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Effect of experimental *Ornithobacterium rhinotracheale* infection along with live infectious bronchitis vaccination in broiler chickens

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ABSTRACT *Ornithobacterium rhinotracheale* (ORT), a bacterium causing respiratory tract infection, has led to a significant problem in the intensive poultry production in Egypt. Polymerase chain reaction-amplified 784-bp specific ORT DNA fragments were found in 7 ORT isolates from lungs, air sacs, and tracheas of commercial broilers or layers in Egypt in 2015. The objective of this study was to investigate the role of the live variant IBV 4/91 with ORT infection. A total of 120 14-d-old broiler chickens (Cobb 500) were equally divided into 4 groups for experimental infection in a complete randomized design. Group 1 was infected with ORT strain and live infectious bronchitis vaccine (IBV 4/91) simultaneously; group 2 was infected with the bacterial strain alone; group 3 was vaccinated only with IBV 4/91, and group 4 was the non-vaccinated and non-infected control group. The respiratory signs, post-mortem lesions (tracheitis and pneumonia) and histopathological findings of lungs, trachea, and air sacs in the experimentally infected broiler chickens appeared to be more prominent in the chickens of group 1 than group 2. With respect to body weight, weight gain, feed conversion rate, and *Ornithobacterium* re-isolation, there was a difference ($P \leq 0.05$) among the chickens of group 1 and the other groups. This reveals that the use of live infectious bronchitic vaccines, which is a common practice in the local Egyptian field of production, may concomitantly increase the pathogenicity of ORT in broiler chickens.

Key words: *Ornithobacterium rhinotracheale*, infectious bronchitis virus, broiler chicken, experimental infection

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INTRODUCTION

Ornithobacterium rhinotracheale (**ORT**) is a relatively recently named bacterium, associated with respiratory disease in avian species. ORT is a pleomorphic, gram-negative, rod-shaped bacterium first classified by Vandamme et al. (1994).

High economic losses in poultry production may be caused by ORT as it is accompanied by increased mortality rate, retarded growth, higher medication cost, increased condemnation rate, drop in egg production, reduction in eggshell quality, and decreased hatchability. The severity of clinical signs, duration of the disease and mortality with confirmed ORT outbreaks was found to be extremely variable and was influenced by many environmental factors, such as poor management, inadequate ventilation, high stocking density, poor litter conditions, poor hygiene, high ammonia levels, concurrent diseases (or live vaccine strain), and the type of secondary infection (van Empel and Hafez, 1999).

Clinical signs and post-mortem lesions associated with ORT infection include tracheitis, pericarditis, sinusitis, exudative pneumonia, and yogurt-like exudate in the abdominal air sac (Banani et al., 2001).

However, as these lesions are not sufficiently specific to diagnose the disease, laboratory tests are needed for definitive diagnosis. Though microbiological isolation and identification were done by several investigators,

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Table 1. History of positive ORT flocks.

Flock (isolate) no.	Locality	Type of flock	Total no.	Age	Mortality % (last 3 d)	Organ of isolation
17	Elbehera, Egypt	Broiler	2,300	30 d	2.3	Lung
22	Elbehera, Egypt	Broiler	6,350	32 d	0.23	Trachea
30	Marsa Matrouh, Egypt	Broiler	6,000	37 d	0.66	Air sac
31	Marsa Matrouh, Egypt	Broiler	6,000	37 d	3.6	Lung
51	Elbehera, Egypt	Layer	30,000	35 weeks	0.11	Air sac
52	Elbehera, Egypt	Broiler	1,500	28 d	0.26	Lung
54	Elbehera, Egypt	Broiler	4,000	28 d	6.3	Air sac

currently, many reports discuss the diagnosis of ORT by using polymerase chain reaction (**PCR**) and 16S ribosomal gene sequencing (Ozbey et al., 2004; Koga and Zavaleta, 2005).

The recent incidence of severe respiratory disease complex syndrome in Egypt and the associated economic losses have made it necessary to investigate the role of the live variant IBV 4/91 with ORT infection.

MATERIALS AND METHODS

ORT Isolates

In 2015, 7 ORT isolates were collected from diseased broiler and layer flocks in the Elbehera and Marsa Matrouh province (Table 1). All the examined birds of layer flock had a history of respiratory disease manifested as cough, sneezing, rales, nasal discharge, conjunctivitis, swollen head, and decreased egg production. Post-mortem examination showed yogurt like air sacculitis (Figure 1a and b) and pneumonia.

