

## **SOME STUDIES ON ORNITHOBACTERIUM RHINOTRACHEALE (ORT) IN BROILER CHICKENS**

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### **ABSTRACT**

Affected 100 flocks revealed chronic whitish diarrhea. Consistent gross pathological lesions recorded were congestion and consolidation of lungs, hemorrhages in trachea, congestion and hemorrhages in liver, splenomegaly and necrotic foci on the kidneys, mortality in various flocks ranged from 3.7 – 15% detailed isolation and biochemical studies revealed that *Ornithobacterium Rhinotracheale* was responsible for this wide spread respiratory problem. Five ORT isolate positive PCR implications were pre elicited to be the size of 784 bp – finally it was confirmed that sequence analysis of 16 sr RNA and ORT isolates from Gen bank with identified from 94% to 98%. Antibacterial sensitivity revealed that most of the isolates were sensitive to lincospectin (lincomycin + spectinomycin) and doxycycline. Many of the isolates showed resistance to kanamycin, norfloxacin, enrofloxacin, tetracycline and chloramphenicol. Experimental infection of broiler orally with ORT evoked respiratory signs with mortality reached 20% lesions of moderated sacculitis mild tracheitis and unilateral pneumonia were recorded during the observation period. On other hand of broiler infected with ORT revealed that the infected and treated broiler were lower significance than infected and treated with lincospectin. The microscopical examination revealed pathological changes can be seen in lung, trachea, liver, spleen and brain.

### **INTRODUCTION**

*Ornithobacterium Rhinotracheale* (ORT) is a pathogen best known for causing respiratory tract infections, such as airsacculitis and pneumonia, in birds all over the world. ORT can be a primary or secondary etiological agent depending on strain virulence, adverse environmental factors, the immune state of the flock, and the presence of other infectious agents (**van Empel, et al 1999**). The pathogen may cause systemic diseases such as hepatitis, joint lesions, and cerebrovascular pathology or could lead to economic losses due to growth retardation and the rejection of carcasses for consumption (**Hafez 2008**).

*Ornithobacterium Rhinotracheale* (ORT) has been firstly isolated from broiler chickens (**Du Preez, 1991**). Recently ORT has been isolated from ducks, goose, ostrich, pheasants, pigeons, quails, rook and turkey (**Hafez, 2002**).

In many countries of the world, ORT has been incriminated as a possible additional causative agent in respiratory disease complex in poultry. The organism causes substantial

financial losses due to high rates of condemnation up to 50% in slaughtered affected flocks (**Hafez and sting, 1999**).

*Ornithobacterium Rhinotracheale* was defined as Gram-negative, highly pleomorphic, non-motile, non sporulating bacteria (**Vandamme et al, 1994**).

In Egypt, many studies were carried out in this problem (**Youssef, and Ahmed 1997 and Awaad et al., 2002**)

The diagnosis of ORT infection is based on isolation, identification, serology, and polymerase chain reaction studies. ORT is a difficult bacterium to culture. It grows slowly and needs special growth conditions and so attempts at isolation are often negative and plates are overgrown by other bacteria. Infections with ORT can be treated with antibiotic successfully; however, the bacterium rapidly develops resistance to antibiotics. The outbreak of respiratory disease associated with ORT has been reported in the USA, France, the Netherlands, Belgium, Spain, Germany, Hungary, Israel, Korea, Japan, Taiwan, Turkey, and South Africa (**Hafez, 2008; Van Empel et al., 1994; Erganis et al., 2002; Charlton et al., 1993 and Devreise et al., 1995**).

This work was planned to study clinical signs and pathological findings in flocks of broiler naturally and experimentally infected with ORT, moreover isolation and attempts of treatment conducted.

A wide spread respiratory problem was recorded in broiler chickens of different age groups in various poultry raising areas of Sharkia Governorate. There was high mortality as well as morbidity and poor response to antimicrobials. It created havoc in the poultry industry and many farms were closed. The present investigations were initiated to ascertain the etiology of the syndrome, its various epidemiological factors and susceptibility to various antimicrobials.

## MATERIALS AND METHODS

Four hundred samples (liver, lung, trachea, spleen and bird heads) were collected from 100 of different ages from different farms in Sharkia Governorate, suffering from respiratory signs (sneezing, nasal discharge, dyspnea and sinusitis).

Samples were transported to the laboratory on ice and processed immediately for bacterial isolation studies.

