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STUDIES ON *PASTEURELLA MULTOCIDA* AND OTHER BACTERIAL PATHOGENS ASSOCIATED WITH SOME PROBLEMS IN DUCK FARMS IN ASSIUT GOVERNORATE

(With 2 Tables and 8 Figures)

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دراسات عن الباستيريلا ملتوسيدا وبعض البكتريا المرضية المصاحبة لبعض المشاكل فى مزارع البط بمحافظة اسيوط

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عـند استقصـاء مـدى تواجد ميكروب الباستيريلا ملتوسيدا والميكروبات المصاحبة لها فى بعض مزارع البط الحكومية والخاصة بمحافظة اسيوط ، تم اخذ عينات من الطيور المريضة اكلينيكـيا وذلـك من الاعضاء المحتوية على علامات باثولوجية للعدوى البكتيرية مثل الكبد والقلـب والقصـبة الهوائـية والرئتين والطحال. خضعت جميع العينات للفحوص البكتيرية والفيروسـية والمـيكوبلازما. تـم عـزل عدة عوامل بكتيرية وكانت النسبة الكلية للبكتريا المعـزولة (٥١,٧%) ، باسـتيريلا ملتوسـيدا (٢٥%) ، الميكروب القولونى (١٦,٧%) ، كامبيلوباكتر جوجيناى (٣,٣%) ، باستيريلا ملتوسيدا + الميكروب القولونى (٦,٧%). ومن ناحـية اخـرى أعطت المحاولات المبذولة للعزل الفيروسى والميكوبلازما نتائج سلبية. تم إجـراء العـدوى الصناعية فى بط عمر ٣ أسبوع بالبستيريلا ملتوسيدا. هذا وقد تم ملاحظة الأعـراض الاكلينيكـية والصـفة التشريحية التى تشبه إلى حد كبير تلك التى لوحظت فى العـدوى الطبيعية بعد ٧٢ ساعة من العدوى. تم ايضا عزل البستيريلا ملتوسيدا مرة أخرى مـن الطـيور المصابة تجريبيا. عند اجراء اختبار الحساسية للمعزولات معملياً ضد بعض المضـادات الحـيوية، قـد تبين ان جميع المعزولات شديدة الحساسية لكل من الجنتاميسين والانروفلوكساسين والاوكسيتتراسيكلين. بالاصافة الي ذلك تم اخذ بعض العينات من الطيور المصابة تجريبيا للفحص الهستوباثولوجى الذي اسفر عن وجود بعض الافات الباثولوجية في كـل مـن الكـبد والقلـب والمخ والرئتين. ومن تلك الدراسة يتضح مدى تواجد ميكروب البستيريلا ملتوسيدا كمسبب لبعض المشاكل المرضية فى مزارع البط بمحافظة اسيوط ومدى حساسيته لبعض المضادات الحيوية.

SUMMARY

Pasteurella multocida (*P. multocida*) and several bacterial agents associated with some problems in ducks were investigated in different governmental and private farms at Assiut Governorate. Samples were taken from different tissues showing pathological evidence of infection such as liver, heart, trachea, lungs and spleen as well as from blood. All samples were subjected for bacterial, mycoplasma and viral isolation. It was found that the total percentages of isolated bacteria were (51.7%), *P. multocida* (25%), *E. coli* (16.87%), *Campylobacter jejuni* (3.3%), and mixed infection with *P. multocida* and *E. coli* (6.7%). On the other hand, all examined samples were negative for mycoplasma and viruses. Moreover, experimental infection was carried out with isolated *P.multocida* in two weeks old ducks. After 72 h of infection, samples were taken for both bacterial isolation and histopathological examination. *P. multocida* was also isolated from experimentally-infected ducks. When they subjected to the *in vitro* sensitivity tests, it was shown that they were highly sensitive to gentamycin, norfloxacin and oxytetracyclin. Among examined tissues of experimentally-infected ducks, liver, heart, brains and lungs showed multiple pathological changes. Thus, the present study has shown the role of *P. multocida* as a causative agent of some problems in duck farms at Assiut Governorate and their sensitivity for some antibiotics.

Key words: *Pasteurella multocida, duck, pathology, pathogenicity*

INTRODUCTION

Avian cholera is a highly contagious disease caused by the gram-negative bacterium *P. multocida* which is responsible for widespread disease that affects about 100 wild avian species (Botzler, 1991). Epizootics caused by *P. multocida* occur almost in waterfowl populations and cause annual mortality in various waterfowl areas (Samuel *et al.,* 2003). Avian cholera usually appears as a septicemic disease results in high morbidity and mortality rates among affected birds. Sometimes, the disease occurs in the form of epornitics due to rapid spread and extraordinary virulence of the organism (Samuel *et al.,* 2003). The important sources of infection with *P. multocida* in waterfowl are including carrier birds and wetland sediments and water. Also, contamination of the environment particularly water after

epizootics facilitates transmission of *P. multocida* among dense populations of waterfowl either by ingestion and/or the inhalation of aerosols (Botzler, 1991). Additionally, bird-to-bird contact may be considered an important component of transmission (Wobeser, 1992).