An ORT isolate (no. 17) from the lungs of broiler chickens was used for experimental infection along with live variant IBV 4/91.

Bacteriological Examinations

The samples were streaked on blood agar base enriched with 5%–10% defibrinated sheep blood, with the addition of gentamycin 10 μL/mL (van Empel and Hafez, 1999). Pinpoint grayish white small colonies indicated possible presence of ORT. Biochemical tests revealed that ORT isolates were catalase negative and oxidase test positive. All isolates were cultured on MacConkey's and Frey's agar medium and revealed negative results except for pathogenic *E. coli* and *Mycoplasma* (van Empel and Hafez, 1999).

Molecular Identification and Sequencing of ORT

Using PCR, extraction was done according to manufacturer information of Thermo Scientific Gene jet genomic DNA purification kit. The amplification was carried out using condition modification by van Empel and Hafez (1999), in which primers can amplify a 784-bp DNA fragment within the 16 s ribosomal rRNA region.

Sequencing of ORT Isolates

Sequencing was done for 2 ORT isolates (no. 17 and 51) using QIAquick PCR purification kit and the purified reverse transcription (RT) PCR product was sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA), as per manufacturer instructions (Zehr et al., 2014).

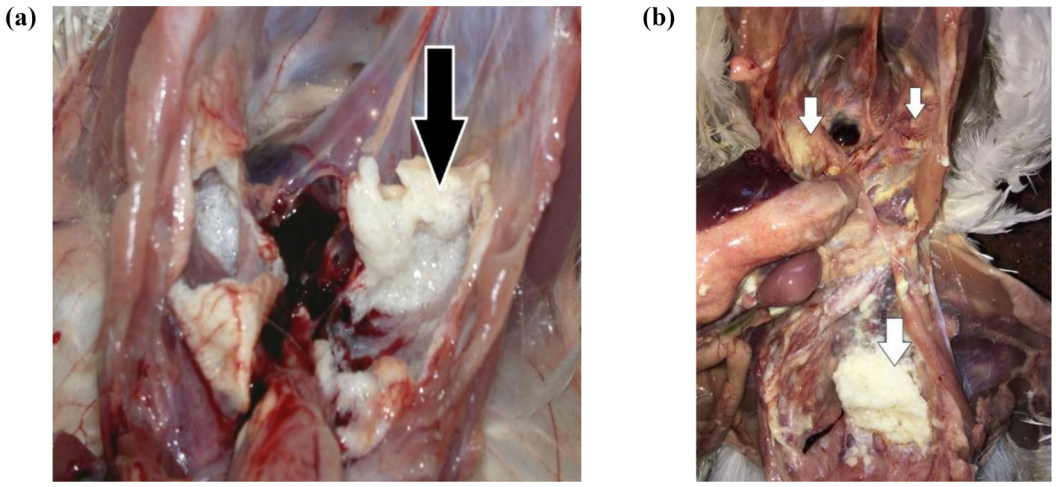

Figure 1. (a and b) Sacrificed field broiler chicken showing air sacculitis (thoracic or abdominal) with accumulation of yoghurt-like creamy foamy exudates (arrow).

Table 2. Experimental design.

Group no.	No. of birds	Age of ORT experimental infection	Age of vaccination (IB 4/91)
1	30	14 d old	1 and 14 d old
2	30		Not done
3	30	Not done	1 and 14 d old
4	30	Not done	Not done

Experimental infection was via intra-tracheal route with a dose of 200 μL of l.2 $\times$ 10^9 CFU/mL and IBV vaccine 4/91 administered as eye drops.

A BLAST analysis (Basic Local Alignment Search Tool) was initially performed to establish sequence identity to the GenBank accessions. In addition, the phylogenetic analysis was performed using the CLUSTAL W multiple sequence alignment program (MegAlign module of Lasergene DNAStar software Pairwise), which was designed by Thompson et al. (1994).

Experimental Infection (Table 2)

The present study was reviewed by the Committee on the Ethics of Animal Experiments of Damanhour University, Egypt, and in compliance with all mandatory laboratory health and safety procedures.