For isolation and identification of different tissues were cultured on Nutrient broth (Difco), Nutrient agar (Difco), Blood agar (Difco), MacConkey's agar (Difco), Typtose Soya agar, PPLO broth (Oxide) and PPLO agar (Oxide). After 24 to 48 hours of incubation at 37°C, various colonies were picked and detailed biochemical investigations were undertaken (**Cruickshank, 1975 and Quinn et al., 1994**). PPLO broth and agar were observed up to a week. Various isolates were subjected to oxidase and  $\beta$ -galactosidase tests for confirmation.

The susceptibility of the isolates to ten different antibacterials was determined by the Kirby-Bauer disc diffusion method described by **Bauer et al., (1966)**. The commercially available antibiotic discs included lincospectrin, neomycin, kanamycin, norfloxacin, chloramphenicol, tetracycline, doxycycline, erythromycin, flumequine, enrofloxacin and oxalinic acid.

PCR assay DNA was extracted both from ORT isolates. Samples were streaked from the frozen stock onto 5% sheep blood agar with 10 µg/mL of gentamicin. Plates were incubated in a moist chamber with 7.5% CO<sub>2</sub> at 37 °C for 24-48 h. The pinpoint, circular, small opaque to grayish and non-hemolytic colonies with 1-3 mm diameter, were selected (**Van dammic et al., 1999**).

Colonies with characteristics of ORT were used by various identification methods such as staining by Gram's method, biochemical identification tests, and finally genetically identified by polymerase chain reaction (PCR) and DNA sequencing (**Van – Emple et al., 1997 and Tasi et al., 2006**).

Suspected ORT isolates were stored in brain heart infusion (BHI) broth with 30% glycerol at -80°C. Polymerase chain reaction a-DNA extraction from isolates were presented by the colonies suspended in 300µL of pure water and heated at 100°C for 10 min and then were centrifuged for 10 min at 11, 600 rpm. The supernatant fluid was used for DNA extraction and frozen at -20°C until further use. The pellet of bacterial cells was resuspended and used for DNA extraction.

#### **PCR assay:**

The PCR assay was performed with the DNA Thermo Cycler (TC-512, England). Primers used in our study were those reported by **van Emple and Hafez, (1999)**, OR16S-F1 (5'- GAG AAT TAA TTTACG GAT TAA G) and OR16S-R1 (5'- TTC GCTTGG TCT CCGAAGAT), which amplify a 784-bp fragment within the 16S ribosomal RNA. These actions were performed in a final volume of 25 µL containing 5 µL of template DNA, 2.5 µL of reaction buffer, 1 µL of deoxynucleoside triphosphates (dNTPs), 1 µL of MgCl<sub>2</sub>, 0.3 µL of TaqDNA polymerase, 13.2 µL of distilled water, and 1 µL of each primer for ORT. Initial denaturation was at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 52 °C for 60 s, and extension at 72°C for 90 s, with a final extension at 72°C for 7 min.

#### **Experimental infection:**

Two hundred broiler chick 15 day old were classified 4 equal groups:

**Group (1):** was remained as control negative (not infected not treatment).

**Group (2):** inoculated orally with 1 ml /10<sup>9</sup>cfu local isolates of ORT at 15 days old.

**Group (3):** was inoculated orally with 1 ml /10<sup>9</sup>cfu local isolates of ORT at 15 day old and treated with lincospectine 1gm /l for 5 successive days.

**Group (4):** treated with 1g/L lincospectine for 5 successive days after inoculation. All chicks were kept under observation for 14 days. The mortality, morbidity rate and re-isolation of ORT were recorded.

The birds had been vaccinated against Newcastle disease and infections bursal disease during 1st and 2<sup>nd</sup> week of age, respectively.

Tissue section from lung, trachea, liver, brain and spleen were prepared, processed, sectioned and stained by H&E according to **Bancroft and Gamble, (2008)**.

### RESULTS AND DISCUSSION

In most of the affected flocks, purulent nasal discharge, swelling of head, weakness and respiratory distress were noted. On postmortem, congestion and consolidation of lungs (unilateral or bilateral), congestion of liver and enlargement of spleen were the consistent lesions observed. Variable mortality was recorded in different affected flocks, the mortality started suddenly and it peaked within 4–5 days and then gradually declined. Mortality ranged from 4 to 15% on various farms.

#### **Bacteriological isolation:**

The result of bacteriological isolation from examined freshly dead broiler was manifested in table (1). ORT was detected in lung 100% trachea 80%, liver 80%, spleen 60% and heads 40%.

In the present study, only 5 ORT bacteria were isolated from the respiratory tract of broiler chickens and one of the main reasons for low isolation could be the overgrowth by rapid-growing bacteria masking the ORT colonies, as reported earlier by many authors (**Van Empel et al., 1999; Hafez, 1998 and Canal et al., 2005**).

