Incidence and Significance of *Aeromonas hydrophila* In Non Hatched Chicken Eggs

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ABSTRACT

This work was designed to investigate incidence of *Aeromonas hydrophila* in non hatched chicken eggs and study its pathogenic effect on embryonated chicken eggs. Four hundred non hatched chicken eggs were collected, (200) samples from mechanical hatchery and (200) from balady hatchery in Assiut governorate . Thirty (7.5%) *A. hydrophila* isolates were isolated, 14 (7%) and 16(8 %) from the mechanical hatchery and balady hatchery respectively. Inoculation of *A. hydrophila* strain via yolk sac route in a dose of 1.5x 10^7/ ml induced 100% embryonic mortalities after 3 days. Re-isolation of *A. hydrophila* from liver and yolk with a percentage 90% and 95% respectively . Groups of 5 days –old and 17 days –old ECE dipped in media contain *A. hydrophila* in a dose 6.1X 10^9 /ml induced 25%and 30% embryonic mortalities after 7 and 4 days respectively , the re-isolation of *A. hydrophila* was 60%,33.3% from liver and 80% ,83.3% from yolk respectively. Hatchability was 75% and 70% in dipped groups. The rate of *A. hydrophila* re-isolation from organs of sacrificed hatched chickes (intestine, liver, heart and yolk sac) were 93.1,68.9,17.2 and 51.7% respectively. Histopathological changes in liver, heart and intestine were hepatic degeneration ,myocardial congestion and intestinal heamorrhages.

Key words: A. hydrophila, embryonated chicken eggs, pathogenicity, hatcheries.

INTRODUCTION

Hatchery is one of the greatest areas of disease risk in the whole cycle of poultry operation .A large proportion of embryos die at different stages of incubation *(1)*. Bacterial and fungal contamination of the hatching eggs is important as they not only prevent more than 10 percent of fertile eggs from hatching but may also give rise to cross contamination and subsequent mortality or poor performance of chicks in the subsequent stages of production. *(2)*. Bacterial pathogens which find their way into eggs by different means are one of the main factors which lower hatchability due to spoilage of incubating eggs and death of embryos *(3)*.In recent years ,concerted efforts were made in several countries to identify more bacterial pathogens responsible for embryonic mortalities in hatcheries, *Aeromonas hydrophila* is one of these bacterial pathogens.*(4)*. Bacteria of the genus *Aeromonas* and family Aeromonadaceae are ubiquitous Gram negative bacilli ,asporogenous and non-lactose fermenting organisms .Up to fourteen *Aeromonas* species have been discovered and *Aeromonas hydrophila* is the most commonly occurring member of the genus *Aeromonas* (5) *Aeromonas hydrophila*, either alone or in combination with other organisms, can cause localized and systemic infection in avian species including poultry *(6,7)*. *Aeromonas* was recorded in chickens and turkeys suffering from enteritis and watery feces *(8)* as well as in ducks suffering from salpingitis, Septicemia and / or airsaculitis *(9)* *Aeromonas hydrophila* and other motile aeromonads (*A. sobria and A.caviae*) are

receiving increasing attention as a human pathogens especially as causative agents of gastroenteritis, wound infections and septicaemia ***(10,11)***. The broad spectrum of infection with *A. hydrophila* and its pathogenicity is paralleled by a range of virulence factors including adhesions, cytotoxins, haemolysin, and various enzymes. However, most strains of *A. hydrophila* produce enterotoxins, regardless of the source ***(12)***. From pathological point of view, there were little available literatures cover the pathogenicity of this microorganism in chickens and other poultry, ***(7, 13, 14)***.

No available literature dealing with the transmission of *Aeromonas* through the ovary (ovo transmission of *Aeromonas*) while , there were scanty literature on isolation and significance of *A. hydrophila* on embryonated chicken eggs. *Aeromones* has been recovered from dead – in – shell embryos and weak chicks ***(4,15- 17)***,and its pathogenicity studied in embryonated chiken eggs ***(18-20)***.Our goal for this study was to identify the incidence of *A. hydrophila* in hatchery eggs and study its pathogenic effect on the embryonated chicken eggs.