All commercial broiler chickens used for this study revealed negative ORT isolation through tracheal swabs collected at age 1 and 14 d, before experimental infection. A total of 120 chicks, used for this trial, were divided into 4 groups (30 birds each) and each group contained 3 replicates (10 birds each), divided as follows: group 1, injected simultaneously at age 1 and 14 d with live variant IBV 4/91 (Nobilis IBV 4/91, Intervet, Netherlands) through intra-ocular route and ORT isolate no. 17 at a dose of 200 μl of 1.2 $\times$ 10^9 CFU/ml by intratracheal route (Thachil et al., 2009); group 2 chickens were experimentally infected only with ORT at 14th day of age with the same dose; group 3 were vaccinated only with live variant IBV 4/91 on same days of age through intra-ocular route and finally, group 4 remained non-vaccinated and non-infected as the control group.

Feed and water were supplied ad libitum and kept under daily observation. All birds were vaccinated with inactivated vaccines such as H_9N_2 (MeFluVac AI H_9N_2, United Biomed, Egypt), administered on the 5th day of age and a bivalent ND+ H_5N_1 (MeFluVac ND+AI H_5N_1, United Biomed, Egypt), administered on the 8th day of age by intramuscular route.

Histopathology

Specimens from the trachea, air sac, and lung of 3 birds of each group, sacrificed using intravenous injection of sodium pentobarbital (50 mg/kg), were collected 3 and 10 days post infection (dpi) for histopathological examination (Bancroft and Layton, 2013).

Statistical Analysis

The experimental design used was a complete randomized design. The statistical analysis for final body weight **(Bwt.)**, total weight gain **(WG),** and final feed conversion rate **(FCR)** at 35 d was performed using SPSS (2008) (Statistical Package for the Social Sciences, ver. 17.0) program to test the effect of ORT infection alone and along with live IBV 4/91 as a triggering effect. The statistical model used was:

$$Y_{ij} = \mu + T_i + e_{ij},$$

where Y_{ij} = an observation, μ = the overall mean, T_i = fixed impact of treatment and e_{ij} = random error associated to each observation.

RESULTS

PCR and Sequencing

A PCR-amplified 784-bp specific ORT DNA fragment of the 7 ORT isolates from lungs, air sacs, and tracheas of 6 broiler flocks and 1 layer flock from the Elbehera and Marsa Matrouh provinces was detected and their history indicated in Table 1. Phylogenetically, the 2 examined ORT isolates (no. 17 and 51) were closely related (98%–100%) to Asian, European and American ORT strains (Figures 2 and 3).

Results of Experimental Infection (Table 3)

A.1. The effect of ORT infection + IB 4/91 vaccination on final Bwt., total WG, and final FCR:

ORT infection triggered by IB 4/91 vaccination caused a decrease ($P \leq 0.05$) in final Bwt. by 335.41 gm (14.1%) compared to the control chicken group. In

Table 3. Effect of Ornithobacterium strain on the final body weight, total weight gain, and final feed conversion rate in the experimentally infected groups at 35 d.

Parameters/group no.	1	2	3	4
Final body weight (g)	2054.73 $\pm$ 57.59^{c}	2168.43 $\pm$ 46.25^{bc}	2226.67 $\pm$ 50.33^{b}	2390.14 $\pm$ 56.78^{a}
Total weight gain (g)	1857.93 $\pm$ 54.78^{c}	1975.36 $\pm$ 43.74^{bc}	2026.2 $\pm$ 47.38^{b}	2189.07 $\pm$ 53.49^{a}
Final feed conversion rate (g feed/g gain)	1.73 $\pm$ 0.05^{a}	1.67 $\pm$ 0.03a^{b}	1.68 $\pm$ 0.04^{ab}	1.60 $\pm$ 0.04^{b}

Means within the same row carrying different superscripts are significantly different at $P \leq 0.05$.