Different antibacterial like neomycin, doxycycline, norfloxacin, trimethoprim and enrofloxacin had been tried but with poor response.

Regarding the etiology of the organism, the majority of ORT isolates are resistant to gentamycine (**Hafez, 1998**), and this antibiotic has to retard the growth of other bacteria in media cultures. However, in our study sometimes gentamicin did not prevent the growth of all other bacteria species, which might be due to overuse of antibiotics such as gentamicin in commercial broiler flocks as suggested earlier (**Canal et al., 2005**). The biochemical characteristics observed in our ORT isolates were similar to those in earlier reports authors (**Van Empel et al., 1999 and Canal et al., 2005**).

### **Biochemical characterization:**

The typical colonies, 1–2 mm in diameter, circular, opaque to grey and convex with round edges were seen. These colonies were picked and streaked on MacConkey's agar, Nutrient agar and Tryptose soya agar. From many of the flocks, there was no growth on nutrient and MacConkey's agar. However, on tryptose soya agar, pinpoint, round circular colonies were observed. The isolates grew poorly on TSI slants.

The organisms were Gram-negative were suspicious for *Ornithobacterium rhinotracheale* as the causative organism. Various biochemical tests including indole, catalase, nitrate reduction, urease, methyl red, oxidase,  $\beta$ -galactosidase, lysine decarboxylase and sugar fermentation tests were performed for confirmation. The results of these various tests are summarized in Table (2).

Under optimal conditions, all 5 isolates were positive for oxidase and urease tests; however, they were negative for catalase, triple sugar iron agar, MacConkey agar, indole, and other properties table in accordance with the results of **Canal et al. (2005)**. Four out of 5 isolates, taken from glucose, galactose and lactose cultures, were positive for acid production; however, no acid was produced from these 4 sugars by the fifth ORT isolate. **Vandamme et al. (1994)** observed no acid production from glucose, in agreement with the results of **Canal et al. (2005)**. One out of 4 isolates, taken from maltose, was positive for acid production. Nevertheless, no acid production was observed from sucrose, which was not in accordance with the results of **Canal et al. (2005)**. The variability of biochemical results obtained here was compatible with the literature reports and probably reflected the great genetic variability found indifferent regions (**Van Empel et al., 1999; Hafez, 1998 and Canal et al., 2005**), even in one country.

### **PCR:**

All 5 ORT isolates and oisitive control DNA extracted from ORT vaccine were positive in PCR. All positive PCR amplicons were predicted to be the size of 784 bp. Nonetheless, the ORT PCR assay did not detect the distilled water as a negative control (Figure 1).

### **DNA sequencing:**

The DNA sequencing were obtained and were deposited under Gen Bank Accession nr: E4730706. The sequence of 16 rRNA of ORT isolated in this study and other sequences obtained from Gen Bank were analusied and the sequence of our isolates showed the identify to be ranging from 94% to 98% from Gen Bank.

Five ORT isolates based on the sequence analysis of 16S rRNA, ORT isolates in our study was close to the isolates from Gen Bank with identity ranging from 94% to

98%. DNA sequences obtained here showed the identity to be according to the fall of sequences in cluster ORT isolated from sequences of U87100 and AY162321. Sequences were obtained from strains that were isolated from a turkey flock in Minnesota (Amonsin et al., 1997) broiler flocks in Brazil (Canal et al., 2005), and were obtained from broiler chickens and pigeons.

#### **Results of pathogenicity of local isolates ORT to broiler chicks:**

ORT isolates proved high pathogenic to 15 day old broiler chicks via oral inoculation. Results shown in Table (4) highly morbidity and low mortality rate in group 2. Inoculated broiler of group 3 and treated with lincospectine showed low morbidity and low mortality.

Symptom of P.M. lesion in group 2 showed chronic whitish diarrhea. Consistent gross pathological lesions recorded were congestion and consolidation of lungs, hemorrhages in trachea, congestion and hemorrhages in liver, splenomegaly and necrotic foci on the kidneys, mortality in group 2 (infected and non- treated) was 20%.

Experimental infection with ORT was able to reduce the respiratory disease alone (group 2) which is the test important of the same characteristics as seen in natural outbreak.

Group (2) infection with ORT alone show nasal discharge, coughing and reduce appetite during first week post-infection, while group (3) broilers infected with ORT and treated with lincospectine 1g/L 5 successive days showed decrease mortality and morbidity. The result are in agreement with other ptrviously cited studies (Hafez et al., 1993 and Back et al., 1996).