MATERIALS AND METHODS

Collection of samples

A total number of 400 samples where taken from one mechanical and one balady hatcheries in Assiut governorate (200 samples of each) .Liver, heart and yolk sac samples of dead –in-shell embryo as well as swabs from egg shell surface, cracked and piped eggs were not collected to avoid extraneous contamination. Beside swabs were taken from the incubators and hatcheries(egg room, egg dishes and other equipments).

Isolation and identification

The collected samples and swabs were inoculated under aseptic condition , into Trypticase soya broth(Biolife) and brain heart infusion broth (Biolife) containing 10mg/ml ampicillin, tubes incubated aerobically at 28-30 °C for 24 hr. ,a loopfull from the resultant growth was streaked onto Aeromonas agar medium(idg) and other was streaked onto MacConkey -mannitol agar plus ampicillin (Biolife), the plates were incubated aerobically at 28-30°C for 24hr. Suspected colonies(translucent pink colonies with darker centers on Aeromonas agar, and pale yellow non lactose fermenter on MacConkey agar were selected and purified on the nutrient agar plates for further identification.

Films from pure colonies were stained by Gram's stain and examined microscopically. The identification of the isolates was carried out by determining their morphological, cultural and biochemical characteristics according to criteria of ***(21, 22)***.

Pathogenicity of *A. hydrophila* :To study the significance of *A. hydrophila* isolated from the two hatcheries on the viability of the chicken embryo and its effect on hatched chicks.

Experimental Design

Aeromonas hydrophila Strains recoverd from embryonated chiken eggs during this work were used for the Pathogenicity experiment .

Embryonated chiken eggs

A total of Ninty embryonated chicken eggs (ECE) obtained from Commercial poultry hatchery. Eggs used in this experiment were 55(ECE) of 5 days –old and 35(ECE) of 17 day-old. Five of each age were subjected to bacteriological examination, which proved to be free from bacterial contamination. The remaining eighty (ECE) were divided into 3groups containing ECE of 5 days –old ,and 2 groups containing ECE of 17 days –old .

Group No.1, containing , 20 ECE of 5 days – old were inoculated via yolk sac with 0.2ml suspension of 1.5 X 10^7*A. hydrophila* organism /ml in brain heart infusion broth .

Group No.2, containing 20 ECE of 5 days – old were dipped in 18 hrs. chilled broth

bacterial culture containing 6.1X 10^9 CFU/ml for five minutes.

Group No.3, containing 10 ECE of 5 days –old were kept without infection and put in a separated incubator as a negative control group.

Group No.4, containing 20 ECE of 17 day-old were dipped in 18 hrs. chilled broth bacterial culture containing 6.1X 10^9 CFU/ml of *A. hydrophila* for five minutes.

Group No.5, containing 10 ECE of 17 day -old were kept without infection and put in a separated incubator as a negative control group.

ECE of all groups were incubated at 37 °C and humidity 50 % with daily candling for recording viability or mortality till hatch. Livers and yolks of the dead embryos from each group were subjected to reisolation of *A.hydrophila*. The hatched chicks from each group were killed. Livers,hearts and intestines were collected for reisolation of *A. hydrophila* and histopathological examination.

Histopathological examination

This examination was carried out according to the method of ***(23)***. Representative samples from livers, hearts and intestines of dead embryos as well as sacrificed hatched chicks, were immersed and fixed in neutral buffered formalin . These samples were dehydrated, cleared, embedded and cut to 5um then they were transferred to glass slides and stained with hematoxylin and eosin stains. Then they were examined by ordinary light microscope.