Regarding *P. multocida* infection in ducks, it was reported that pasteurellosis is an important septicemic disease of growing ducklings (Gordan and Jordan, 1982). For instances, Bayoumi *et al.* (1988) reported epornitics of pasteurellosis in duck farms caused 5-30% mortalities with higher rates in birds of more than 4-weeks of age. Rhoades and Rimler (1988) reported that *P. multocida* infection is a disease of young ducklings and ducks younger than two weeks old usually die 1-2 days after appearance of clinical signs and older birds may survive a week or more. Moreover, Ibrahim (1991) isolated *P. multocida* from ducks over 10 weeks of age. Nakamine *et al.* (1992) and Takahashi *et al.* (1996) recovered *P. multocida* from 25% of 200 Muscovy ducks in a farm died of an acute disease in Japan. Mariana and Hirst (2000) isolated 10 strains of *P. multocida* and two strains of *P. gallicida* and *P. septica* from ducks in Indonesia.

Clinical signs of *P. multocida* infection in ducks were including ocular and nasal discharges, mild coughing, greenish diarrhea, ataxia, tremors of head and neck followed by coma (Chaudhury and Mahanta, 1985). Subacute disease was characterized by lameness, corneal turbidity, dystasia and depression (Takahashi *et al.*, 1996).

Necropsy findings due to acute *P. multocida* infection in ducks were congested lungs and enlarged liver and spleen (Gordon and Jordan, 1982). In less acute disease, fibrinous pericarditis, periheptatitis, airsaculitis and meningitis were observed (Rhoades and Rimler, 1988). Histopathologically, ducks died acutely of avian cholera showed lesions of hemorrhagic septicemia with widespread vascular damages and focal necrosis in the liver and other organs (Hunter and Wobeser, 1980; Nakamine *et al.*, 1992). Moreover, Songserm *et al.* (2003) reported that *P. multocida* played a role in induction of sinusitis in ducks as they isolated *P. multocida*, group B and serotype 3, from sinusitis in the affected ducks.

This work deals with the investigation of the cause of death of some outbreaks suspected to be due to *P. multocida* and some associated infectious agents in duckling in some governmental and private farms at Assiut Governorate. Investigations were done through isolation and identification of the etiological agents using morphological and biochemical properties and studying the pathogencity of *P. multocida* to

experimentally-infected ducklings. Moreover, the in vitro sensitivities of the isolated *P. multocida* strains to different chemotherapeutic agents were also studied.

MATERIALS and METHODS

A total of 120 freshly dead and sacrificed ducklings (3-10 weeks old) were obtained from different governmental and private farms at Assiut Governorate. Those birds showed depression, diarrhea and morbidity and mortality rates reached to 10 and 25%, respectively. Clinical signs manifested by diseased birds and necropsy findings encountered in dead birds were studied and described.

Experimental infection

Experimental infection was done for testing the pathogenicity of the recovered *P. multocida* from naturally infected birds. Twenty five ducklings (3 weeks old) were obtained from the Farm of Faculty of Agriculture, Assiut University, Assiut for testing. It was proved that they were free from *P. multocida* infection through cultural and serological examination.

Isolation of etiologic agents

a) Bacterial isolation

Bacterial isolation was carried out from different organs of freshly dead ducklings including liver, heart, lungs, trachea and spleen as well as from nasal and eye exudates. Isolates were inoculated into tryptose broth, brain heart infusion broth, nutrient broth and Campylobacter enrichment broth. Tryptose broth and nutrient broth tubes were incubated at 37°C for 18-24 h. Brain heart infusion broth was incubated at 31°C for 3 days. Campylobacter enrichment broth was incubated at 42°C for 48 h in an atmosphere of 5% O_2, 10% CO_2 and 5% N_2 by using an anaerobic jar and Campylobacter gas generating kits. Thereafter, subculturing was done on solid media.

***P. multocida* examination**

For characterization of *P. multocida*, blood agar or dextrose starch agar plates as selective medium for *P. multocida* were used and incubated at 37°C for 24 h. They were examined for non-hemolytic and dew drop like colonies. Suspected colonies were cultured in broth for 6 h at 37°C for storage at -80°C (Lee *et al.*, 1991; Rimler and Glisson, 1997).

***E. coli* examination**

Broth cultures were spread on MacConkey's agar and incubated at 37°C for 24 h. Suspected *E. coli* colonies were selected and purified.

Then, pure colonies were picked and stained by Gram stain (Cruick-Shank *et al,* 1975).

***Campylobacter jejuni* examination**

Campylobacter blood agar base supplemented with skirrow Campylobacter selective supplement were incubated at 42°C for 48 h in microaerobic atmosphere of 5% O_2, 10% CO_2 and 5% N_2 using Gas-pak anaerobic jar and Campylobacter gas generating kits (Skirrow and Benjamin, 1980; Aarestrup *et al.*, 1997).

Mycoplasma examination

Brain heart infusion agar plates were incubated at 37°C in moist candle jar under reduced oxygen tension for detection of mycoplasma according to Sabry and Ahmed (1975) and Ball *et al.* (1994).