#### **Microscopic examination:**

Lung section of ORT infected broiler showing slight heamorrhage and emphysema (Fig. 2); pneumonia with massive heterophelic infiltration (Fig. 3); focal pulmonary congestion and hemorrhage (Fig. 4) and replacement of pulmonary tissue with aggregation of lymph cyst and heterophils beside extravasted erythrocut (Fig. 5).

Trachea of broiler infected with ORT showing inflamed mucosa, submucosa, congested capillaries round cells heterophils aggregation in mucosa (Fig. 6).

Spleen of broiler infected with ORT showed hemocidrium and lymphocytic necrosis (Fig. 7).

Liver of broiler infected with ORT showing congestion and mononuclear cell infiltration (Fig. 8).

Brain of broiler infected with ORT showing submeningeal leukocytic aggregation (Fig. 9).

The aforementioned signs were in accordance with **Vanven et al., (2005); Sharhar, (2008); Sanaa, (2002) and Vanveen et al., (2004)** who was observed the ORT is one of the serious respiratory diseases which cause lung congested, catarrhal bronchitis with metaplasia of the lining epithelial and few leucocytes infiltration osseous surrounded by numerous lymphocyte.

**Table (1):** Incidence of ORT in examined broilers

Organ examined broiler	Total		Lung		Trachea		Liver		Spleen		Brain	
	No	%	No	%	No	%	No	%	No	%	No	%
400	18	4.5	5	1.25	4	1	4	1	3	.75	2	.5

**Table (2):** Biochemical characterization of *Ornithobacterium rhinotracheale*

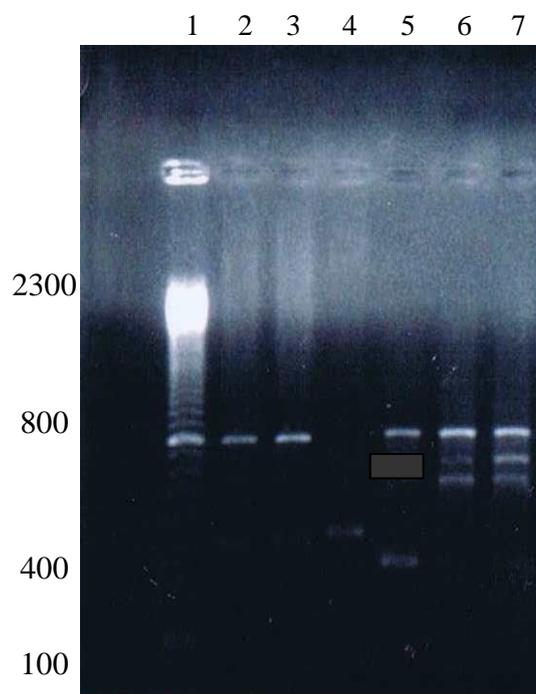
Tests	Results
Indole	-
Voges-Proskauer test	+
Methyl red test	V
Urease	+
Oxidase	+
Nitrate reduction test	-
$\beta$ -galactosidase	+
Catalase test	-
Glucose	-
Fructose	+
Sucrose	+
Maltose	+
Sorbitol	-
Zylose	-
Mannitol	-
Arabompse	-
Inositol	-
Lysine Decarborylase	-

