

ULTRASTRUCTURAL STUDY OF THE TRACHEA IN EXPERIMENTALLY INFECTED BROILERS WITH IBV SEROTYPE 4/91

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Summary: Avian infectious bronchitis virus (IBV) is a globally distributed Coronavirus that causes tremendous economic loss in the poultry industry. The primary target cells of the virus are located in the respiratory tract. Strain-dependent, IBV may spread to other epithelia and cause nephritis or drop in egg production. Subcellular changes in broilers trachea induced by infectious bronchitis virus serotype 4/91 were examined by transmission electron microscopy (TEM). Seventy 1-day-old commercial broiler chicks were divided randomly into two groups (control and experimental), and at the age of 21 days, all birds in the experimental group were challenged intranasally with the virus. Four birds from challenge group and two birds from the control group were euthanized at 1, 2, and 4 days post inoculation (PI) and tracheae were isolated after necropsy and examined with TEM. Mild tracheal rales, coughing and gasping were seen in the experimental group. As gross lesions, hyperemia and edema in tracheal mucosa were observed. IBV infection resulted in hypertrophy of goblet cells, their rupture, and the formation of excess mucus. At the level of the ciliated cells, complete deciliation of the tracheal surface was observed. Swelling and increase in the amount of endoplasmic reticulum was seen in infected birds.

Key words: transmission electron microscope; infectious bronchitis virus; serotype 4/91; trachea; broilers

Introduction

Avian infectious bronchitis virus (IBV), an enveloped, positive-sense single stranded RNA virus, is a member of the *Coronaviridae* family of the order *Nidovirales* that causes highly contagious disease of poultry, which poses a major threat to the poultry industry. The avian coronavirus IBV has a unique morphology. It is an envelope virus that is spherical to pleomorphic with evenly dispersed spike-like projections on the

surface of the virion. Clinical cases of infectious bronchitis (IB) are associated with respiratory, reproductive, digestive and renal infections in domestic poultry and in various other avian species (1). Although effective vaccines are available and utilized routinely in commercial poultry production, the virus tends to mutate frequently (2). Little or no cross-protection occurs between different serotypes of infectious bronchitis virus (3), therefore, continuous determination of the epidemic serotype and production of new generations of vaccines are crucial for controlling IB in each geographic region or country. The IBV

serotype 4/91 (also named 793/B and CR88), was first reported in Britain in the early 1990s (4, 5), and is one of the most common IBV serotypes worldwide (3). In Iran, the presence of Massachusetts serotype as the major circulating IBV was confirmed in 1994 (6). By isolation and serologic identification of some field strains, VASFI MARANDI and BOZORGMEHRI FARD (7), suggested the presence of IBV variant(s) in Iran. Serotype 4/91 was isolated in Iran by MOMAYEZ *et al.* (8) and a recent study revealed that it has been the dominant IBV serotype throughout 1994-2004 in Iran (9). In the present study, ultrastructural changes in the trachea due to infection with IBV serotype 4/91 were investigated.

Materials and methods

Virus preparation

IBV serotype 4/91 isolated from broiler flocks in Iran (10) was used in this study. Virus propagation was performed in 10-day-old embryonated chicken eggs. Eggs were obtained from a respiratory disease-free flock. The embryo lethal dose (ELD₅₀) of infected allantoic fluid was calculated according to the REED and MUENCH'S (11) formula. In the present study, allantoic fluid containing 10^{6.5} ELD₅₀/0.1ml of the virus was used to induce the disease.

Experimental Design

This study was performed after receiving approval from the Animal Ethics Committee of the Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran. Also, it was conducted with respect to the International Guidelines for research involving animals (Directive 2010/63/EU). Seventy, 1-day-old commercial broiler chicks (Ross breed) were divided randomly into two groups (fifty chicks in the experimental and twenty chicks in the control group). Experimental and control groups were housed in separate experimental rooms. Food and water were supplied *ad libitum*. No vaccine was used in either of the two groups. Prior to virus challenge, all birds were found free from IBV antibodies (Flock Check IBV ELISA test kit, IDEXX Laboratories Inc., Westbrook,