RESULTS

Based on the identification methods used in our study, a total of 30 (7.5%) *A. hydrophila* strains were isolated from 400 embryonated chiken eggs collected from two hatcheries in two consecutive cycles of each hatchery. Out of 30 isolates 14 (7%) were isolated from embryonated chiken eggs of the mechanical hatchery, and 16(8 %) isolates were from the balady hatchery as shown in table (1). Bacteriological identification of these isolates revealed that colonies of *A. hydrophila* on Aeromonas agar were translucent pink colonies with darker centers, while on MacConkey - mannitol agar plus ampicillin,were pale yellow , non lactose fermenter colonies. Microscopic characters were gram negative ,straight, rod shape to coccoid with rounded ends, singly or in pairs and non-sporulated. Biochemicaly the isolated *A. hydrophila* were oxidase , catalase, and indole positive, and they produced acid and gas in triple suger iron agar. Obtained results about pathogenicity test are shown in tables (2-3-4-5). Dead embryos (from 5 and 17-day-old ECE) which infected by dipping in 6.1X 10^9 CFU /ml of *A. hydrophila* broth culture exhibited severe congestion of the liver, myocardium and yolk sac. There was one - hatched chick died after one day of hatching, it was suffering from dullness, diarrhea. At necropsy it showed moderate congestion of liver, heart and yolk sac , severe enteritis with precipitation of urates in the ureters.The (ECE) treated with *A. hydrophila* either by inoculation via yolk sac route or dipping , were greatly affected. In group 1 which inoculated via yolk sac route the embryo mortality was 100% (3 days post inoculation), while in group 2 which dipped in *A.hydrophila* broth culture, the mortality rate was 25% (7 days post dipping). The embryonic mortalities were 30% (4days post dipping) in *A. hydrophila* infected group 4 which was 17 days old (ECE), while it was 0% in groups number 3 and 5 which were non infected. The hatchability of the infected ECE was 0% in group 1while it was 75%, 70% in groups number 2,4 respectively, while it was 100% in groups number 3and 5 which were non infected control groups. *A. hydrophila* was re-isolated from internal organs of hatched chick. Re-isolation of *A. hydrophila* from liver and yolk of dead embryos of group 1 inoculated by yolk sac route were 90% and 95% while it was 60% and 80% in group 2 which dipped in *A.hydrophila* broth culture, group 4 which had dipped in media containing *A. hydrophila*

showed, 33.3% and 83.3% for re-isolation of *A.hydrophila*, from liver and yolk of dead embryos, as shown in table(4).The rate of re-isolation of *A.hydrophila* from internal organs of sacrificed hatched chicks were 93.1, 68.9, 51.7 and17.2 % from intestine, liver, yolk sac and heart, respectively as shown in Table (5).

Histopathological examination results of dead embryos and sacrificed hatched chicks from *A. hydrophila* infected eggs revealed that changes in the liver in form of hydropic degeneration , dilatation in the hepatic blood vessels, in addition to congestion of the coronary veins, Figure [1(a,b)].In some cases there was dispersion of hepatocytes and hepatocellur necrosis with hemorrhages, Figure (2). The cardiac muscles showed vacuolar degeneration in myocardial muscles and loss of its striation, Fig. [3(a, b)] .Also there was congestion in the myocardium Fig .[3(c,d)].While the intestinal lesions were , sever hyperplasia in the intestinal epithelium with sloughing of the epithelial cells in the lumen of the villi,Figure (4), some cases showed severe enteritis represented by hemorrhages , necrosis and presence of inflammatory cells, Fig. [5(a, b)].

Table 1. Incidence of *A.hydrophila* isolation from embryonated chicken egg

Source of samples	No. of samples	No. of isolates	%
Mechanical hatchery	200	14	7
Balady hatchery	200	16	8
Total	400	30	7.5%

Table 2. Results of *A. hydrophila* infection in 5- day old chicken embryo by inoculation and dipping

Group No.	No. of infected ECE	Embryonic mortalities per day post infection															Rate of embryonic mortality %	Chick hatching	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		No.	%
1	20 (inoculated via yolk sac route)	10	5	5													100%	—	0%
2	20 (dipped in broth culture)				1	1	1	2									25%	15	75%
3	10 (control)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%	10	100%

Table 3. Results of *A. hydrophila* infection in 17- day old chicken embryo by dipping

Group No.	No. of infected ECE	Embryonic mortalities per day post infection				Rate of embryonic mortality %	Chick hatching	
		1	2	3	4		No.	%
4	20	1	1	2	2	30%	14	70%
5	10 (control)	-	-	-	-	0%	10	100%

