Pathological examination and bacterial re-isolation by culture and PCR of experimental *Ornithobacterium rhinotracheale* infection in broiler chickens

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**SUMMARY**

The objective of the present study was to investigate the pathogenicity of the *Ornithobacterium rhinotracheale* (ORT) B3263/91 (serotype A) strain inoculated alone (4.8x10^8 CFU) to 14 day old broiler chickens (n=30) by the aerosol route whereas 30 not treated birds served as negative controls. After inoculation, birds were clinically examined and weighted and histological, microbiological (culture) and molecular (PCR) analyses were performed on different parts of the respiratory tree (sinuses, larynx, trachea, lungs and air sacs) and on visceral organs collected from 10 birds of each group on the weeks 1, 2 and 3 following experimental ORT inoculation. The clinical signs (rales, gasping and mucus discharge) peaked between the 5th and the 9th days in 10% of treated birds. Focal epithelial hyperplasia associated with necrosis and inflammatory lesions (oedema, blood vessel congestion and moderate inflammatory infiltration) of the *lamina propria* of the upper parts of the respiratory tract and of air sacs, as well as around bronchioles and in BALT in some lung areas were observed within the first 3 weeks, lungs being more belatedly affected than upper parts. In parallel, ORT was successfully re-isolated by culture and PCR from trachea and lungs. These results show that this ORT strain acts as a primary infectious agent of the respiratory tract even if the induced lesions were transient and mainly located in air sacs and suggest the involvement of other viral and bacterial infections for disease amplification.

**Keywords:** *Ornithobacterium rhinotracheale*, broiler chickens, culture, PCR, histopathological findings.

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**Introduction**

Respiratory diseases cause heavy economic losses in poultry. The germ *Ornithobacterium rhinotracheale* (ORT), named by VANDAMME et al. [19], is a slow growing, pleomorphic, gram negative, rod shaped, recently described pathogen associated with poultry diseases [15], particularly in respiratory diseases characterized by decreased growth rate and increasing mortality rate in poultry [6, 9]. It has recently been isolated in many countries of the world from chicken, duck, goose, guinea fowl, gull, ostrich, partridge, pheasant, pigeon, quail, rook and turkey [3, 12, 14] and has been incriminated as a possible additional causative agent for respiratory diseases [17, 19]. The clinical symptoms observed during the infection included weakness, gasping, severe dyspnoea, mucus discharge and mortality [2, 16]. Necropsy showed fibrinopurulent pneumonia and fibrinous inflammation of the thoracic air sacs [8, 16]. At post-mortem examination, the most striking feature was a foamy white, ‘yoghurt-like’ exudate in the air sacs, predominantly in the abdominal air sac, although pneumonia was also found [10]. These symptoms can disappear within one week but can also be deteriorated...
by succeeding infections with other pathogens and although they may not be recognised as an ORT infection anymore [15].

The aims of the study were to reproduce the disease syndrome associated with ORT in broiler chickens, to describe the clinical, gross and histopathological findings and to develop culture and PCR assays for the diagnosis of experimental ORT infections.

Materials and Methods

BACTERIAL STRAIN

The ORT B3263/91 (serotype A) strain, kindly supplied by Dr. HAFEZ, was used for this study. Bacteria were grown at 37°C on sheep blood agar plates in a 5-10% CO2 atmosphere, or in brain heart infusion broth (BHI, Oxoid).

ANIMALS AND PROTOCOL DESIGN

All animal studies were approved by the committee for animal experiments of the Elazig Veterinary Control and Research Institute, according to international regulations.

Two groups of 30 commercial 14 day old male broiler chickens which did not receive any vaccination or treatment were used and were housed in two separate rooms. Birds of the first group were infected by aerosol route with 1 ml of a pure culture of ORT strain B3263/91 (serotype A) (4.8x10^8 CFU/mL), whereas the second group was non-infected and served as a control group. During aerosol challenge, the bacterial culture was administered as a fine spray to the chickens in an isolator, using a commercial paint sprayer; the developed mist was maintained for at least 10 min without air circulation.

After inoculation, birds were clinically examined every day and weighted weekly. Ten chickens of each group were killed by cervical dislocation at the 1st, 2nd and 3rd weeks after inoculation and samples from sinuses, larynx, trachea, lungs, air sacs and other visceral organs were collected immediately after death and examined by bacteriological, molecular and pathological methods.

BACTERIOLOGICAL ANALYSIS

For microbiological analysis, swabs from the lung and trachea samples were aseptically inoculated on blood Agar supplemented with 7% sheep blood and 10µg/mL of gentamicin. The plates were incubated in a 5-10% CO2 atmosphere at 37°C for at least 48h. After 24 hr of incubation at 37°C in 5% CO2, suspicious colonies were sub-cultured and the identities confirmed biochemically [18].