Identification of isolates

Identification of different microorganisms was carried out according to Cruick-shank *et al.* (1975) and Collins and Lyne (1991) as followings:

1- Cellular morphology.
2- Colonial morphology including color, shape, size, odor and pigment production.
3- Biochemical reactions such as carbohydrate fermentation test using sugar media (1% peptone water + dextrose, sucrose, lactose, maltose), indole production, catalase test, urease test, citrate utilization, motility, MacConkey's agar growth and hemolysis on blood agar.

b) Virus isolation

Bacteria free suspension was prepared from tissues obtained from liver, spleen and kidneys in antibiotics-containing phosphate buffered saline. Samples were centrifuged at 4000 rpm for 20 min. The supernatant was inoculated into allantoic sac of 10 day old embyronated chicken eggs and incubated for 6 days. Embryos were candled daily and fluids were harvested and tested for haemagglutination activity (Yamaguchi *et al.*, 1981).

Pathogenicity test in mice

Five mice of 3-4 weeks old were obtained from Animal House Unit, Department of Pharmacology, Faculty of Medicine, Assiut University, Assiut. Animals were inoculated subcutaneously with 0.2 ml of 10^6 çfu of *P. multocida* obtained from diseased birds. Mice died 24-48h after inoculations were subjected to postmortem examination. Re-isolation of *P. multocida* was carried out from infected mice (Cruick-shank *et al.*, 1975).

Pathogenicity test in ducklings

Twenty five healthy ducklings of 3 weeks old were used for testing the pathogenicity of recovered *P. multocida* from naturally infected birds. Birds were inoculated intramuscularly with 0.1 ml of overnight broth $7x10^7$ *P. multocida*/duck. Five ducklings were kept in parallel as uninfected controls (Ibrahim, 1991; Pehlivanoglu *et al.*, 1999).

Antibiotic sensitivity test

The sensitivity of isolated *P. multocida* to different antibiotics was done by Disc diffusion method using dextrose starch agar according to Cruick-shank *et al.* (1975). Methods according to Finegold and Martin (1982) using Muller Hinton agar and Bopp *et al.* (1985) using Brucella agar base supplemented with 5% sheep blood were used for *E. coli* and *Campylobacter jejuni*, respectively. Used antibiotic discs were ampicillin (10 µg), streptomycin (10 µg), gentamycin (10 µg), chloramphenical (30 µg), nalidixic acid (30 µg), norfloxacin (5 µg), erythromycin (15 µg), neomycin (30 µg), colistin sulphate (25 µg) and oxytetracycline (30 µg).

Histopathology

Tissue specimens were taken from liver, heart, brain and lungs of experimentally-infected ducklings. Specimens were fixed in 10% neutral buffered formalin and processed routinely for paraffin embedding techniques. Embedded tissues were sectioned at 3 um and stained with hematoxylin/eosin (Bancroft and Stevens, 1982).

RESULTS

Clinical examination

Diseased ducks of different ages showed depression, ruffled feathers, anorexia, mucous discharges from body orifices and diarrhea. Necropsy findings were represented by congestion of the carcasses, necrotic foci and multiple petichiae on liver and spleen, congestion and edema of the lungs and hemorrhages on the intestine particularly the duodenum.

Bacterial isolation

Among 120 samples from different organs of duck flocks, 62 samples (51.7%) were found positive for bacterial isolation. It was also found that 30 sample (25%), 20 samples (16.7%), 4 samples (3.3%) and 8 samples (6.7%) were positive for *P. multocida, E. coli, Campylobacter*

jejuni and mixed infection of *P. multocida* and *E. coli* infections, respectively (Table 1).

Identification of isolated organisms

P. multocida

When *P. multocida* examined morphologically, they appeared as gram negative, short cocco-bacilli, non-motile and bipolar-stained organism. Biochemically, the organism was non-hemolytic on blood agar and did not grow on MacConkey's agar. Moreover, all isolates fermented dextrose, sucrose, mannitol and galactose. But they did not ferment maltose, lactose and arabinose. When the isolates were injected subcutaneously into both mice and ducklings, they produced characteristic lesions for *P. multocida*.

E. coli

Isolated strains of *E. coli* were observed as gram negative bacilli, smooth glossy and rose-pink in color on MacConkey's media.

Campylobacter jejuni

Campylobacter strains appeared as small, gram negative and curved or spiral rods. They were motile in semi-solid Brucella agar, non-hemolytic when grown on Brucella blood agar and grown at 42°C. They were also oxidase and catalase positive, hydrolyse hippurate and produced inhibition zone of 12-14 mm in nalidixic acid sensitivity test.

Mycoplasma detection

There was no evidence for mycoplasma infection in examined samples.

Viral isolation

No viral agents were recovered from the examined specimens.

Pathogenicity of *P. multocida* in ducklings

Clinical symptoms in experimentally-infected ducklings with *P. multocida* appeared 72 h after intramuscular inoculation. They were including emaciation, drowsiness, fever, respiratory signs, lacrimation and dysponea. Birds exhibited morbidity rate about 80% and mortality rate reached 75%. Postmortem lesions were arthritis, congestion of the liver, spleen and lungs and peticheal hemoorhage on the liver and coronary fat with presence of minute necrotic foci on the liver surface. Surviving birds showed incoordination and ataxia. Re-isolation trials were adopted from liver and heart. No lesions were observed in control birds which found also negative for isolation of the organisms.

Antibiotics sensitivity test

The *in vitro* sensitivity test showed that the three isolated microorganisms were highly sensitive to gentamycin, norfloxacin and oxytetracyclin. The obtained results were illustrated in Table (2).