ME). Tracheal swabs were taken from chicks for detection of other respiratory pathogens such as avian influenza virus (H9N2), Newcastle disease virus and *Mycoplasma spp.* by molecular assay before and after IBV inoculation (12) and the results were negative. At the age of 21 days, all birds in the experimental group were challenged intranasally with 0.2 ml of allantoic fluid virus suspension (titre 10^{6.5} EID₅₀ per 0.1ml). Control birds were sham inoculated with an equal volume of normal saline. Four birds from the challenge group and two birds from the control group were euthanized with chloroform at 1, 2, and 4 days post inoculation (PI) and tracheae were isolated after necropsy.

Electron microscopy

Tracheal specimens were fixed in 2.5% glutaraldehyde-2% formaldehyde, washed in sodium cacodylate buffer solution (pH 7.4), post-fixed in 1% OsO₄, dehydrated in ascending concentration of ethanol, and embedded in medium Spurr's resin. Tracheal sections (1 µm) stained with toluidine blue were examined for trimming of the blocks to areas of interest. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined in a Philips CM10 transmission electron microscope (13).

Results

Clinical signs and gross lesions

No clinical signs and gross lesions were observed in control group. In experimental group mild tracheal rales, coughing and gasping were seen at 24 hours PI. Severity of the signs was less after 5 days PI. Slight hyperemia and edema in tracheal mucosa were noticed in the euthanized birds. No mortality was observed in either group.

Electron Microscopy

In chicks from the control group the tracheal surface was lined by a pseudostratified columnar epithelium. The surfaces of control group tracheas were covered with abundant cilia and some goblet cells. The following changes were seen in chicks of experimental group at different days

post inoculation. In day 1 PI, virus particles were localized in the tracheal epithelial cells (Figure 1). In day 2 PI, limited infiltration of heterophils and lymphocytes to the tracheal epithelium, inflammation and congestion, hypertrophy of goblet cells, mild edema and partial loss of cilia were seen. In day 4 PI, deciliation of the tracheal epithelium was increased and complete deciliation was seen at some sections. Other changes in this time were: infiltration of plasma cells, and

appearance of vacuoles containing lymphocytes and heterophiles (Figure 2), edema in sub mucosal layer, severe degeneration of the epithelial cells and deformation of the mucous gland. Viral particles were observed outside surface cytoplasmic membranes and occasionally within cytoplasmic vesicles (Figure 3). Swelling and increase in the amount of endoplasmic reticulum were seen at 4 PI (Figure 4).

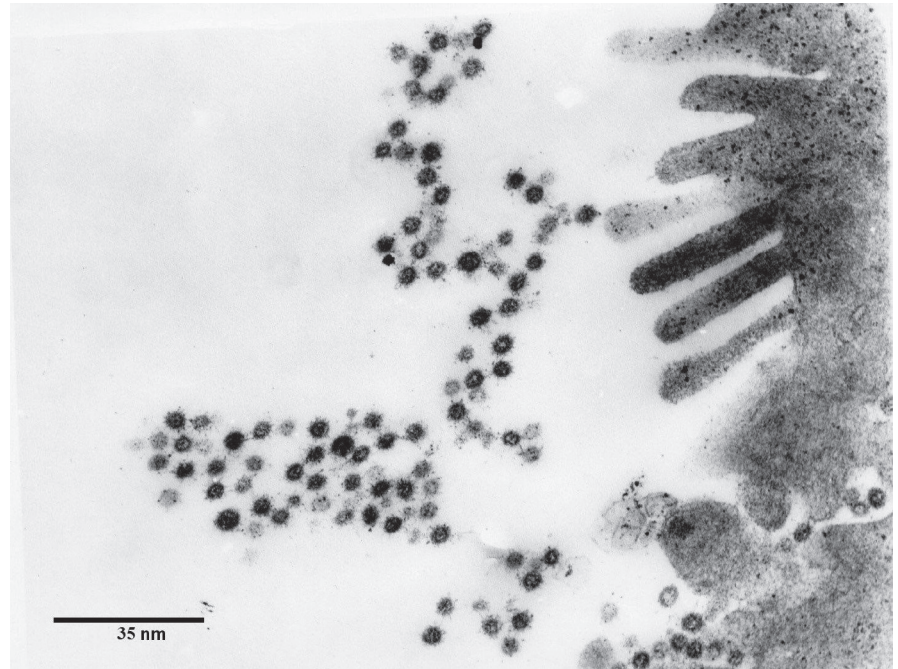


Figure 1: Viral particles in epithelial cells of chicken trachea at day 1 after infection. $\times 28500$

