

Molecular and Serological Diagnosis of Newcastle and *Ornithobacterium rhinotracheale* Broiler in Chicken in Fars Province, Iran

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Abstract—Respiratory diseases are the most important problems in the country's poultry industry, particularly when it comes to broiler flocks. *Ornithobacterium rhinotracheale* (*ORT*) is a species that causes poor performance in growth rate, egg production, and mortality. This pathogen causes a respiratory infection including pulmonary alveolar inflammation, and pneumonia of birds throughout the world. Newcastle disease (ND) is a highly contagious disease in poultry, and also, it causes considerable losses to the poultry industry. The aim of this study was to evaluate the simultaneous occurrence of *ORT* and ND and NDV isolation by inoculation in embryonated eggs and confirmed by RT-PCR in broiler chicken flocks in Fars province. In this study, 318 blood and 85 tissue samples (brain, trachea, liver, and cecal tonsils) were collected from 15 broiler chicken farms. Survey serum antibody titers against *ORT* by using a commercial enzyme-linked immunosorbent assay (ELISA) kit performed. Evaluation of antibody titer against ND virus is performed by hemagglutination inhibition test. Virus isolation with chick embryo eggs 9-11 and RT-PCR method were carried out. A total of 318 serum samples, 135 samples (42.5%) were positive for antibodies to *ORT* and titer of HI antibodies against NDV in 122 serum samples (38/4%) were 7-10 (log₂) and 61 serum samples (19/2%) had occurrence antibody titer against Newcastle virus and *ORT*. Results of the present study indicated that 20 tissue samples were positive in embryonated egg and in rapid hemagglutination (HA) test. HI test with specific ND positive serum confirmed that 6 of 20 samples. PCR confirmed that all six samples were positive and PCR products of samples indicated 535-base pair fragments in electrophoresis. Due to the great economic importance of these two diseases in the poultry industry, it is necessary to design and implement a comprehensive plan for prevention and control of these diseases.

Keywords—ELISA, Newcastle disease, *Ornithobacterium rhinotracheale*, seroprevalence.

I. INTRODUCTION

RESPIRATORY infections are the most important diseases affecting birds, especially industrially produced poultry. These diseases inflict heavy economic losses on poultry industry because they kill large numbers of poultry, entail

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considerable treatment costs, and reduce production. Various viral and bacterial agents may be involved in respiratory infections either singly or simultaneously with other microorganisms [1]. Respiratory infections may also be influenced by non-infectious factors such as poor management, insufficient ventilation, high-density poultry flocks, unsuitable conditions, and poor sanitation that prolong the duration of these infections and of the inflicted losses. *ORT* infections are among the infections that have attracted the interest of researchers in recent years and play a very important role in respiratory complexes [2]. *ORTs* are Gram-negative, polymorphic, non-motile, rod-shaped, and non-spore forming bacteria that grow slowly, belong to the superfamily of RNA containing bacteria [3], and cause respiratory infections including inflammation of air sacs and pneumonia in birds all over the world. *ORTs* are often reported in chickens and turkeys, but they have also been reported in other bird species [4]. Depending on the intensity of pathogenicity in the related strain, environmental conditions, immune status of the birds, and presence of other infectious agents, *ORT* can act as the primary or secondary pathogen [5]. Van Veen et al. described *ORT* as a primary pathogen in broilers [6]. Nevertheless, it seems infectious and non-infectious agents are involved as initiator or intensifier of *ORT* infections on poultry farms [7]. In recent years, prevalence of *ORT* infections has been reported from all over the world including the United States, France, Holland, Belgium, Spain, Germany, Hungary, Israel, Korea, Japan, Taiwan, Turkey, Brazil, Iran, and South Africa. Furthermore, reports have been received that concurrence of *ORT* infections with those caused by other pathogenic agents in poultry may intensify the pathogenicity of *ORT* [8]. Despite results obtained from laboratory infections caused by *ORT* in broilers without any of the common infection symptoms or environmental stresses, the role played by *ORT* as a primary infectious agent is still unclear and uncertain. In many reported cases of respiratory infections in chickens and turkeys, *ORT* has had a complementary role to other agents, including Newcastle disease virus (NDV), that cause respiratory diseases. ND is one of the viral respiratory diseases that inflict heavy economic losses on poultry industry every year. In investigated of molecular characterization and phylogenetic analysis of NDVs isolated from recent outbreaks (2009 to 2011) in Fars province, Iran shown that ND was considered as the most important infection that leads to substantial production losses [9]. It is one of the most dangerous diseases

of poultry, is extremely contagious, has global importance due to the extensive and substantial damage it causes, has long since been identified in most countries of the world, and has always been troublesome given its widespread expansion [9]. NDV is a negative sense RNA virus, belongs to the genus *Avulavirus* of the family *Paramyxoviridae*. It varies from sub-clinical or mild infections of the upper respiratory system to severe and fatal infections [10]. These two diseases have been studied separately numerous times, but not concurrently so far, in Iran. Therefore, this research investigated the concurrence of *ORT* infection and ND in broilers in Fars Province, and isolated and identified NDV using the PCR method and egg inoculation.