Histopathology

The most common histopathological findings were observed in the liver, heart, brain and lungs. HE-stained hepatic sections revealed congestion and dilatation of blood sinusoids with red blood cells (Fig.1), vacuolar degeneration of the hepatocytes (Fig. 2), focal areas of necrosis (Fig. 3) and mononuclear cellular infiltration in the portal areas (Fig. 4). The lesions in the heart were including congestion of blood vessels, interstitial edema and degeneration of the myocardial fibers (Fig. 5). There were congestion of blood vessels, perivascular edema and degeneration of some neurons in the brain (Fig. 6). Lungs showed congestion and thrombosis of blood vessels (Fig. 7), interstitial edema and necrosis of the epithelial lining of parabronchiols (Fig. 8).

Table 1: Incidence of bacterial isolates from examined ducks.

Examined samples	Positive samples		*P. multocida*		*E. coli*		*C. jejuni*		Mixed infection	
	No.	%	No.	%	No.	%	No.	%	No.	%
120	62	51.7	30	25	20	16.7	4	3.3	8	6.7

Table 2: Antibiotic sensitivity test for *P. multocida*, *E. coli* and *Campylobacter jejuni* isolates from ducks.

Antibacterial agents	Isolates		
	P. multocida	*E. coli*	*C. jejuni*
Ampicillin	R	R	R
Streptomycin	++	R	R
Gentamycin	+++	+++	+++
Chloamphenicol	R	R	+
Nalidixic acid	++	R	++
Norfloxacin	++	+++	+++
Erythromycin	R	R	+++
Neomycin	R	++	++
Colistin sulphate	+	+++	R
Oxytetracycline	+++	+++	+++

+++ High sensitivity. + Low sensitivity.
++ Intermediate sensitivity R Resistant.

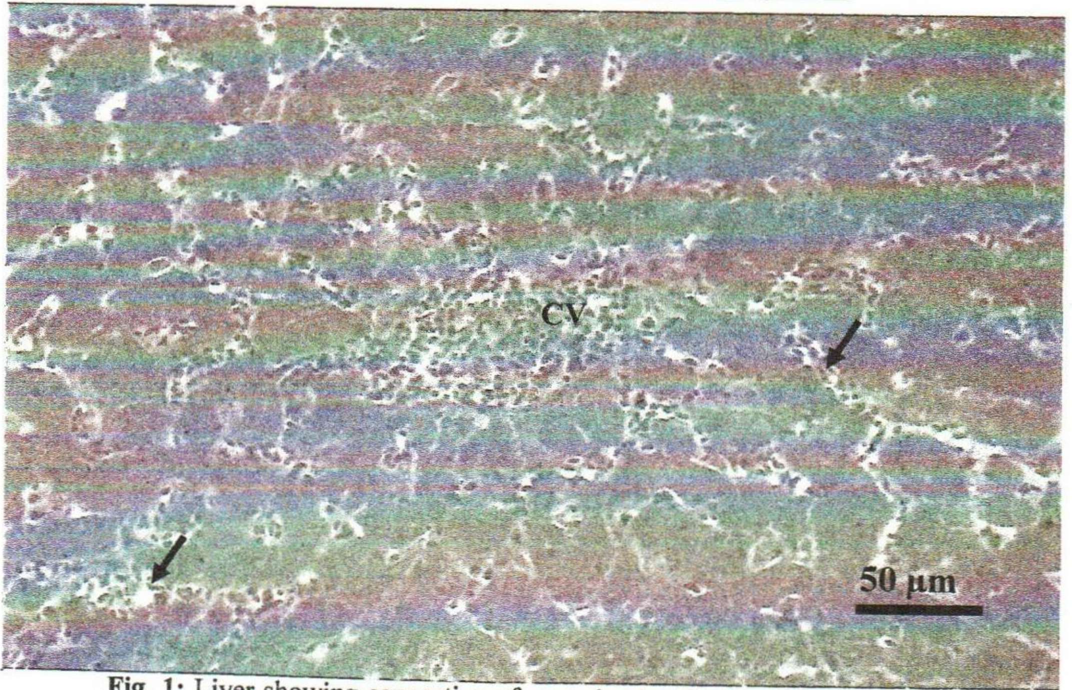

Fig. 1: Liver showing congestion of central vein (CV) and dilatation of blood sinusoids and their engorgement with red blood cells (arrows). (H&E)