**Table (3):** In vitro susceptibility of 18 isolates of *Ornithobacterium rhinotracheale* to different antimicrobials

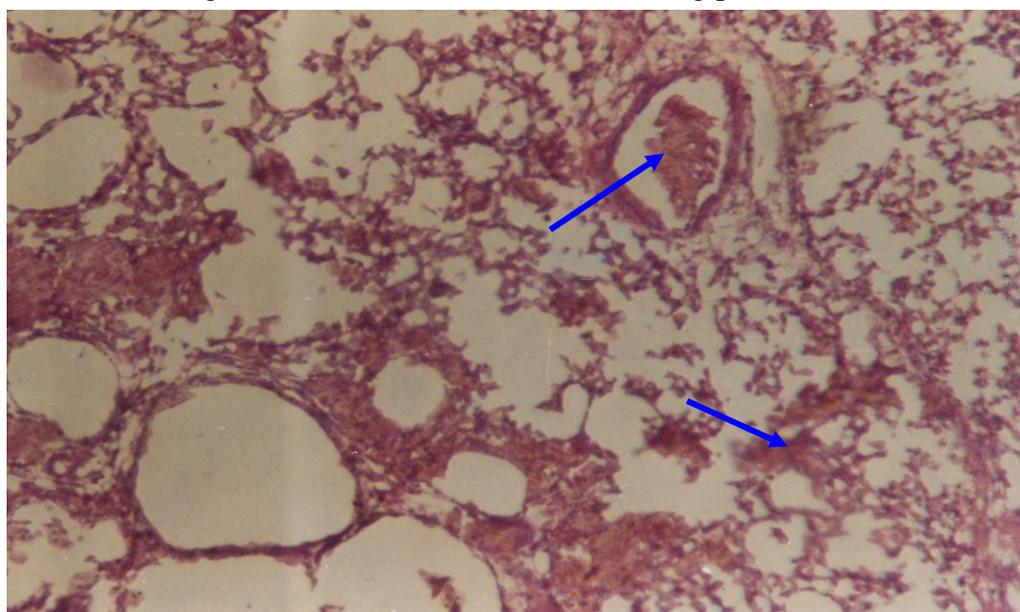
<b>Anitmicrobials</b>	<b>No. susceptible</b>	<b>No. resistant</b>
Doxycycline	17	1
Tetracycline	6	12
Kanamycin	12	6
Enrofloxacin	1	17
Norfloxacin	1	17
Chloramphenicol	3	15
Spectinomycin+Lincomycin	18	-
Flumequine	10	8
Erythromycine	0	18
Oxalinic acid	1	17

**Table (4):** Result of pathogenicity of ORT isolate of broiler chicks

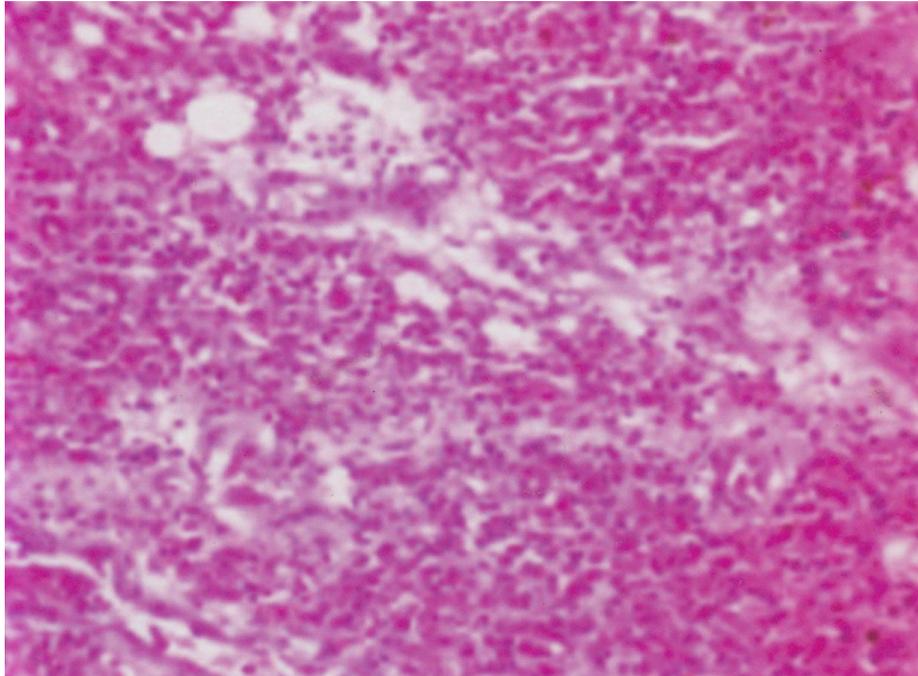
<b>Groups</b>	<b>No. of chicks</b>	<b>Morbidity</b>	<b>Mortality</b>	<b>Reisolation</b>
(1) Non infected non treated	50	-ve	-	0/5
(2) Infected with ORT	50	6%	20%	5/5
(3) Infected with ORT and treated with Lincospectine	50	10%	2%	1/5
(4) Treated with Lincospectine and non-infected	50	-	-	0/5