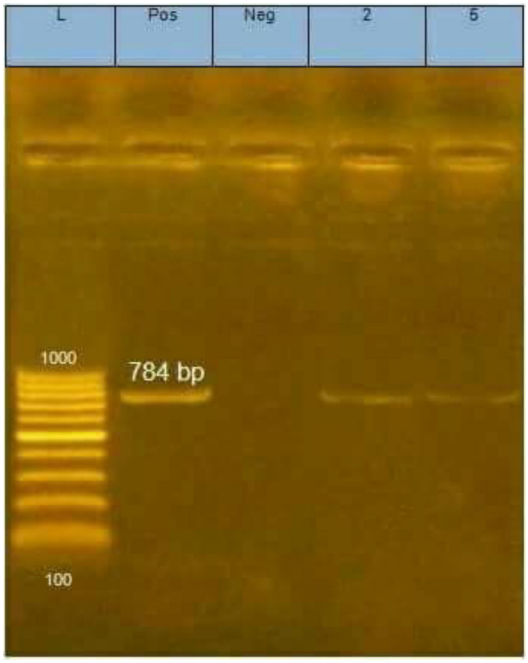

Figure 2. Reverse transcription polymerase chain reaction positive band at 784 bp (2: isolate no. 17 and 5: isolate no. 51).

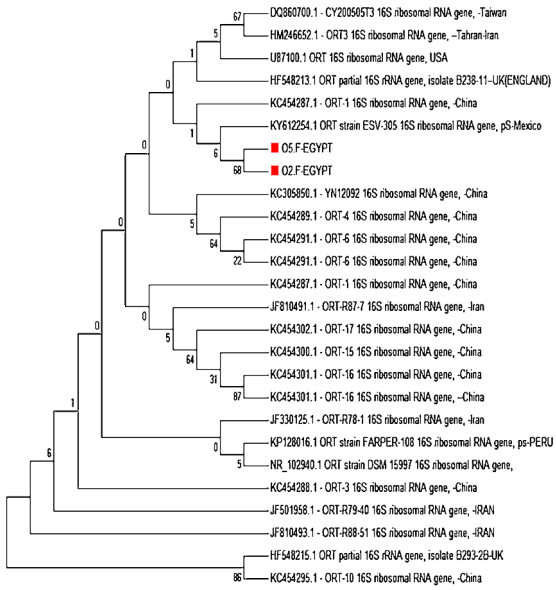

Figure 3. Phylogenetic analysis of 2 ORT isolates (2: isolate no. 17 and 5: isolate no. 51).

total WG, there was a decrease ($P \leq 0.05$) compared to the control group by 331.14 gm (15%). Final FCR in chickens of group 1 was higher ($P \leq 0.05$) than all other groups.

A.2. Clinicopathological effect of ORT infection + IB 4/91 vaccination:

Moderate clinical signs in the form of dullness, ruffled feathers, and rales were observed at 3 to 6 dpi without any mortality. Post-mortem examination of the 3 sacrificed birds revealed moderate tracheitis with presence of exudates, congestion in the lung, and bilateral air sacculitis at 3 and 10 dpi.

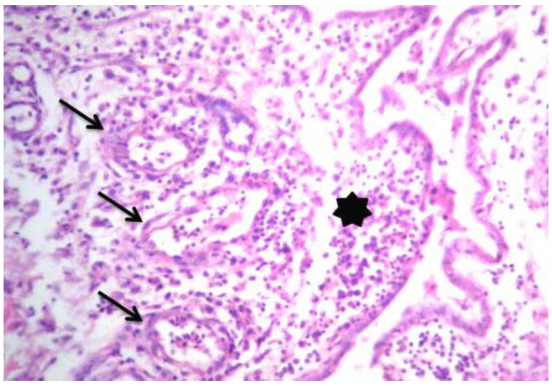

Figure 4. Air sac of a chicken of group 1 on the 17th day of the experiment, showing great thickening with mononuclear cell infiltration (*) as well as congestion of lamina propria blood vessels (arrows). HE ×400.

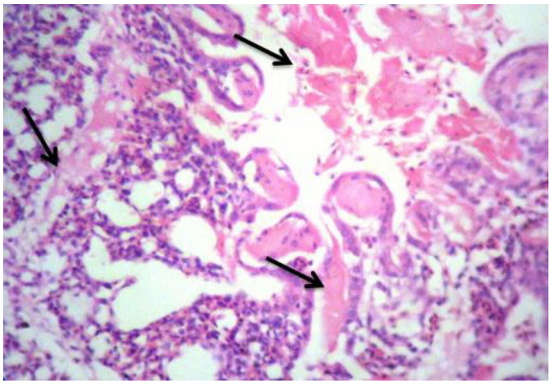

Figure 5. Lung of a group 1 chicken on the 24th day of the experiment, showing fibrinous deposits in the interstitial tissue, para bronchi, and atria associated with inflammatory cells (arrows). HE ×400.