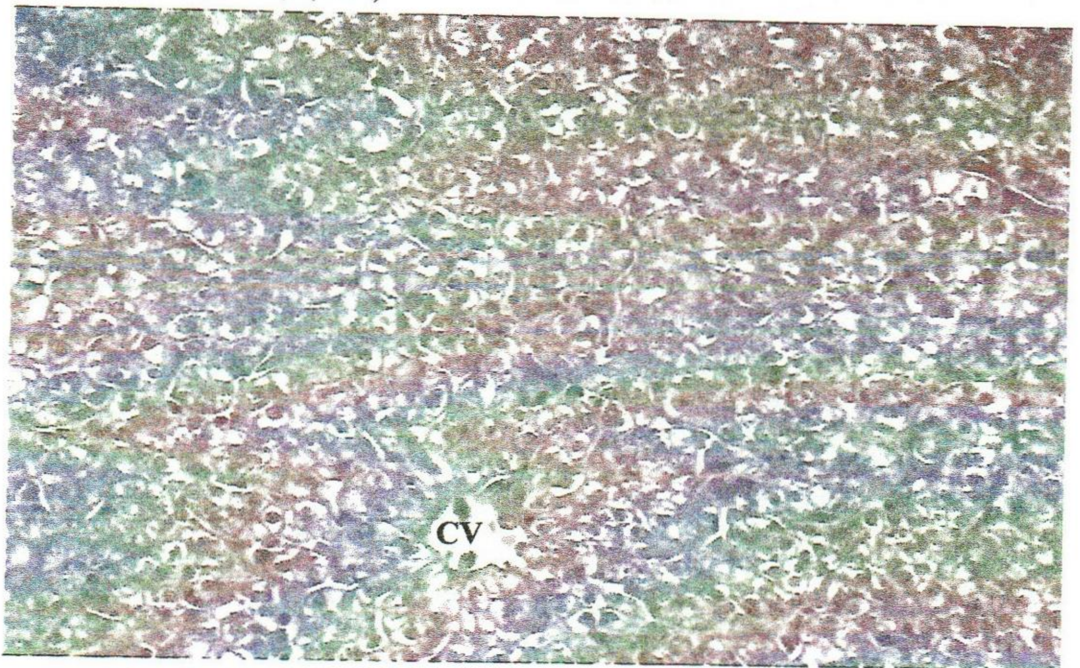

Fig. 2: Liver showing vacuolar degeneration of the hepatocytes. Central vein (CV). (H&E)

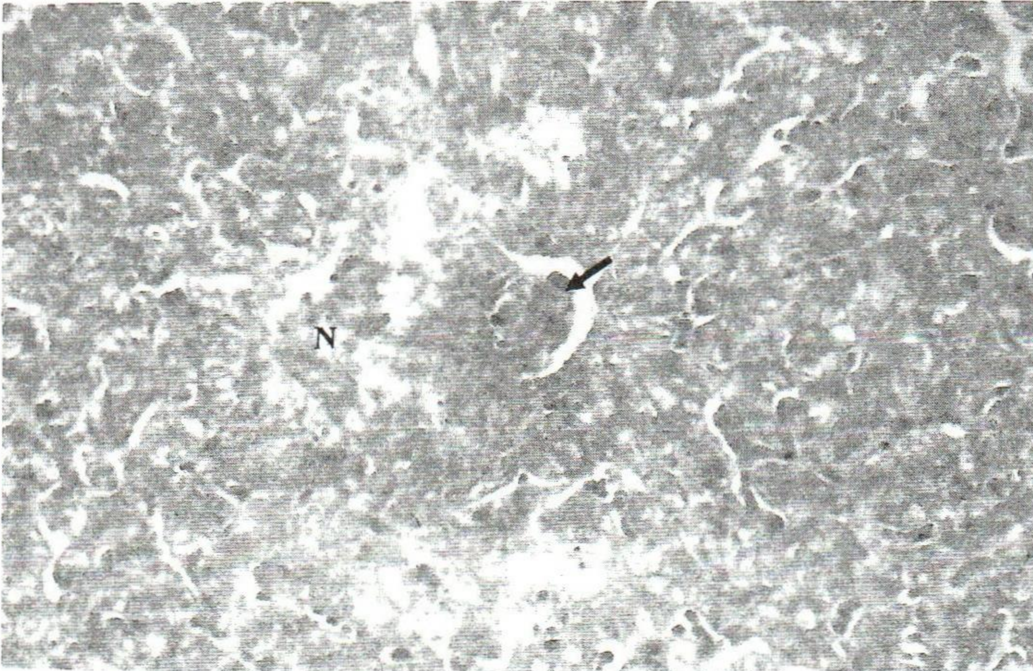

Fig. 3: Liver showing thrombosis of a blood vessel (arrow) and area of necrosis (N). (H&E)