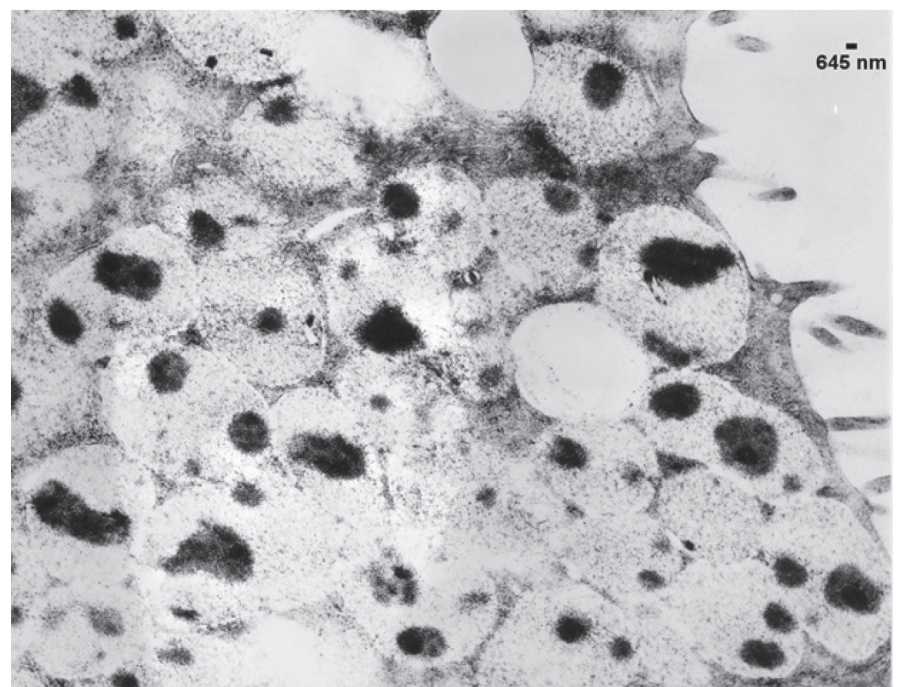


Figure 2: Trachea at 4 days post infection. Electron-dense particles in hypertrophied Goblet cells. Partial deciliation of the tracheal epithelium. $\times 1550$