Table 4. Re-isolation of *A. hydrophila* from liver and yolk of dead embryos

Group No.	Total No. of ECE	No. of dead embryos	Re-isolation from liver and yolk			
			Liver	%	Yolk	%
1	20	20	18	90%	19	95%
2	20	5	3	60%	4	80%
4	20	6	2	33.3%	5	83.3%
3	10	-	-	-	-	-
5	10	-	-	-	-	-

Table 5. Re-isolation of *A. hydrophila* from sacrificed survived chicks

Group No.	No. of hatched chicks	Re-isolation from internal organs							
		Intestine	%	Liver	%	Heart	%	Yolk	%
2	15								
4	14	27/29	93.1	20/29	68.9	5/29	17.2	15/29	51.7
Total	29								

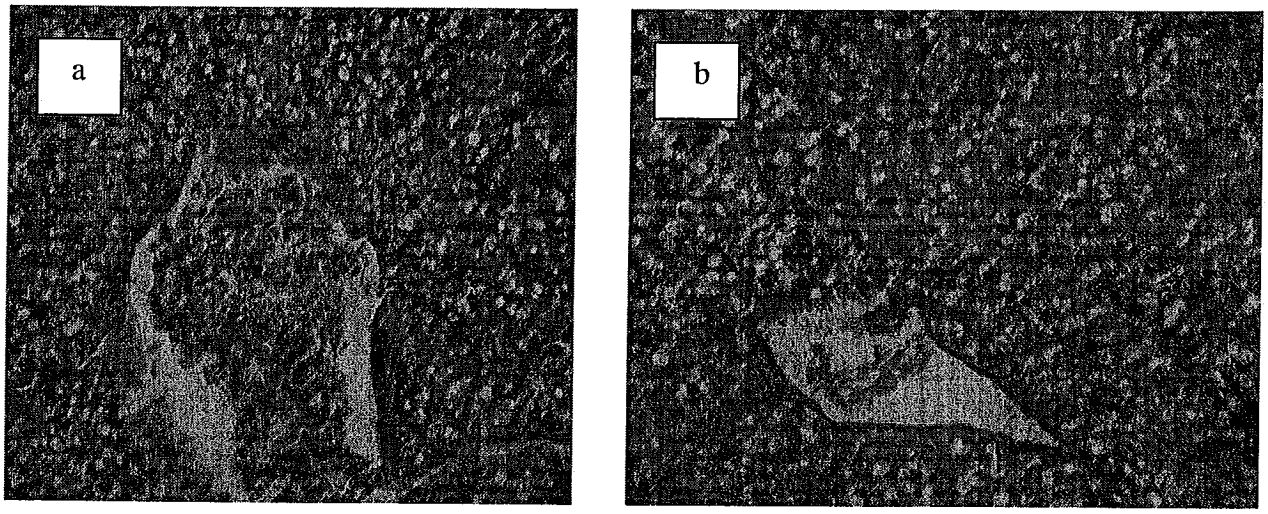

Fig. 1. **(a)** Liver of sacrificed hatched chick showing vacuolar degeneration H&E (X400).
(b) Liver of sacrificed hatched chick showing congestion in central vein and vacuolar degeneration H&E (X400).

Fig. 2. Liver of sacrificed hatched chick showing hepatocellular necrosis with heamorhages, H&E (X400).