PCR

Colonies from suspected isolates grown on selective agar were suspended in Eppendorf tube containing 300µL distilled water. Bacterial lysis was accomplished by the addition of 300µL of TNES buffer (20mM Tris pH 8.0, 150mM NaCl, 10 mM EDTA, 0.2% SDS) and 200µg/mL proteinase K following by incubation at 37°C for 2 hours then the mixture was boiled for 30 min. Bacterial DNA was extracted by phenol / chloroform / isoamylalcohol (25:24:1), then precipitated with ethanol and 0.3 M sodium acetate at -20°C for overnight and centrifugation (7 000g, 5 minutes, 4°C). The DNA pellet was washed twice with 300µL of 90% and 70% ethanol, respectively, each step followed by centrifugation (7 000g, 5 minutes, 4°C). The pellet was dried, and dissolved in 50µL of distilled water, then used as template DNA for PCR.

The PCR was performed in a touchdown thermocycler (Hybaid, Middlesex, England) in a total reaction volume of 50µL containing 5µL of 10X PCR buffer (10mM Tris HCl pH 9.0, 50mM KCl, 0.1% Triton X-100), 5µL 25mM MgCl2, 250mM of each deoxynucleotide triphosphate, 2U of Taq DNA Polymerase (MBI, Fermentas) and 40 pmol of each of primers [OR 16s-F1 (5΄-GAG AAT TAA TTT ACG CCG AGT TAA G-3΄) and OR16S-R1 (5΄-TTC GCT TGG TCT CCG AAG AT-3΄)] derived from 16S rRNA gene [4] and 5µL of template sample DNA. Amplification was obtained with initial denaturation step at 94°C for 5 min, followed by 45 cycles at 94°C for 30 seconds, 52°C for 1 min and 72°C for 7 min. The amplified products were visualized by ethidium bromide (0.5µg/mL) staining after electrophoresis at 70 volts for 1 hour in 1.5% agarose gels. PCR products with the molecular size of 784 bp were considered for identification as Ornithobacterium rhinotracheale.

PATHOLOGICAL EXAMINATION

Tissue samples were fixed in 10% neutral formalin and embedded in paraffin, and then cross sections were made and stained with haematoxylin-eosin.

Results

CLINICAL FINDINGS

None of the chickens died during the entire experiment. with “Mild rales, gasping and mucus discharge were observed in 3 infected chickens. The findings peaked between the 5th and the 9th days in 10% of treated birds. Compared with the control group, there was a decrease of the weekly live weight gain, particularly at the 2nd week after inoculation. No clinical sign was found in control birds.

PATHOLOGICAL FINDINGS

Accumulation of small amounts of mucus in the lumen of the sinuses, larynx and trachea associated with various degree of mucosal hyperaemia was noted in 5 chickens between the 1st and 2nd weeks after inoculation. Air-sacs were cloudy in appearance and sometimes lungs appeared focal grey red in chickens with macroscopic findings.

Histopathological lesions were observed in the sinuses, larynx, trachea, lungs and air sacs from experimentally infected birds. No histopathological lesion was evidenced in control birds.

Lesions seen in the sinuses, larynx and trachea were almost similar each other and were found in 50% for sinuses and 40% for the other parts of the upper respiratory tree of infected birds 2 weeks after inoculation. The frequency of these lesions decreased on the 2nd week and they have totally disappeared on the 3rd week after inoculation (Table I). Few small foci of hyperplasic epithelium were found in the posterior part of the sinuses, larynx and upper trachea. Slight epithelial degeneration, necrosis and desquamations were seen in some areas of mucosal surface. In the lamina propria, there were mild oedema, congestion of blood vessels and haemorrhage at various degrees. Cellular reaction was generally moderate, consisting of lymphocytes, macrophages, heterophils and a few plasma cells in the lamina propria (Figure 1). Focal areas of lymphoid accumulation were seen in the lamina propria of the sinuses, larynx and trachea. There was exudate containing few degenerating and necrotic epithelial cells, heterophils and red blood cells in the lumen of the sinuses, larynx and trachea.

Infiltration with inflammatory cells, haemorrhage and necrosis of bronchial associated lymphoid tissue (BALT) were seen in some areas of the lungs (Figure 2). Focal epithelial hyperplasia was observed in the primary bronchiole, associated with mild peribronchial oedema, congestion of blood vessels and moderate infiltration by lymphocytes, macrophages, few heterophils and plasma cells. In the air sacs, focal epithelial hyperplasia (Figure 3), degeneration, necrosis and desquamation were seen whereas an inflammatory reaction (oedema, blood vessel congestion, inflammatory infiltration) was evidenced in the lamina propria (Figure 4). The inflammatory lesions were detected in 40% and 30% of infected birds in lungs and in air-sacs respectively on the 1st week after ORT inoculation. Thereafter, the frequency of lung and air sac injury rose on the 2nd week reaching 50% and 60% of infected birds respectively, whereas inflammatory reaction was completely abolished on the 3rd week (Table I).