II. MATERIALS AND METHODS

A. Sample Collection

From late August 2014 to late April 2015, 318 blood sample and 85 tissue samples were collected from the brains, tracheae, and broilers presenting symptoms of the diseases from 15 poultry farms in Fars Province in which the broilers exhibited respiratory symptoms and where losses were high. All samples were placed in ice and transferred to the laboratories at the Razi Vaccine and Serum Research Institute as fast as possible. After separating the sera from the blood samples, the samples were coded and kept at -70°C to be tested. Moreover, similar tissue samples from each poultry farm were mixed together, antibiotic containing culture medium was added to them, they were centrifuged for 15 minutes at 1500 g, and the supernatant was inoculated into the allantoic cavities of eggs with 9-11 day old specific pathogen-free embryos (SPF).

B. Serological Study of *ORT*

Using commercial ELISA kits (IDEXX), all serum samples were studied with respect to their antibody titers against *ORT*. These kits can recognize antibodies in chicken and turkey serum. All samples were prepared and studied based on the instructions of the manufacturing company. The plates were read at 650 nm wavelength using an ELISA microplate reader. The titer of each serum sample was calculated based on the OD of the sample in each plate using SPSS and the following formula:

$$\text{S/P} = (\text{OD of the serum sample} - \text{Average of negative controls}) / (\text{Average of the positive controls} - \text{average of the negative controls})$$
$$\text{Log}_{10} \text{ titre} = 1.09 \times (\text{Log}_{10} \text{ S/P}) + 3.36$$

$\text{Anti Log}_{10} = \text{Antibody titre of the serum}$

Based on the recommendation of the company that manufactured the kits, serum samples with S/P ratios equal to or lower than 0.4 were considered negative and those S/P ratios higher than 0.4 positive.

C. Hemagglutination Inhibition (HI) Test against NDV

All sera taken from each poultry farm underwent the hemagglutination inhibition test separately to determine their antibody titers against NDV, and the obtained results were

analyzed.

D. Isolation of NDV

To isolate NDV, tissue samples were first prepared. Similar tissues from each poultry farm were then mixed and mashed in a steel mortar that included a medium containing antibiotics (penicillin, streptomycin, and amphotericin) in the weight ratio of 20%. The samples were then centrifuged for 15 minutes at 15000 g. The supernatant of centrifuged samples was passed through 0.22μ filter paper and was used to inoculate eggs having 9-11 day old specific pathogen free embryos. The aminoallantoic fluids were harvested and analyzed for HA [11]. HA negative titers were passaged once again in embryonated chicken eggs. HI test was used on HA positive sample for virus isolate subtyping, and was performed using ND anti-sera obtained from Istituto Zooprofilattico Sperimentale delle Venezie [11].

E. RNA Extraction

The viral RNAs were extracted directly from infectious allantoic fluids using VeTeKTM viral gene spin kit (Intron Biotechnology Inc., Seongnam, Korea), according to the protocol. The viral RNA extracted was resuspended in DNase/RNase free water and stored at -70°C until use [9].

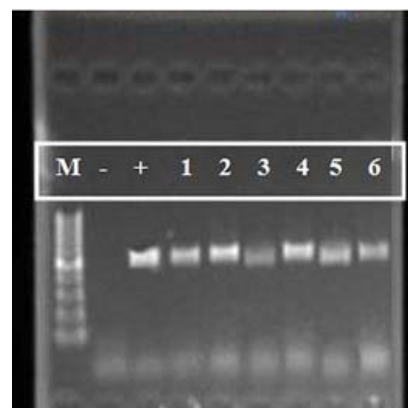


Fig. 1 RT-PCR products of F gene (NDV), an expected size PCR product, and 535 bp of F gene were detected. Lane 1=Marker 100 bp DNA ladder, Lane 2=Negative control, Lane 3= positive control, Lane 4-9=field samples

F. Polymerase Chain Reaction. (PCR)

Standard RT-PCR was carried by use of the one-step RT-PCR kit (INtRON Biotechnology Inc., Seongnam, Korea). The PCR amplification and sequencing were performed using degenerative primers ($5'$ -ATGGGC(C/T)CCAGA(C/T)CTTCTA-3'; and $5'$ -CTGCCACTGCTAGTTGTGATAATCC-3'). The primers generated an expected amplicon size of 535 bp (47-581 of the F gene). RT-PCR was carried out in $8\mu\text{L}$ reaction mixture using Vetek viral gene-spin Viral DNA/RNA Extraction Kit (Intron Biotechnology Inc., Seongnam, Korea) containing (dNTP, enzyme mix, reaction buffer and stabilizing buffer), $1\mu\text{L}$ from each forward and reverse primer ($25\mu\text{M}$), $2\mu\text{L}$ of template RNA, 0.25RNase inhibitor and $8\mu\text{L}$ of RNase free water was added to make a reaction volume of $20\mu\text{L}$. The cycling parameters for PCR

reaction were 48 °C for 45 min at RT. PCR reactions were subjected to 35 cycles consisting of denaturation at 94 °C for 2 min, annealing at 56 °C for 2 min, and extension at 72 °C for 1 min, followed by 72 °C for 10 min [9]. The amplicons were separated in 1.5% agarose gel electrophoresis and visualized under ultra-violet light after being stained with ethidium bromide.