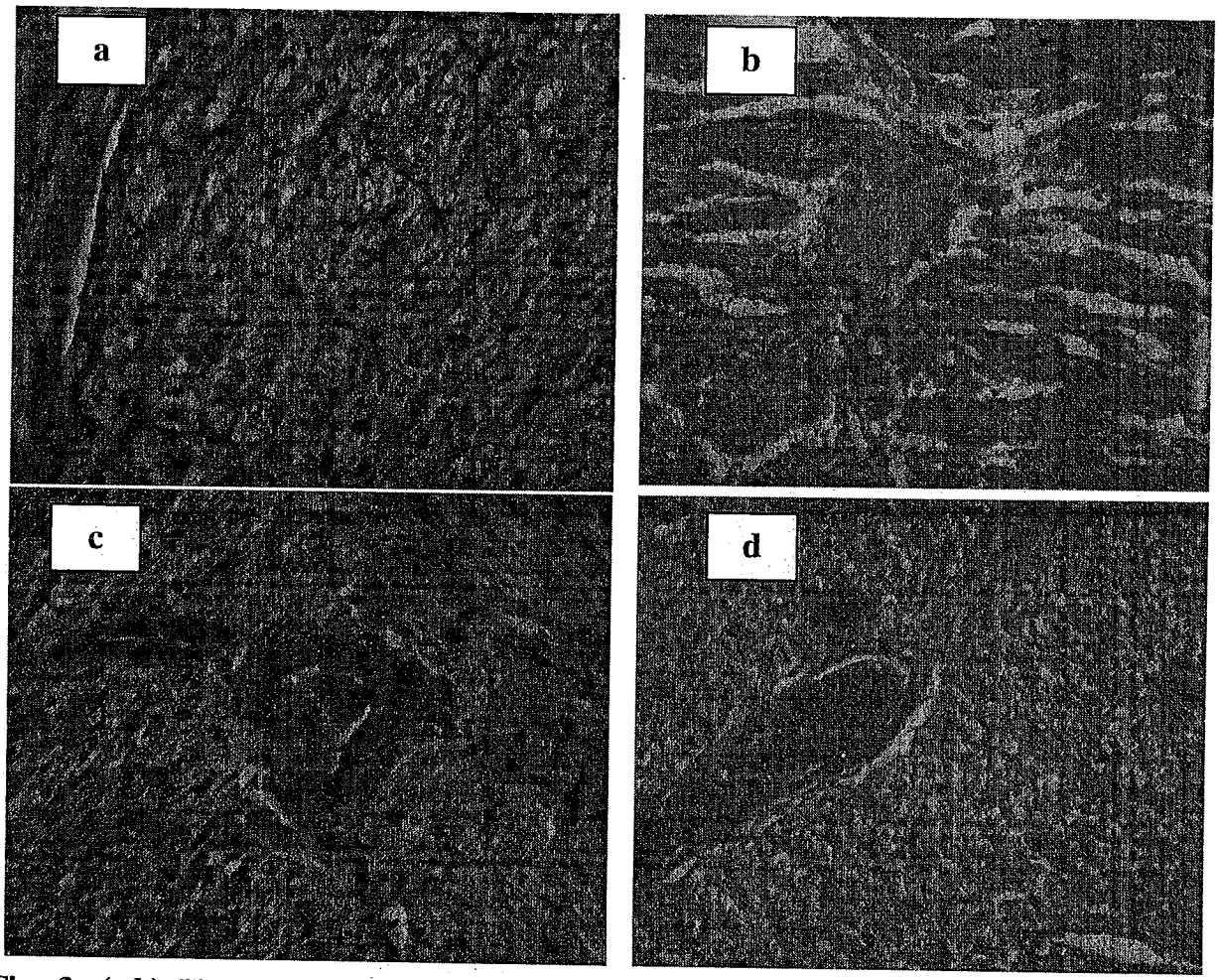

Fig. 3. **(a,b)** Heart of sacrificed hatched chick showing vacuolation in myocardium and congestion , H&E (X400).
(c,d) Heart of sacrificed hatched chick showing congestion, H&E (X400).

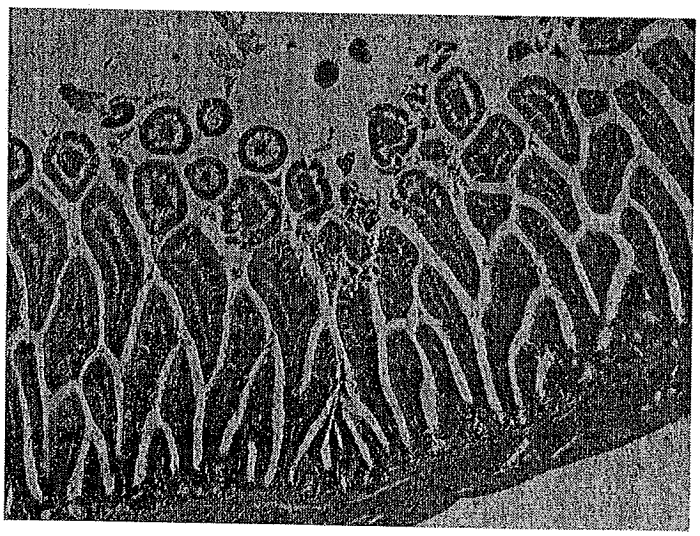

Fig. 4. Intestine of sacrificed hatched chick showed hyperplasia of intestinal crypts and sloughing of the epithelium inside the lumen H&E (X100).