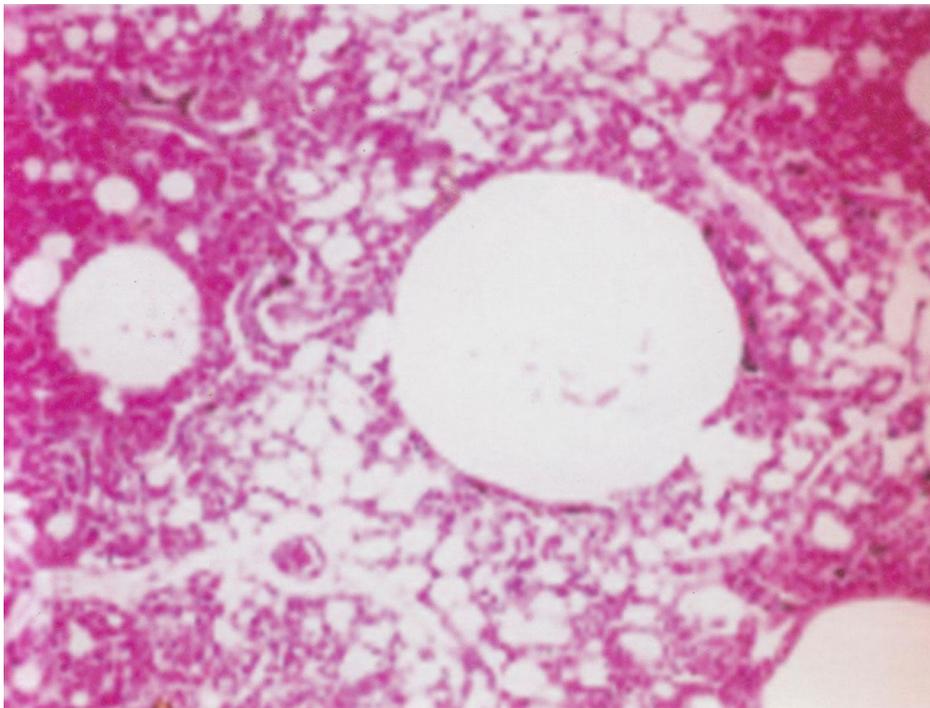
**Figure (1):** Electrophoresis of PCR products on a 1% agarose gel stained with ethidium bromide. 100bp molecular weight marker (lanes 1) positive control, (lane 4) negative control (lane 2, 3, 5, 6 & 7) lung positive isolates



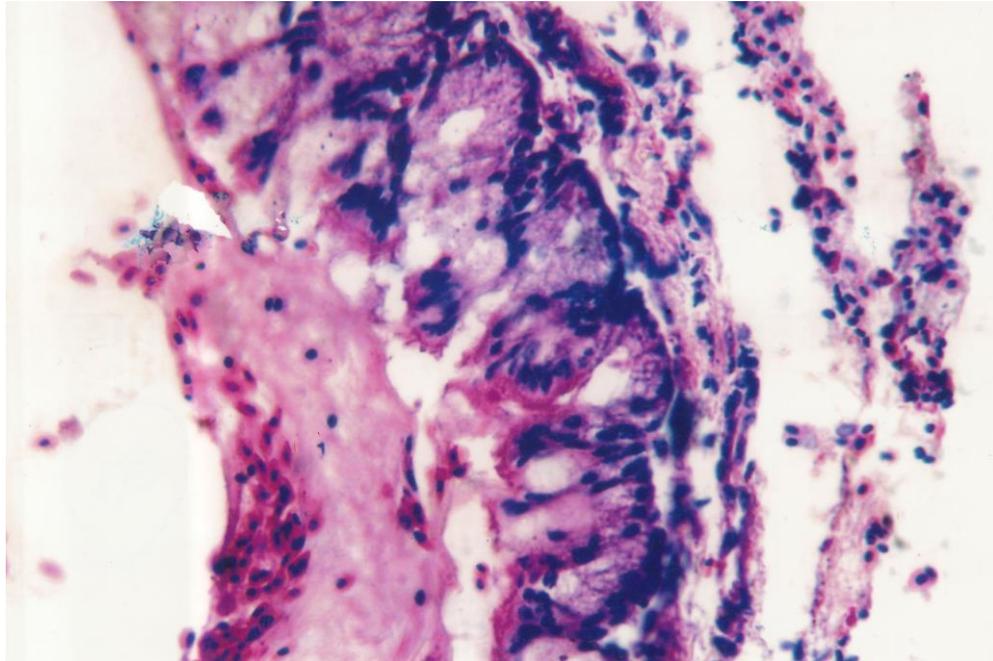
**Fig. (2):** Lung section of ORT infected broiler showing slight heamorrhage and emphysema (H&E X 100)