III. RESULTS

Of the 318 serum samples taken from 15 broiler farms in Fars Province, 135 (42.5%) had positive antibody titers against *ORT* in ELISA tests (Fig. 1) and 122, or 38.4%, possessed antibody titers of 7 to 10 against NDV in the HI test. Moreover, using the HI method, 61 serum samples (19.2% of the total) included antibody titers against *ORT* and antibody titers higher than seven against NDV. Of the total tissue samples taken from the poultry farms to isolate the virus through inoculation of eggs with embryos, 20 samples had rapid hemagglutination activities, and six of them were considered Newcastle positive in the HI test using the standard antibody against NDV. Of the 20 tissue samples with positive HI test results, only six samples amplified the 535 bp segment and were identified as samples containing NDV (Fig. 2). It must be mentioned that all samples that were identified positive using the isolation method were also identified as positive in the PCR test. Therefore, considering the disease symptoms and isolation of the virus from the mentioned tissue samples, this virus cannot be a lentogenic NDV and, hence, cannot be a vaccine. Results indicated that, in half (50%) of the poultry farms that had antibody titers against *ORT*, antibody titers against Newcastle were higher than 7, and results of their NDV isolation and of the PCR test were positive for Newcastle.



Fig. 2 The rate of *ORT* among broiler flocks in Fars Province. Blue refer to positive samples and red refer to negative samples

IV. DISCUSSION

Respiratory syndrome is one of the important problems in the poultry industry of Iran during the growing period of flocks, especially of broilers. Various viral and bacterial agents including NDV, avian influenza virus, Infectious Bronchitis virus, pneumoviruses, and the bacteria *Escherichia coli*, *ORT* and mycoplasmas, can play roles in the development of this syndrome [11]. One of the bacterial agents in the respiratory disease complex in poultry is the *ORT* bacterial species that was first isolated and identified in Iran in 2000 by Babani et al. at the Razi Vaccine and Serum Research Institute from a broiler flock and a pullet flock that exhibited respiratory symptoms [12]. Bacteria of the *ORT* species grow very slowly, and they grow after being incubated for 48 hours

under microaerophilic conditions and are quickly covered by rapid-growing bacteria such as *Escherichia coli* and *Proteus* or *Pseudomonas* bacteria. This makes the isolation and identification of *ORT* very difficult, and many researchers use serological techniques to diagnose diseases caused by this bacterial species. Some researchers have reported the ELISA test is useful in detecting *ORT* antibodies in day old chicks and in egg yolk. Furthermore, another researcher compared results obtained from ELISA tests with those observed in the rapid agglutination test, and reported the agglutination test detected the specific antibodies only in 56% of experimental infections in turkeys during the first two weeks of the infections. However, the ELISA test could detect these antibodies in up to 100% of the infected birds during the period leading to 8 weeks after infection [2]. The ELISA test has been used in many Iranian Provinces to detect *ORT*. Based on research conducted in Tehran and Chaharmahal and Bakhtiari Provinces, the extents of infection with *ORT* were 44.5 and 10.2%, respectively, which indicates differences in the extent of infection in the various Provinces. Results of the present research indicated 42.5% of the samples were positive for antibody titers against *ORT* in Fars Province. These results agree with those obtained in research conducted in the various regions of Iran and in other countries. The observed differences could be due to different ages of the studied broilers, differences in the time the infections occurred, instability of the produced antibodies and, probably, because the studies were carried out in different seasons of the year. Bacteria of the *ORT* species can act as the primary pathogen and do not need to be accompanied by other pathogens to cause diseases. Nevertheless, it seems presence of infectious and non-infectious agents in poultry farms can act as the trigger for or an intensifier of *ORT* pathogenicity. Banani et al. reported concurrence of *ORT* infections with other pathogenic agents of poultry in Iran [13]. One of the basic problems in the poultry industry in Iran results from viral respiratory infections, and the general veterinary office of Fars province reported that about 30% of chickens raised in Fars province develop respiratory problems, one of the causal reasons of which is NDV. This virus inflicts substantial economic losses annually by causing mortality resulting from the ND, by reducing weight of chickens, and through lowering egg production. At present, this virus infects a substantial number of industrially produced poultry every year, which inflicts considerable losses on the poultry industry. Therefore, rapid and timely diagnosis of these diseases, their differentiation from each other, and identification of their viruses are of great importance. Previous studies carried out in Fars province and in other parts of the country suggested NDV was present in broiler poultry farms, and this emphasized the importance of this virus in the development of respiratory diseases in poultry, which was followed by heavy losses inflicted on the poultry industry [9]. Nevertheless, no thorough studies had been carried out on concurrent infection with *ORT* and NDV in Iran. Therefore, considering the prevalence of *ORT* infections in Iran, and given the presence of respiratory infections by NDV, the present study determined antibody

titres against this bacterial species and NDV in broilers, and confirmed the obtained serological results through isolating and identifying NDV from sick broilers.

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