The ORT infective strain was re-isolated from all 10 tracheal swabs as early as 3 and 10 dpi, and confirmed both morphologically and biochemically.

A.3. Histopathological findings of the 3 sacrificed birds of group 1:

Air sacs:

Air sacs at 3 dpi showed severe thickening with mononuclear cell infiltration, as well as diffuse epithelial necrosis and fibrinous exudate covering the epithelial surface (Figure 4). At 10 dpi, air sacculitis showed reduced severity in mononuclear inflammatory cell infiltrations besides congestion.

Lungs:

Pulmonary lesions at 3 dpi appeared as granulomatous pneumonia, represented by aggregation of inflammatory cells with fibrosis pleuritis, desquamated necrotic epithelium and fibrin deposits in the parabronchial space. The same lesions showed marked perivascular edema at 10 dpi (Figure 5).

Trachea:

At 3 dpi, the tracheal mucosa showed catarrhal tracheitis, goblet cell hyperplasia, lymphocytic cell infiltrations; loss of cilia was also observed and continued at 10 dpi, with mucosal and glandular epithelial

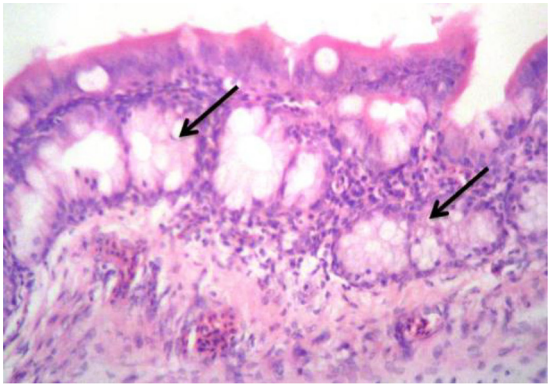

Figure 6. Trachea of a group 1 chicken on the 24th day of the experiment showing mucosal and glandular epithelial hyperplasia (arrows), congestion, and mononuclear cell infiltrations. HE ×400.

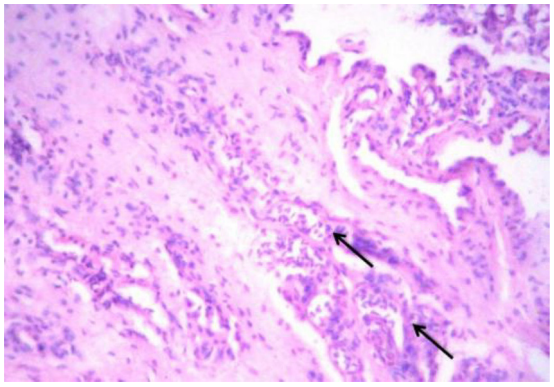

Figure 7. Air sac of a group 2 chicken on the 17th day of the experiment showing congestion (arrows) with infiltration of mononuclear inflammatory cells and few fibroblasts. HE ×400.

hyperplasia, congestion and mononuclear cell infiltrations (Figure 6).

B.1. The effect of ORT infection alone on final Bwt., total WG, and final FCR:

ORT infection alone caused a decrease ($P \leq 0.05$) in Bwt. by 221.7 gm (9.27%) and in total WG by 213.71 g (9.76%) compared to the control group. Total FCR in chicken group 2 was no different ($P \geq 0.05$) from other groups.

B.2. Clinicopathological effect on chickens infected only by ORT:

Mild clinical signs in the form of ruffled feathers and rales were observed at 3 dpi, without any mortality.

Post-mortem examination of the 3 sacrificed birds did not reveal any significant pathological changes in the organs examined at 3 dpi; at 10 dpi, slight exudate in the trachea and unilateral or bilateral lung congestion with mild air sacculitis was recorded.

ORT re-isolation: All 10 tracheal swabs collected at 3 dpi revealed positive results; 7 out of 10 tracheal swabs collected at 10 dpi had successfully been re-isolated and confirmed morphologically and biochemically.

B.3. Histopathological findings of the 3 sacrificed birds of group 2:

Air sacs:

At 3 dpi, air sacs showed focal areas of congestion with infiltration of mononuclear inflammatory cells and few fibroblasts (Figure 7), and mild air sacculitis at 10 dpi.