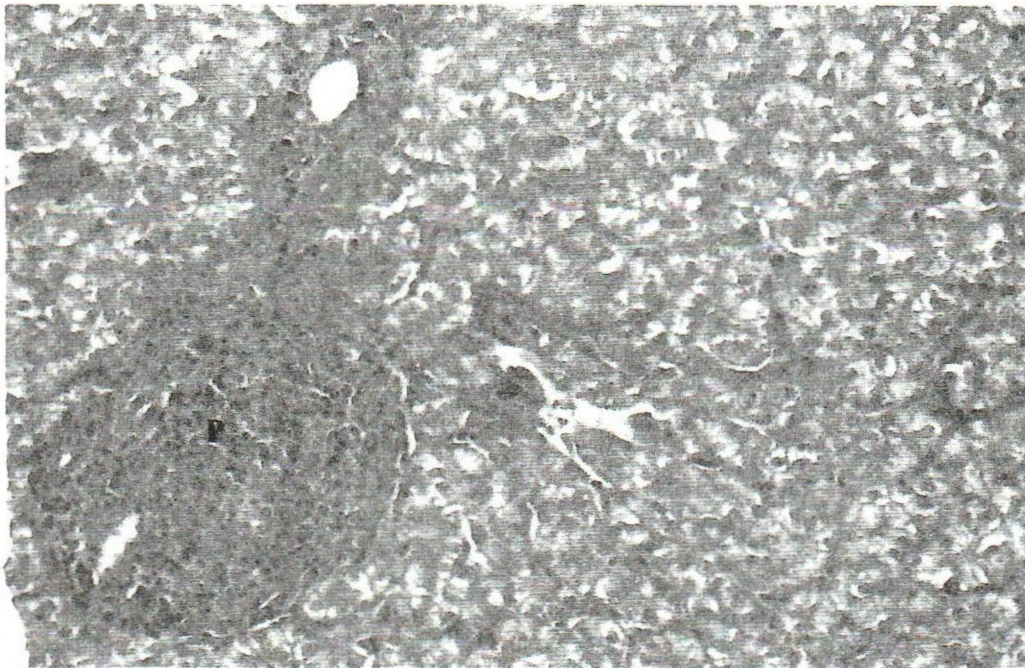

Fig. 4: Liver showing mononuclear cellular infiltration in the portal area (P) and vacuolar degeneration of the hepatocytes. (H&E)

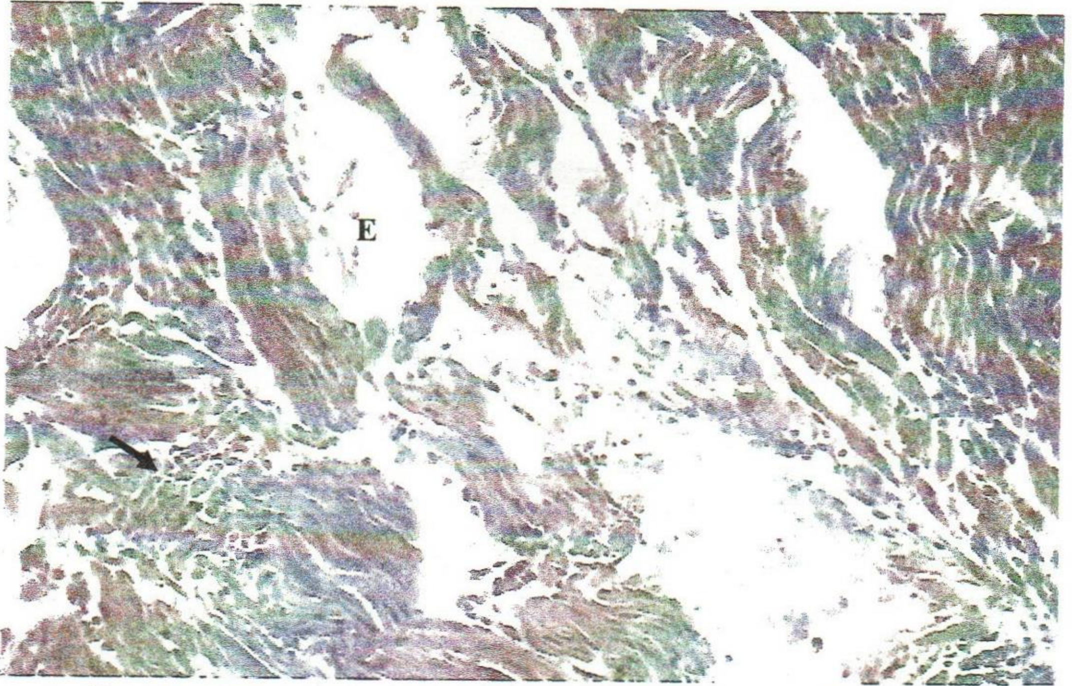

Fig. 5: Heart showing hemorrhage, interstitial edema (E) and degeneration and necrosis of myocardial fibers (arrow). (H&E)

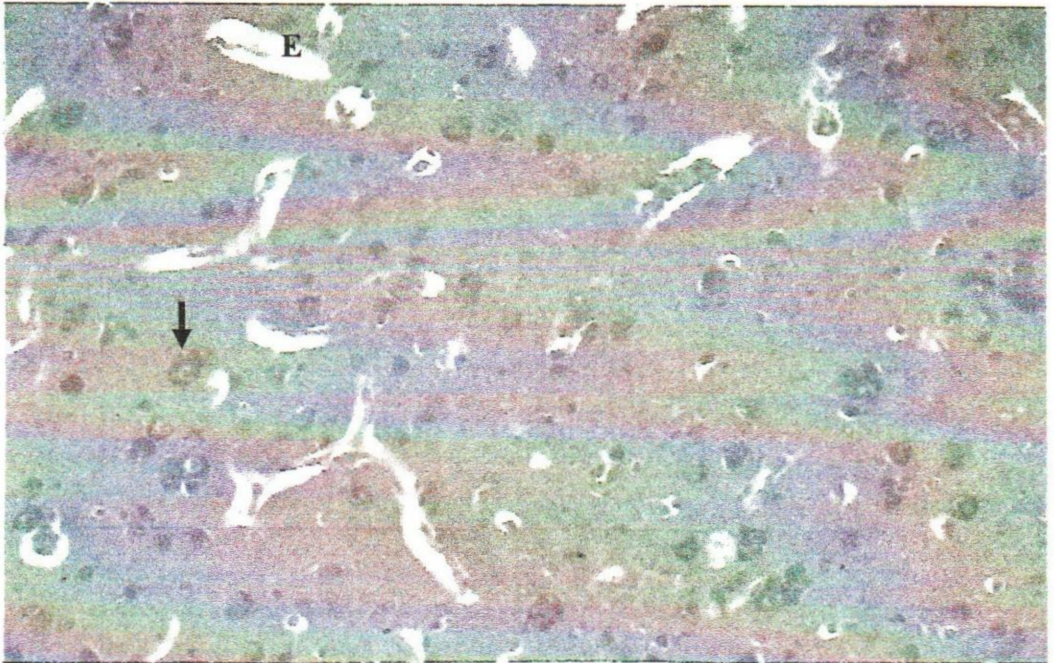

Fig. 6: Brain showing congestion of blood vessels, perivascular edema (E) and neuronal degeneration (arrow). (H&E)