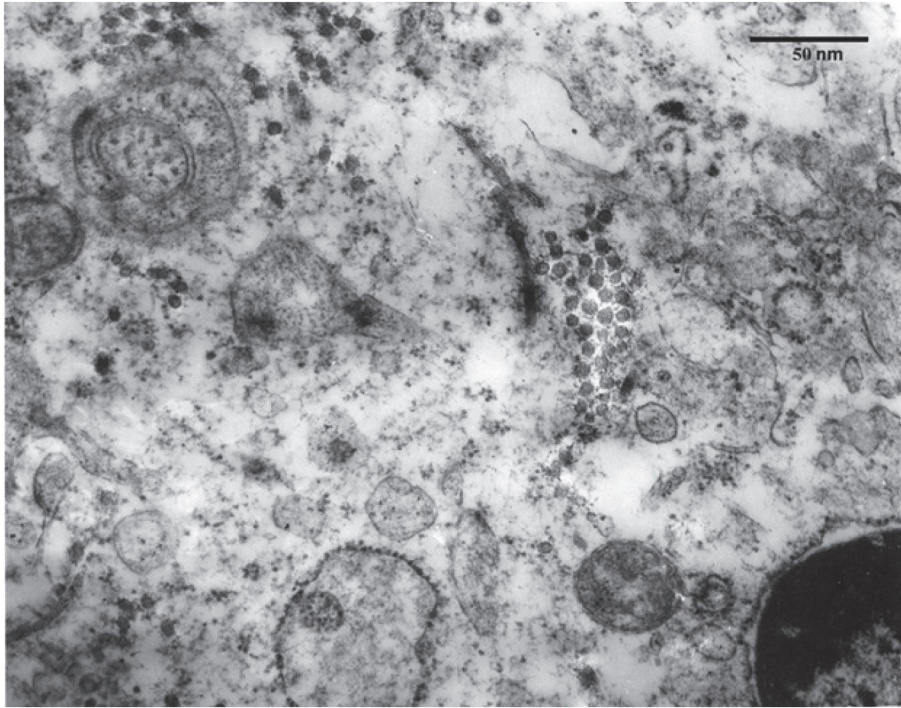


Figure 3: Viral particles are seen in the Golgi apparatus and in electron-dense areas at day 4 after infection. $\times 21000$

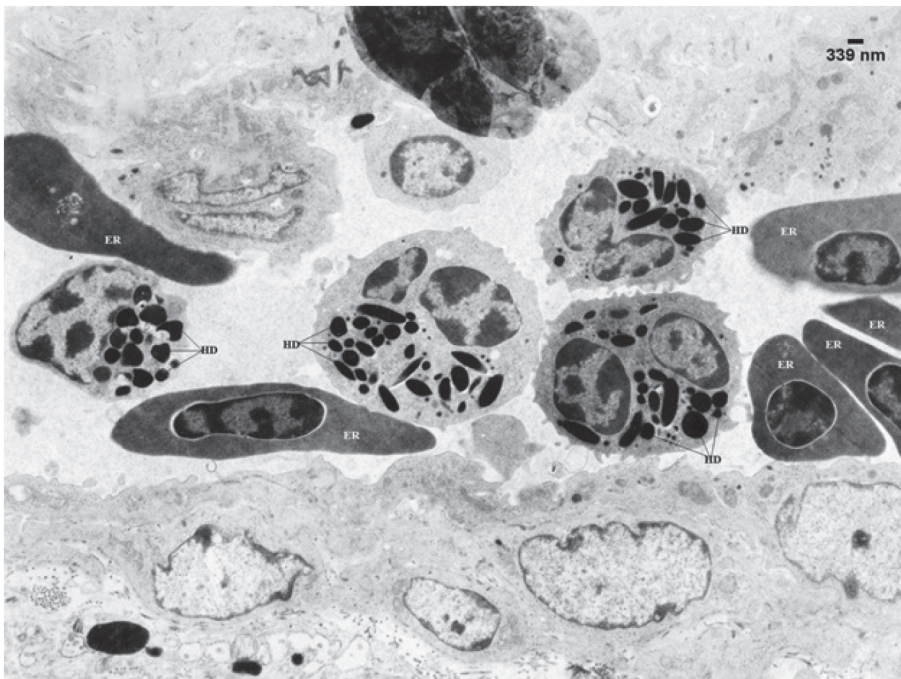


Figure 4: Heterophile degranulation at day 4 after infection. $\times 2950$ (ER: Endoplasmic reticulum, HD: Heterophile degranulation)

Discussion

The progression of tracheal lesions induced by inoculation of commercial broiler chicks with infectious bronchitis virus serotype 4/91 was examined by tracking subcellular changes using transmission electron microscopy. Serotype 4/91 of IBV was first isolated in 1985 in France, and then spread to many countries in Europe, Japan, Saudi Arabia, Thailand and Mexico (5, 14). Despite the regular vaccinations on chicken farms, mostly with Massachusetts (Mass) strains, IB still exerts a severe negative impact on the poultry industry in Iran. Cross-protection between different serotypes of IBV is variable; hence vaccination failure may be due to low homology (26%) between the 4/91 and Massachusetts serotype vaccines such as the H120 strain (15, 16). Therefore, it is very important to understand the effects of this virus on broilers.

Replication of IBV occurs in the ciliated epithelium and mucous cells within 24 hr after intratracheal or aerosol inoculation, and viral particles are confined to small vacuoles of cytoplasm (17, 18). Severity of the tracheal lesions are varied due to different inoculation routes, strains and inoculum sizes of IBV and age of chicken infected (17, 19, 20). In our experiment, tracheal epithelial changes such as hyperemia and edema were similar to those described previously (21, 22).