No pathologic finding was seen in any other visceral organs from infected chickens.
PATHOGENICITY OF ORNITHOBACTERIUM RHINOTRACHEALE IN BROILER CHICKENS

BACTERIOLOGICAL FINDINGS

ORT was isolated from trachea samples of 5 chickens showing clinical and pathological signs during the 1st week after ORT exposure and from 3 lung samples with pathologic signs on the 2nd week after inoculation. By contrast, no ORT culture was obtained from trachea or lungs removed on the 3rd week (Table I). In parallel, PCR amplification of genomic bacterial DNA using OR16S-F1 primer pairs gave bands of 784 bp, corresponding to the expected size of amplified genomic ORT DNA (Figure 5). All isolates positive by ORT culture were also positive by PCR and culture negative samples were also PCR negative. No amplified product was obtained from the negative controls.

<table>
<thead>
<tr>
<th>Infected group</th>
<th>1st week HP</th>
<th>Culture</th>
<th>PCR</th>
<th>2nd week HP</th>
<th>Culture</th>
<th>PCR</th>
<th>3rd week HP</th>
<th>Culture</th>
<th>PCR</th>
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<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<td>3</td>
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<td>0</td>
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<tr>
<td>Lungs</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>5</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>0</td>
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<td>-</td>
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</table>

Table I: Frequencies of histopathological findings (HP) and *Ornithobacterium rhinotracheale* (ORT) isolation by culture and PCR in respiratory tree from control negative broiler chickens and from experimentally infected broiler chickens 1, 2 and 3 weeks after aerosol ORT B 3263/91 (serotype A) administration (4.8x10⁸ CFU).

Discussion

ORT has been isolated from various bird species (chicken, chukar, partridge, duck, goose, guinea fowl, ostrich, partridge, pheasant, pigeon, quail, rook and turkey). Within this bacterial species several serotypes and isolates with different virulence seem to be exist [3, 12, 14]. Several ORT strains, isolated from turkey, chicken and partridge, were used for aerosol challenges for both turkeys and broilers of various ages [14]. The most prominent clinical sign after experimental ORT infection appeared to be growth retardation [16]. Decreased fed and water intake were often associated with the isolation of *Ornithobacterium rhinotracheale* in field clinical outbreaks [16]. However, other clinical signs such as weakness, dyspnocia, mucus discharge, an even mortality were also noted [8, 10].

The *O. rhinotracheale* bacterium is the most commonly germ isolated from trachea and lungs of naturally or experimentally infected chickens. In the present study, positive ORT isolation from trachea and lungs were obtained during the first two weeks after inoculation, the lung bacterial infection being delayed compared to tracheal colonisation by bacteria. The presence of ORT was therefore confirmed by PCR using the OR 16S-F1 and 16S-R1 primer combination for specifically amplifying a 784 bp DNA fragment from the 16 rRNA ORT gene.

Disease in broilers, particularly development of airsacculitis and pneumonia, was aggravated by a prior administration of experimental inoculation with some viruses such as Newcastle disease virus, infectious bronchitis virus or turkey rhinotracheitis virus [7, 14]. In the present study, commercial chickens exposed to ORT didn’t show severe pneumonia lesions and mortality, but macroscopic and microscopic lesions in the air sacs characteristic of sacculitis were observed. By contrast, in other studies, airsacculitis, pneumonia and increased mortality were observed in commercial broilers and in turkeys after aerosol, intra-tracheal, intravenous and intra-thoracic ORT infection without a primer [3, 11-13]. These discrepancies would result from the probable low virulence of the strain used (depending from the growth medium, the number of passages and the dose of bacterial inoculum) and from the uncertain and heterogeneous microbiological and immunological status of commercial birds. Consequently, these different and contradictory results question the importance of *Ornithobacterium rhinotracheale* as a primary pathogen. Most experimental studies have concluded that experimental inoculation with *O. rhinotracheale* alone causes minimal pathogenic lesions in chickens and turkeys and that the severity of lesions are enhanced when there is a concurrent infection with respiratory viruses or bacteria [1, 4, 5, 14]. It is probable that viral or bacterial priming is often required for exacerbating pathogenicity of low virulent ORT strains.

Nevertheless, as in clinical outbreaks associated with the...
isolation of *Ornithobacterium rhinotracheale*, fibrinous airsacculitis occurred during experimental infection, sometimes accompanied by pneumonia and tracheal exudate [2, 16]. Immunohistochemical investigations have confirmed that aerosol ORT exposure of chickens without viral priming induced significant pathological changes in the respiratory tract [10, 14], with at least partly the same characteristics as seen in clinical outbreaks.

As a conclusion, the present study showed that *O. rhinotracheale* could be considered as a relatively efficient and primary infectious agent in broiler chickens. All three postulates of Koch are fulfilled: i) this agent was isolated from naturally diseased chickens and not from healthy chickens, ii) the agent was grown to purity and the pure agent was able to reproduce the disease in chickens after experimental infection and iii) it was recovered from experimentally diseased birds. Furthermore, differences in pathogenicity of the ORT strains occur and further studies should be needed to clarify the potential pathogen role of various ORT strains.

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**References**