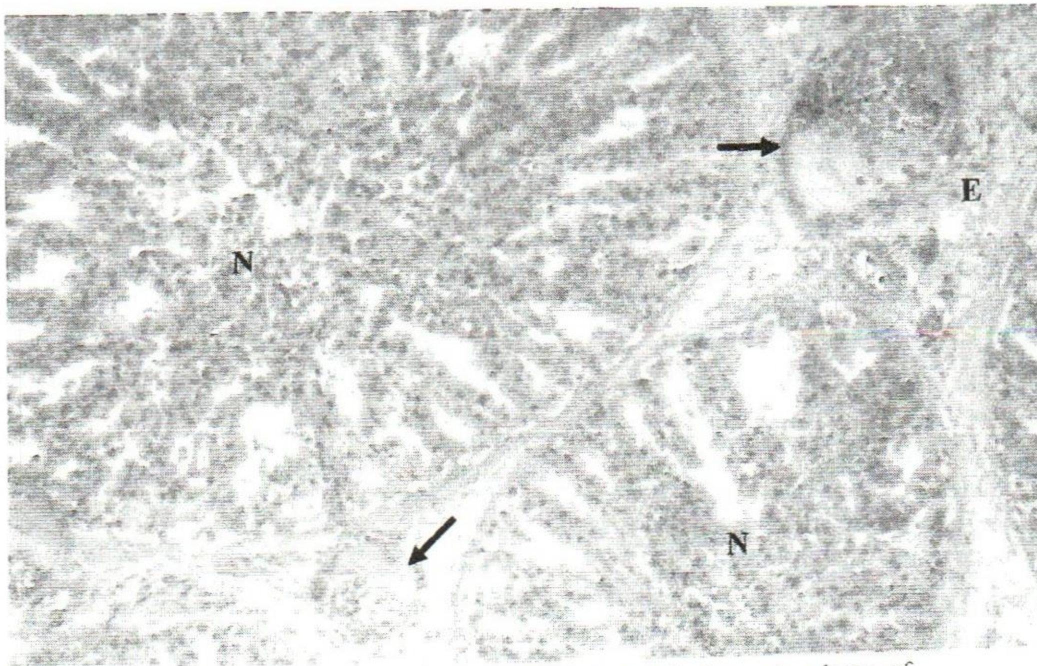

Fig. 7: Lung showing thrombosis of blood vessels (arrow), edema of interstitial tissue (E) and necrosis of the epithelial lining of parabronchiols (N). (H&E)

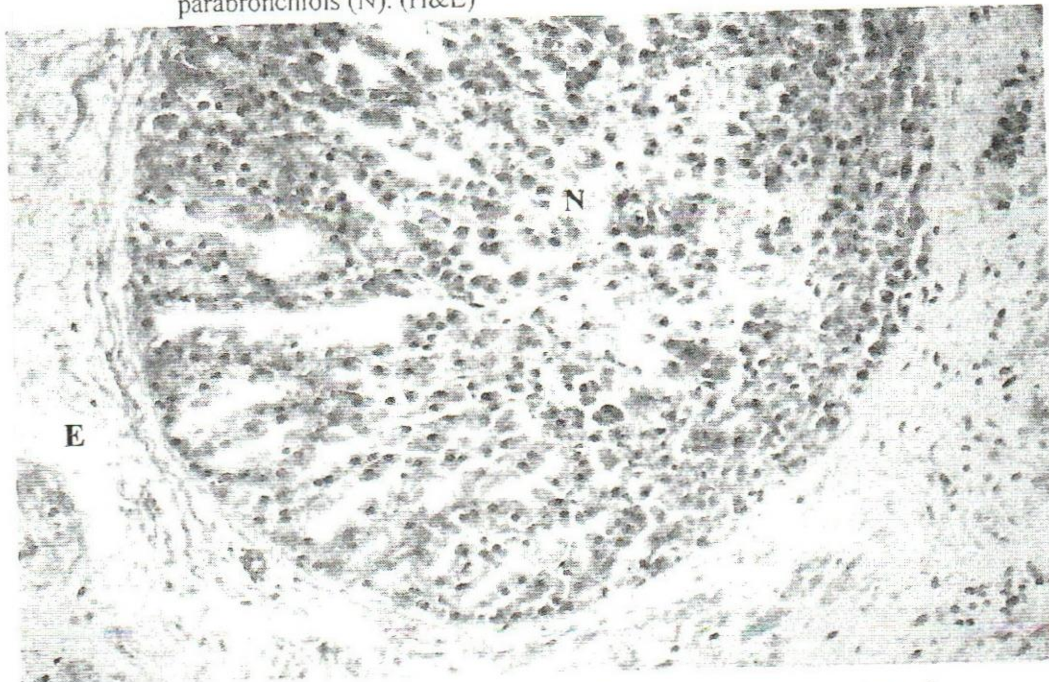

Fig. 8: Lung showing interstitial edema (E) necrosis and sloughing of the epithelial cells in the lumen of a parabronchiol (N). (H&E)

DISCUSSION

Duck cholera is considered the most important bacterial disease threatens duck breeding in Egypt (Gorgy, 1992) and there were some recent reports discussed the role of *P. multocida* as a causative agent of the disease in ducks (Samuel *et al.,* 2003; Songserum *et al.,* 2003).