According to the results, complete deciliation was seen at 4 PI, which is in agreement with results of UPPAL and CHU (23), NAKAMURA *et al.* (20), and ABD EL RAHMAN *et al.* (24). Loss of cilia was observed at day 2 PI and was complete at day 4 PI. The increase in the damage to ciliated cells destruction between 1 and 4 days PI reflects the replacement of the highly differentiated, pseudostratified epithelium containing ciliated cells by a simple squamous to cuboidal epithelium without cilia. Replacement by cuboidal cells is best interpreted as the regeneration phase during which the basal cells at the periphery of a lesion slide across the underlying fibroblastic connective tissue to close the lesion. An early strong activation of the goblet cells was observed in inoculated chicks which most likely reveal the initial IBV infection of these cells. The subsequent decrease in the goblet cells activation score can be attributed to the goblet cells exhaustion and elimination due to virus replication as severe ultrastructural decay and rupture of these cells was observed

after the initial activation. Cilia are responsible for propelling the entrapped particles (bacteria, virus, dust etc.) for disposal. Reduced ciliary motility or disrupted ciliated epithelium could be expected to adversely affect the resistance of birds to microorganisms that normally enter their bodies via the respiratory system (25). On the other hand, this study confirmed that IBV initially infects the upper respiratory tract, where it is restricted to the ciliated and mucus-secreting cells (26).

In Iran, the most prominent lesion in respiratory disease infected flocks is severe exudation in trachea, which leads to tubular cast formation in the tracheal bifurcation and extending to the lower bronchi (27). This is followed by destruction of the cilia by factors such as infectious bronchitis virus.

In normal conditions in which the goblet cells are not activated, the mucus layer that covers the tracheal epithelium is largely washed away during the reparative steps. After virus inoculation goblet cells were ruptured and deformed. This finding is in line with results of MAST *et al.* (28).

In conclusion, although no mortality was seen in infected group in experimental condition, IBV serotype 4/91 can increase the susceptibility to the respiratory disorders by tracheal hyperemia and deciliation in field status.

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ULTRASTRUKTURNA RAZISKAVA SAPNIKA V POSKUSNO OKUŽENIH BROJLERJEIH Z IBV SEROTIPA 4/91

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Povzetek: Aviarni virus kužnega bronhitisa (IBV) je globalno razširjen korona virus, ki povzroča ogromno gospodarsko škodo v perutninski proizvodnji. Primarne tarčne celice virusa so v dihalnem traktu. IBV se lahko razširi tudi na druga epiteljska tkiva in povzroči npr. vnetje ledvic ali padec proizvodnje jajc.

S transmissijsko elektronsko mikroskopijo (TEM) smo pregledali znotrajcelične spremembe sapnika brojlerjev po okužbi z virusom kužnega bronhitisa serotipa 4/91. Sedemdeset enodnevnih brojlerskih piščancev je bilo naključno razdeljenih v dve skupini (kontrolna in poskusna). Pri starosti 21 dni so bili vsi piščanci v poskusni skupini okuženi z virusom preko nosu. Štiri piščance iz poskusne skupine in po dva iz kontrolne smo usmrtili v 3 časovnih točkah po okužbi, natančneje po enem dnevu, po dveh in po štirih dneh. Ob obdukciji smo izolirali sapnike in jih pregledali s TEM. V poskusni skupini smo opazili blago obliko hropenja, kašelj in zasoplost. Močneje sta bila izražena hiperemija in edem sluznice sapnika. Okužba z IBV je povzročila hipertrofijo celic goblet, njihovo pokanje in prekomerno tvorbo sluzi. Na ravni ciliarnih celic smo opazili popolno odsotnost migetalk na površini sapnika. Pri okuženih piščancih smo opazili tudi otekel in povečan endoplazemski retikulum.

Ključne besede: transmissijsko elektronski mikroskop; virus kužnega bronhitisa; serotip 4/91; sapnik; brojlerji