Recent observations on the causes of mortality in broiler parent flocks have demonstrated an increased mortality due to E. faecalis (Gregersen et al., 2010). Information on the epidemiology and lesion types associated with this organism in broiler parents, however, is almost non-existent.

In recent years, respiratory disease of unknown etiology has become prevalent in broilers and young laying hens. This disease manifests itself on the first day after hatching and lasts for more than 30 days, resulting in approximately 50-70% morbidity and 30% mortality in young chickens in China. Post-mortem, hemorrhagic pneumonia and swollen kidneys, along with hemorrhagic bronchial tracts were observed. In a pilot study, ORT, H9N2, S. zooepidemicus and E. faecalis were isolated from lung, liver and spleen from commercial birds (Pan et al., 2012a; Pan et al., 2012b). Although ORT and E. faecalis have been isolated and identified in many cases, reports of co-infection with the aforementioned two pathogens and their potential synergistic role in disease pathogenesis have not been recorded.

**MATERIALS AND METHODS**

**Isolation and identification of E. faecalis**: Lungs were aseptically obtained from diseased chickens with severe pneumonia. Streak cultures were performed using standard 1 nutrient agar with 5% sheep blood and incubated at 37°C under aerobic conditions for 24 h. The suspected colonies were identified by Gram stain and biochemical assays (Chadfield et al., 2004; Pan et al., 2012a). DNA samples were extracted from the suspected isolates using the DNeasy Tissue Kit (Qiagen, Germany) following the manufacturer’s instructions. E. faecalis reference strain (#C55614) originated from cattle was purchased from China Institute of Veterinary Drug Control (IVDC), Beijing. PCR was used to amplify a DNA fragment of the 16S rRNA gene of E. faecalis. The amplification was performed using a pair of specific primers (Francois et al., 2004). A 197-bp fragment was amplified and subjected to electrophoresis in a 1% (w/v) agarose gel. In this report, E. faecalis and ORT taxonomic designations are provided as E. faecalis/ species/location/time.

**Determination of the LD50 of ORT and E. faecalis isolate**: The current study was approved by the Animal Welfare and Use Committee of China Agricultural University. The LD50 of ORT/broiler/Shandong/2011 isolates was determined as described previously (Pan et al., 2012b). Then, forty eight 21-day-old SPF chickens were randomly divided into six groups with 8 chickens per group. The chickens were infected intraperitoneally with different dilutions of E. faecalis in 0.5 ml. The inoculates of E. faecalis contained 5.6×10⁶, 5.6×10⁵, 5.6×10⁴, 5.6×10³, 5.6×10² and 5.6×1⁰ CFUs/ml. Chickens were inoculated intraperitoneally with sterile PBS as a control group. Each group was observed daily for 14 days, and the LD₅₀ was determined using the Reed-Muench method (Thakur and Fezio, 1981).

**Experimental infection of SPF chickens with ORT/broiler/Shandong/2011 and E. faecalis/broiler/Hebei/2011**: Forty-eight 21-day-old healthy SPF chickens were randomly divided into six groups with eight birds in each group. All birds were kept in positive pressure isolators and infected intraperitoneally with LD₅₀ of the isolates in 0.5 ml PBS buffer reagents as shown in Table 2. Each group was observed daily and sacrificed on day 14 post inoculation (p.i.). The chickens were euthanized by intraperitoneal injection of sodium pentobarbital. Gross lesions of both the experimentally infected dead chickens and live birds 14 days p.i. were inspected, and the lungs of the survived chickens were collected for pathogen recovery. The ORT colonies were determined by Gram staining and the candidate colonies were further identified by PCR as described previously (Van Empel and Hafez, 1999). The E. faecalis colonies were evaluated by Gram staining and colony morphology on the blood agar plate.

**Detection of E. faecalis antibody**: In this study, 194 serum samples were randomly collected from 204,000 chickens in Tianjin, Hebei and Jiangsu province. These included 39 serum samples out of 39,000 laying hens aged 180-280 days, 112 from 120,000 breeder broilers aged 120-450 days, and 43 from 45,000 healthy finished broilers aged 35-42 days. Serum samples were inactivated at 56°C for 30 min and tested for the presence of E. faecalis antibodies using the ELISA method. Moreover, the sera from 21-day-old SPF chickens were used as negative control. Afterwards, the optical density (OD) was read at 450nm and each sample was performed in two replications. Positive antibody responses were defined as the highest dilution that gave a ratio greater than 2.1 between test serum and the negative control serum.

**RESULTS**

**Isolation and identification of E. faecalis**: Five E. faecalis strains out of 8 Streptococcus spp. isolates were
successfully isolated from the lungs of diseased chickens by single colony purification. After 24h of anaerobic growth on the blood agar, the colonies formed by *E. faecalis* were surrounded by a pronounced zone of hemolysis. The isolates were all Gram-positive stain under the microscope. Moreover, the isolates grew on glucose, sucrose, lactose, galactose, fructose, mannose, mannitol, and maltose, but were negative on sorbitol, arabinose A, and sodium hippurate. In contrast, the two strains could grow onto bouillon medium pH 9.6, with 6.5% NaCl, and Maconkey agar. Based on the growth properties and analyses listed above, five strains were successfully isolated and separately named after *E. faecalis*/broiler/Hebei/2011, *E. faecalis*/layer/Mongolia/2011, *E. faecalis*/broiler/Tianjin/2011, *E. faecalis*/Jiangsu/2011 and *E. faecalis*/broiler/Liaoning/2011. The genomic DNA extracted from the five *E. faecalis* strains produced the expected 197-bp PCR product (Fig. 1).