Lungs:

Thickening of the interstitial tissue and pleura with edematous fluid, fibrin deposits, congested blood vessels, and inflammatory cells was recorded. Parabronchial space and atria containing fibrinous deposits with desquamated epithelium was present in the sacrificed birds at 3 dpi (Figure 8) and 10 dpi.

Trachea:

At 3 dpi, the sacrificed birds showed intact tracheal mucosa with ciliated epithelium and few lymphocytic cell infiltrations. While at 10 dpi, mucosal and glandular epithelial hyperplasia as well as congestion and mononuclear cell infiltrations were observed (Figure 9).

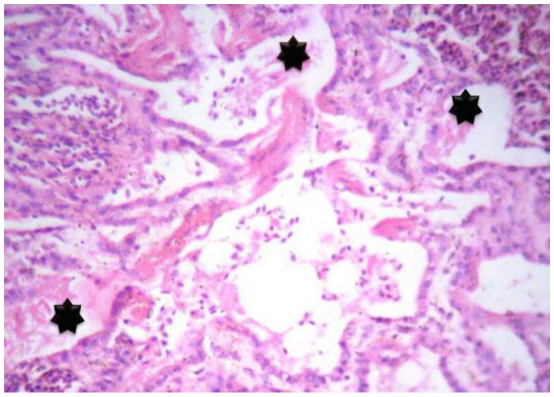

Figure 8. Lung of a group 2 chicken on the 17th day of the experiment showing, parabronchial space, and atria contained fibrinous deposits (*) with desquamated epithelium and erythrocytes. HE ×400.

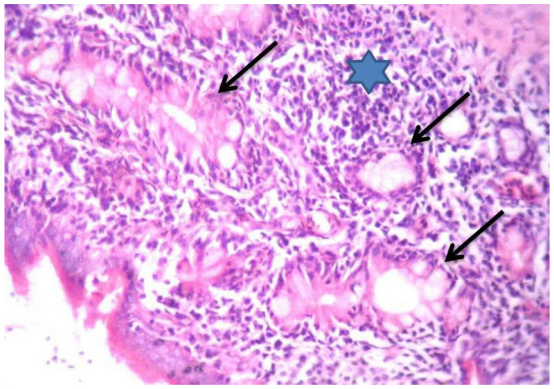

Figure 9. Trachea of a group 2 chicken on the 24th day of the experiment, showing mucosal and glandular epithelial hyperplasia (arrows), congestion and mononuclear cell infiltrations (*). HE ×400.

C. Groups 3 birds, only vaccinated with IB 4/91, revealed no differences ($P \geq 0.05$) in final Bwt., total WG, and final FCR as compared to the control group in this experiment. Both these groups had no mortalities and the post-mortem examination of the 3 sacrificed birds did not reveal any pathological changes in the organs examined at 3 and 10 dpi. ORT was not

isolated from the tracheas at 17 and 24 d age in either group. Histopathologically, no lesions appeared in these groups except for few lymphocytic cell infiltrations in the chickens of group 3 at 3 dpi.

DISCUSSION

Respiratory diseases in poultry cause significant economic losses in the poultry industry, especially in the developing countries. Among the avian species, a new bacterial respiratory disease in chickens was observed in South Africa and named *Ornithobacterium rhinotracheale* (Vandamme et al., 1994).

In this study, 6 positive ORT broiler flocks and 1 positive layer flock were isolated by culturing and confirmed biochemically by PCR which is a fast, sensitive, and specific method to identify and characterize a bacterial strain (Hung and Alvarado, 2001; Ozbey et al., 2004; Hassanzadeh et al., 2010). The PCR procedure is considered to be a useful laboratory tool for the definitive identification of suspected ORT isolates, due to its difficulty in isolation and biochemical characterization as it is usually overgrown by other bacteria (Chansiripornchai et al., 2007; Chin et al., 2008; Churria et al., 2011; Churria et al., 2012).

Owing to these results, the incidence rate of ORT was 11.66%; in broilers, this rate was higher than the previously reported values of 8.5%–10% (El-Gohary and Awaad, 1998; Seyyed et al., 2012) and 7.27% (Elbestawy, 2010). This elevated rate of isolation compared to previous studies may be due to our increased experience with this bacterial pathogen since 2009, and the increased respiratory viral infections such as avian influenza (subtype H5N1 and H9N2), Newcastle disease, and infectious bronchitis virus in the Egyptian poultry production field. This may indicate rapid evolution and spread of ORT.