In the present study, clinical signs and gross necropsy findings manifested by diseased and dead birds were similar to those described by Rhoades and Rimler (1988) and Ibrahim (1991), respectively. They were including marked depression, anorexia, diarrhea and mucous discharges from the body orifices. The postmortem findings were congestion of the carcasses, petechial hemorrhages through out the viscera and necrotic foci on liver surface, edema and pneumonia in the lungs.

When different samples from liver, heart, trachea, lungs, and spleen subjected for bacterial isolation, gram negative, bipolar, non-motile, non spore forming and non-hemolytic bacteria which also did not grow on MacConkey's agar was isolated. In parallel, Lee *et al.* (1991) and Rimler and Glisson (1997) reported agreement results. Moreover, all isolates had sugar fermentation pattern and biochemical properties similar to the general characteristics of *P. multocida* reported by Rhoades and Rimler (1991) and Ibrahim (1996).

P. multocida was isolated from ducks at rate of 25%. Similarly, El-Ghawas (1975), Nakamine *et al.* (1992) and Takahashi *et al.* (1996) isolated *P. multocida* form outbreaks in Muscovy ducks at the same rate. Lotfy *et al.* (1970) isolated *P. multocida* from ducks at a higher rate reaching 42.5%. *E.coli* and *Campylobacter jejuni* were isolated in association with *P. multocida.* They were isolated at rate of 20% and 3.3%, respectively. These results were in consistent with the finding of Harbourne (1962), Songserm *et al.* (2003) and Dobbin *et al.* (2005) who reported that *P. multocida* infection was nearly always accompanied by *E. coli* and *Campylobacter spp.* infection in free-living fowls. Fallacara *et al.* (2001) isolated E. coli at the rate of 67% which much higher than our results.

To reveal the pathogenicity of *P. multocida*, isolates from naturally-infected cases were inoculated intramuscularly in 3 weeks old ducklings. The clinical signs, mortality rate and necropsy findings appeared 72 h post-inoculation and were characteristic for *P. multocida* infection according to Ibrahim (1991) who recorded mortality rate of 80%.

Histologically, dead birds showed marked vascular findings in the liver, heart, brain and lungs. They were including congestion of blood vessels, hemorrhages, thrombosis and edema. In consistent, Hunter and Wobeser (1980) reported prominent vascular lesions in experimentally-infected mallard ducks with *P. multocida*. Hunter and Wobeser (1980) attributed these vascular changes to endotoxin-initiated disseminated intravascular coagulation. Other findings were vacuolar degeneration and necrosis of hepatocytes and mononuclear cellular infiltration in the portal areas in the liver, degeneration of the myocardial fibers in the heart, degeneration of some neurons in the brain and necrosis and sloughing of the epithelial lining of parabronchiols. These lesions were milder than those reported by Hunter and Wobeser (1980) and this may be due to species and age differences. Also, the route of inoculation was also participating (Pehlivanoglu *et al.,* 1999).

P. multocida strains were highly sensitive to gentamycin and oxytetracyclin and highly resistant to ampicillin, erythromycin, chloramphenicol and neomycin. These finding are in agreement with the observation of Shaw *et al.* (1990), Nakamine *et al.* (1992) and Takahashi *et al.* (1996). As illustrated in Table (2), *E. coli* isolates were susceptible to gentamycin, norflaoxacin, colistin sulphate and oxytetracyclin and were resistant to ampicillin, streptomycin, chloramphenical, nalidicic acid and Erythromycin. Erganis *et al.* (1989), Moharana *et al.* (1993) and Jakeen *et al.* (1999) reported agreement results. *Campylobacter jejuni* strains were highly sensitive to gentamycin, norfloxacin, ertythromycin and oxytetracyclin and resistant to ampicillin, streptomycin and colistin sulphate. These finding are in agreement with the results of Nakai *et al.* (1994) and Das *et al.* (1995).

In conclusion, bacterial isolation from freshly dead and sacrificed ducks in some governmental and private farms at Assiut Governorate revealed that the total percentages of isolated bacteria were (51.7%), *P. multocida (*25%), *E. coli* (16.87%), *Campylobacter jejuni* (3.3%) and the mixed infection with *P. multocida* and *E. coli* (6.7%). On the other hand, all examined samples were negative for mycoplasma and viruses. Experimental infection with isolated *P. multocida* in three weeks old ducklings produced the same characteristics of *P. multocida* infection 72 h post-infection. The *in vitro* sensitivity tests have shown that isolated *P. multocida* was highly sensitive to gentamycin, norfloxacin and oxytetracyclin. The present study has shown the role of *P. multocida* as a causative agent of some problems in duck farms in Assiut Governorate and their sensitivity for some antibiotics.

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