**Determination of the LD₅₀ of ORT and *E. faecalis* isolate:** The LD₅₀ of ORT/chicken/Shandong/2011 was determined to be 1.43×10⁸ CFUs/ml. After growth in liquid medium for 24h at 37°C, the concentration of *E. faecalis* was 5.6×10⁹ CFUs/ml. In the chicken experiment, immediate mortality occurred in group 1 after inoculation with the highest concentration. The LD₅₀ of *E. faecalis*/broiler/Hebei/2011 was determined to be 5.6×10⁷.5 CFUs/ml (Table 1) in SPF chickens.

**Experimental infection of SPF chickens with ORT/chicken/Shandong/2011 and *E. faecalis*/broiler/Hebei/2011:** One day post simultaneous inoculation with ORT and *E. faecalis*, 5 chickens out of 8 inoculated birds displayed ruffled feathers, inactivity, poor appetite and respiratory distress. More importantly, significant mortality was observed up to 7 days p.i., reaching a maximum on day 3 p.i. Total mortality amounted to 87.5% of the birds. Moreover, 3 birds inoculated with ORT first and *E. faecalis* 2 days later (ORT/*E. faecalis*) displayed clinical signs and the mortality increased gradually 3 days later while birds infected with *E. faecalis* first and ORT 3 days later (*E. faecalis*/ORT) died swiftly post inoculation with *E. faecalis* with total mortality reaching 62.5% (Table 2). In contrast, 62.5% and 50% mortality was observed in the birds infected with ORT alone and *E. faecalis* alone, respectively. Clinically, a dead peak was observed in chickens with *E. faecalis* infection alone within 2 days and chickens were recovered 3 days later. In comparison with *E. faecalis* infection, mortality occurred 3 days later and lasted for more than 7 days.

At necropsy, hemorrhagic pneumonia, airsacculitis and hemorrhagic nephritis were characteristic of chickens co-infected with ORT and *E. faecalis* at same time (Fig. 2) and in the ORT/*E. faecalis* group in contrast with the *E. faecalis*/ORT group. Moreover, chickens inoculated with *E. faecalis* alone were observed to produce a thick yellow caseous exudate, widely frothy exudates in lungs.
with hemorrhagic pneumonia and severe peritonitis in the simultaneously inoculated with ORT and \textit{E. faecalis} with ORT, then received \textit{ORT} at days 3 p.i. Group 6 received a PBS control. Group 5 was inoculated with \textit{E. faecalis}, then received \textit{ORT} at days 3 p.i. Group 5 was inoculated with \textit{E. faecalis} alone. Group 3 was inoculated with \textit{ORT} alone and 50% with \textit{E. faecalis} infection induced 100% mortality in the \textit{ORT}+ \textit{Streptococcus} group, suggesting that \textit{ORT} might dominate the primary infection instead of viral infections (Pan et al., 2012a). In our study, \textit{E. faecalis} infection triggered swift mortality amounting to 50% loss within 24 hours in comparison with 20% mortality induced by \textit{S. zooepidemicus} infection (Pan et al., 2012a). \textit{E. faecalis} was previously referred to be one of the most dominant bacterial species in the gut flora in 1-day-old chickens (Devriese et al., 1991) and it can cause endocarditis, intra-abdominal infection, pelvic infections as well as pulmonary hypertension in broilers (Tankson et al., 2001). Furthermore, hemorrhagic pneumonia and swollen hemorrhagic nephritis were characterized post infection with \textit{E. faecalis} as compared to progressive bronchial obstruction in the broilers (Pan et al., 2012a) and bronchopneumonia in layer chickens infected with \textit{S. zooepidemicus} (Bisgaard et al., 2013). Although \textit{S. zooepidemicus} has been linked to cases of acute fatal pneumonia in dogs (Priestnall et al., 2010), no hemorrhagic pneumonia was recorded in young chickens. Therefore, our findings suggest that primary infection with \textit{E. faecalis} alone may trigger hemorrhagic pneumonia and co-infection with \textit{ORT} aggravates the respiratory distress and high mortality.