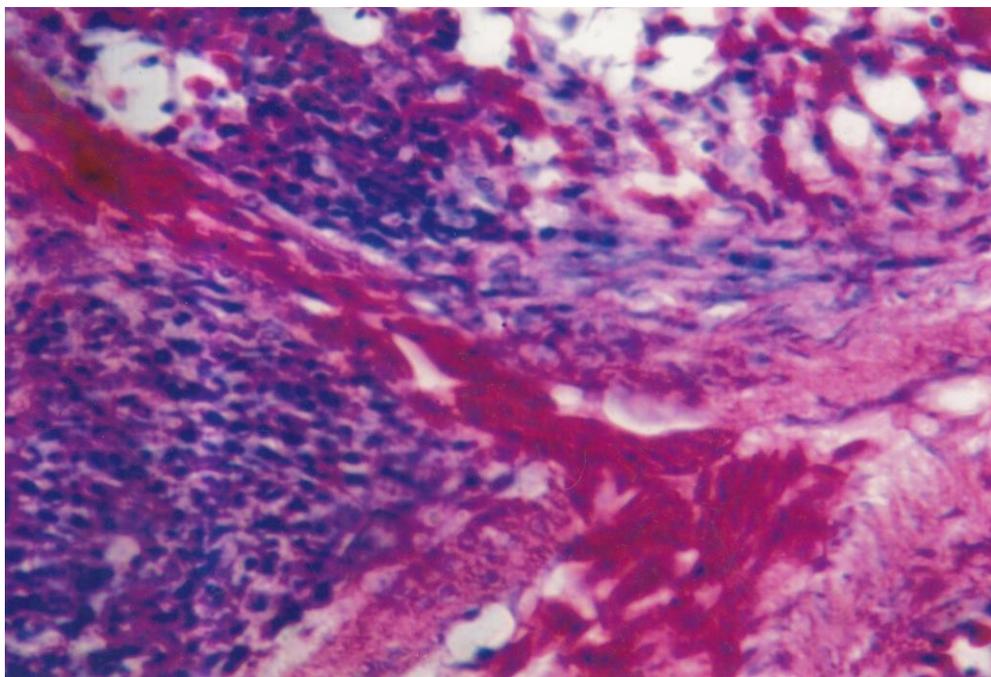
**Fig. (3):** Lung section of ORT infected broiler showing pneumonia with massive heterophelic infiltration (H & E X 400)



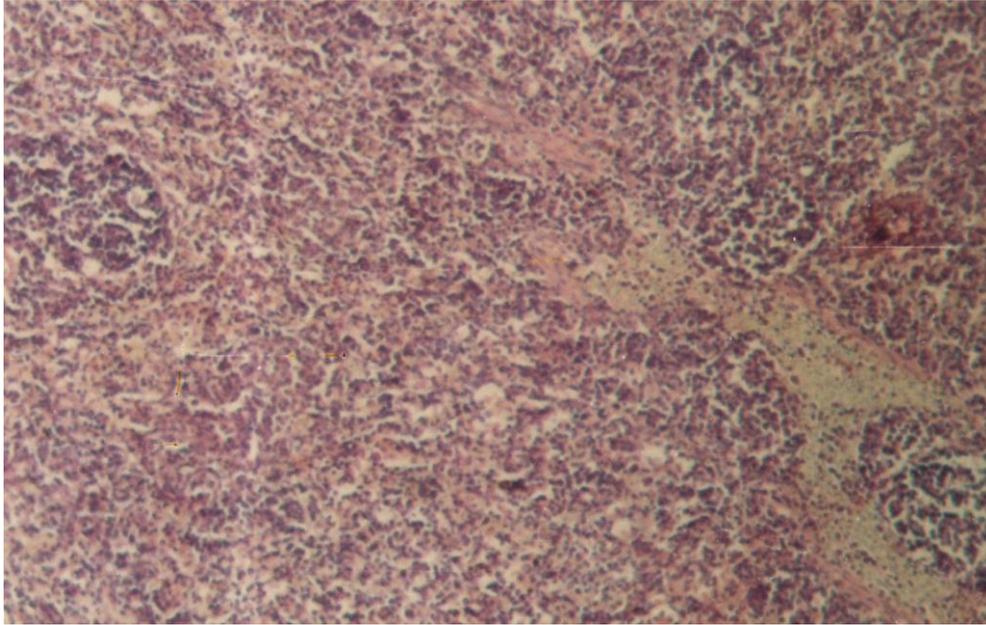
**Fig. (4):** Lung section of ORT infected broiler showing focal pulmonary congestion and hemorrhage (H & E X 100)