Frequent isolation of ORT from various organs indicated that the isolates were commonly recovered from lungs, air sac, and trachea. This was in agreement with van Empel and Hafez (1999) and Welchman et al. (2013) who recorded that the air sacs and lungs were the organs with highest isolation rate of ORT.

Phylogenetically, the 2 examined ORT isolates (no. 17 and 51) were closely related (98%–100%) to Asian, European, and American ORT strains. In addition, Masoud et al. (2015) mentioned that 5 Egyptian isolates showed 94% to 98% similarity to some American and Asian isolates based on the sequence analysis of 16S rRNA.

In 14-d-old chickens, the pathogenicity of the isolated no. 17 ORT strain, alone or combined with live IBV 4/91 vaccine, revealed characteristic findings. ORT infection alone caused only mild respiratory signs and no pathological changes in the examined organs appeared, except at 10 dpi, where slight exudate in trachea and unilateral or bilateral lung congestion was seen with mild air sacculitis. Tracheal ORT was re-isolated as early as 3 dpi in all swabs and at 10 dpi only in 70% of swabs. Histopathologically, the trachea showed epithelial and glandular epithelial hyperplasia and mononuclear cell infiltration only at 10 dpi, which indicated the weak ORT pathogenicity with slow-developing lesions in the absence of triggering factors.

Group 1 chickens infected with ORT and triggered by live IBV 4/91 showed moderate respiratory signs at 3 dpi; post-mortem lesions appeared as tracheitis and bilateral pneumonia. Histopathologically, moderate-to-severe catarrhal tracheitis with goblet cell hyperplasia and loss of cilia were recorded. In addition, bilateral granulomatous pneumonia with lung edema and severe fibrinous air sacculitis was observed at 3 and 10 dpi. In addition, ORT infective isolate was re-isolated at 3 and 10 dpi from all the tracheal swabs.

No mortalities were recorded in any of the groups, as experimentally the environmental conditions were good. However, under field conditions, the severity of the ORT infection's clinical signs, duration of the disease and mortality were extremely variable; they were influenced by many environmental factors such as poor management, inadequate ventilation, high stocking density, poor litter condition, poor hygiene, variable ammonia levels, concurrent disease, and types of secondary infection (Travers, 1996).

These results matched with those of El-Gohary et al. (1998), van Empel and Hafez (1999), and Chin et al. (2008) who reported that the most characteristic ORT histopathological lesion usually can be found as granulomatous pneumonia, tracheitis, and foamy air sacculitis.

In pheasants, Welchman et al. (2013) identified ORT as a part of a complex of other respiratory agents that included: avian paramyxovirus type 2, avian coronavirus, *Mycoplasma* species, *Escherichia coli*, *Pasteurella* species, and *Syngamus trachea*, suggesting synergism with these agents. This was the cause of granulomatous pneumonia and fibrinous air sacculitis that lead to severe respiratory distress.

In addition, one of the most prominent findings at the end of experimental infection was growth retardation in the infected birds of groups 1 and 2. The ORT infection alone and when combined with IBV vaccination had a decrease ($P \leq 0.05$) in final Bwt. by 9.27% and 14.1%, respectively, as compared to the control group.

With respect to total WG, there was a reduction ($P \leq 0.05$) by 15% and 9.76%, in groups 1 and 2 respectively, as compared to the control group. In addition, FCR of the chickens in group 1 was higher ($P \leq 0.05$) than the control group.

This was in agreement with van Empel et al. (1996), Canal et al. (2003), and Hegazy et al. (2015) who determined a positive correlation between the presence of antibodies against ORT and decreased body weight in broilers, without any significant correlation with other parameters such as age, lineage, efficiency index, feed conversion, and mortality. In addition, van Empel et al. (1996) found that ORT caused growth retardation in both turkeys and chickens after experimental

intra-air sac administration, together with air sacculitis and pneumonia after aerosol administration.

CONCLUSIONS

Phylogenetically, the 2 examined ORT isolates (no. 17 and 51) were closely related (98%–100%) to Asian, European, and American ORT strains. It is clear that the ORT causes exaggerated respiratory disease, especially in combination with live attenuated variant IBV 4/91 and led to decreased body weight, weight gain, and increased feed conversion ratio.

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