In this study, 8 layer hens (20.5%), 30 breeder broilers (26.8%) and 22 finished broiler (51.2%) were seropositive against \textit{E. faecalis}. The specific antibodies were detected in 60 (30.9%) of the 194 serum samples. The results of this study indicated that the prevalence of \textit{E. faecalis} antibodies is high in the finished broiler and breeder broilers in Northern China. Outbreaks of \textit{E. faecalis} infection may be associated with the following factors. Firstly, excessive use of antibiotics in chickens’ feed or drinking water may contribute to an overbalance of probiotics in birds’ intestinal tracts, leading to excessive multiplication of \textit{E. faecalis} and opportunistic bacterial infection in the flocks. \textit{Streptococcus} and Enterococcal isolates originating from the host are regarded as commensal organisms. Concurrent enteric infections or infections via an aerosol route that compromise the intestinal villous epithelial integrity may allow penetration of resident \textit{Enterococci} resulting in septicemia and endocarditis (Bisgaard et al., 2013). Secondly, antibiotic abuse might contribute to the infection of \textit{E. faecalis} and \textit{ORT} in poultry. In our pilot study, fosfomycin (MIC>125µg/ml), chlortetracycline (MIC>125µg/ml), spiramycin (MIC>250µg/ml), and penicillin (MIC>125µg/ml) were used as antibiotics in the study.

**DISCUSSION**

In current study, five \textit{E. faecalis} strains out of 8 \textit{Streptococcus} spp. isolates were isolated from the lungs of chickens with hemorrhagic pneumonia. Co-infection with \textit{ORT} and \textit{E. faecalis} induced more than 87.5% mortality with hemorrhagic pneumonia and severe peritonitis in the SPF chickens in comparison with 62.5% mortality with \textit{ORT} alone and 50% with \textit{E. faecalis} infection alone. Interestingly, \textit{E. faecalis} inoculation induced mortality rapidly while \textit{ORT} infection exacerbated persistent loss. Epidemiological survey indicates that seroprevalence of \textit{E. faecalis} is high in the three avian species. The results of this study strongly suggest that co-infection with \textit{ORT} and \textit{E. faecalis} is responsible for the current hemorrhagic pneumonia with high mortality observed in chickens in China.

Although 5 \textit{E. faecalis} strains and 3 \textit{S. zooepidemicus} isolates were identified from the clinical samples, the role of \textit{S. zooepidemicus} in pathogenesis is unclear in the context of \textit{E. faecalis} co-infection with \textit{ORT}. A previous investigation revealed that the combination of \textit{ORT} and \textit{S. zooepidemicus} infection induced 100% mortality in the \textit{ORT}+ \textit{Streptococcus} group, suggesting that \textit{ORT} might dominate the primary infection instead of viral infections (Pan et al., 2012a). In our study, \textit{E. faecalis} infection triggered swift mortality amounting to 50% loss within 24 hours in comparison with 20% mortality induced by \textit{S. zooepidemicus} infection (Pan et al., 2012a). \textit{E. faecalis} was previously referred to be one of the most dominant bacterial species in the gut flora in 1-day-old chickens (Devriese et al., 1991) and it can cause endocarditis, intra-abdominal infection, pelvic infections as well as pulmonary hypertension in broilers (Tankson et al., 2001). Furthermore, hemorrhagic pneumonia and swollen hemorrhagic nephritis were characterized post infection with \textit{E. faecalis} as compared to progressive bronchial obstruction in the broilers (Pan et al., 2012a) and bronchopneumonia in layer chickens infected with \textit{S. zooepidemicus} (Bisgaard et al., 2013). Although \textit{S. zooepidemicus} has been linked to cases of acute fatal pneumonia in dogs (Priestnall et al., 2010), no hemorrhagic pneumonia was recorded in young chickens. Therefore, our findings suggest that primary infection with \textit{E. faecalis} alone may trigger hemorrhagic pneumonia and co-infection with \textit{ORT} aggravates the respiratory distress and high mortality.

![Table 1: Determination of the LD₅₀ of \textit{E. faecalis} broiler/Hebei/2011 in SPF chickens](image1.png)

![Table 2: Experimental infection of SPF chickens with \textit{ORT}/chicken/Shandong/2011 and \textit{E. faecalis} broiler/Hebei/2011](image2.png)

![Table 3: Seroprevalences in broilers and layer hens flock using the \textit{E. faecalis} antigen-based ELISA kit](image3.png)
amoxicillin (MIC>250µg/ml) and tylosin (MIC> 500 µg/ml) were detected to be multi-resistance to both E. faecalis and ORT isolates (Liu et al., 2013). Moreover, hemorrhagic nephritis induced by ORT infection immediately enables E. faecalis to infect other target organs via the blood stream. ORT and E. faecalis infections synergistically aggravate respiratory symptoms in chickens. In the present study, 87.5% mortality was observed in chickens infected simultaneously with both ORT and E. faecalis.

Conclusion: This study shows that co-infection with ORT and E. faecalis may contribute to more severe pneumonia and cause higher mortality than monoinfection with either. Above all, E. faecalis may dominate in a primary infection that may be followed by a secondary infection with ORT. Hence, more in-depth studies are needed to investigate the extent and impact of E. faecalis and ORT co-infection. Moreover, the development of a combined vaccine targeting both pathogens should be prioritized for the benefit of the poultry industry.

Authors’ contribution: PZ contributed to the study and drafted the manuscript. GW performed the statistical analyses. QZ performed isolation. CX YW YD YH collected the samples. JC did ELISA test. CH contributed to the study design and obtained the funding. All authors have read and approved the final manuscript.

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