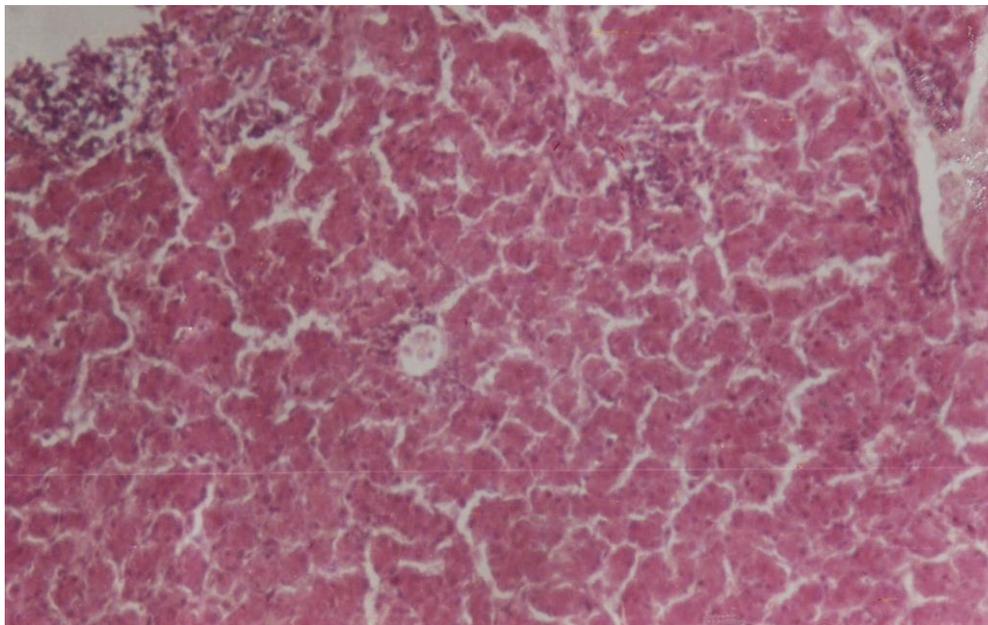
**Fig. (5):** Lung of broiler showing replacement of pulmonary tissue with aggregation of lymph cyst and heterophils beside extravasated erythrocyte (H & E X 150)



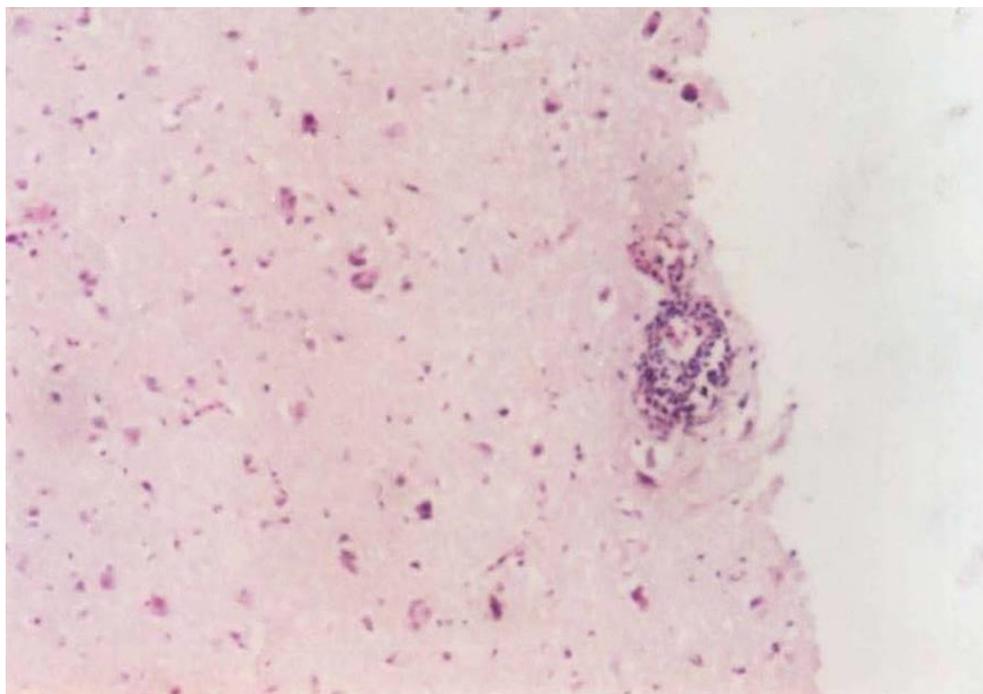
**Fig. (6):** Trachea of broiler infected with ORT showing inflamed mucosa, submucosa, congested capillaries round cells heterophils aggregation in mucosa (H & E X 1200)



**Fig. (7):** Spleen of broiler infected with ORT showed hemocidrium and lymphocytic necrosis (H & E X 100)



**Fig. (8):** Liver of broiler infected with ORT showing congestion and mononuclear cell infiltration (H & E X 100)



**Fig. (9):** Brain of broiler infected with ORT showing submeningeal leukocytic aggregation (H & E X 300)

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