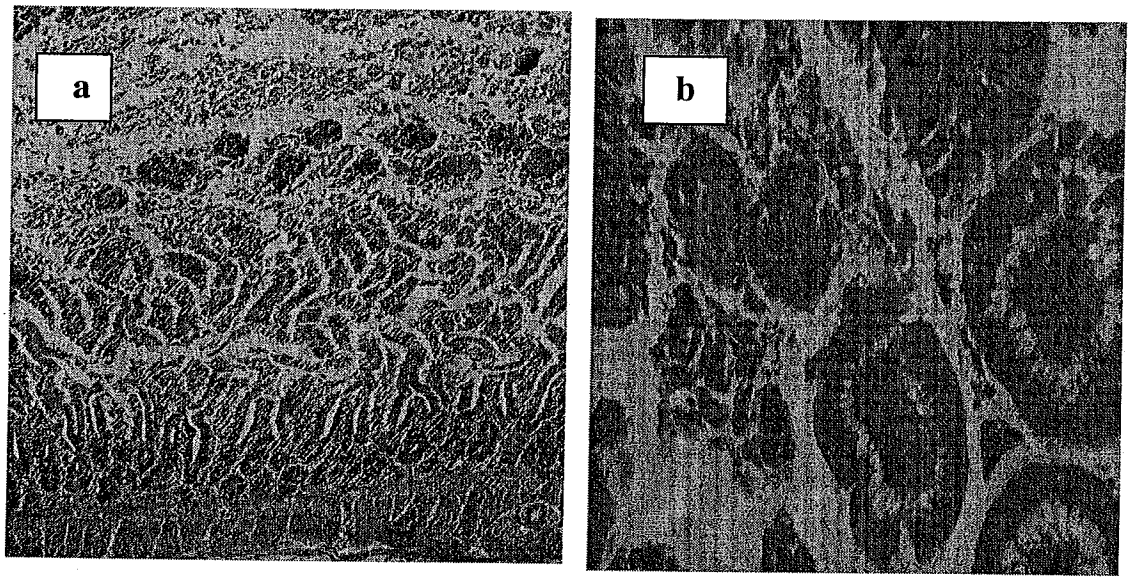

Fig 5. (a) Intestine of sacrificed hatched chick showing heamorrhagic entrities H&E (X100)
(b) Intestine of sacrificed hatched chick showing heamorrhagic entrities represented by necrotic epithelium and luminal inflammatory cells H&E (X400).

DISCUSSION

Increased awareness of *Aeromonas* species in animals, birds and human has stimulated interest about possible existence and distribution of it among different kinds of birds in Egypt. Taking into consideration that *A.hydrophila* infection in birds may constitutes one of the economic problems, so the present work was carried out to investigate the prevalence of *A.hydrophila* in embryonated chicken eggs and study the effect of *A.hydrophila*, on the viability and hatchability of embryonated chicken eggs as well as pathological effect on hatched chicks. Screening made during the present work declared the incidence and prevalence of the organism in embryonated chicken eggs, it is evident from the results that 30 *A.hydrophila* isolates representing an incidence percentage of 7.5% were recoverd from 400 embryonated chicken eggs .This relatively high incidence of *A.hydrophila* in embryonated chicken eggs, this may explained by using uncleaned hatching eggs contaminated with droppings and inadequate disinfection of contaminated incubators and hatcheries. In similar studies *A.hydrophila* have been isolated from embryonated chicken eggs ,dead-in –shell chicken embryo and weak chicks by different investigators,***(15,16,25,26)*** *A.hydrophila* was the predominant isolate 77 isolates out of 125 *Aeromonas* species isolates in poultry eggs, ***(24)***. Also our results nearly come in accordance with results of ***(4)*** who isolated 3(0.78%) *A.hydrophila* strains from dead-in – shell chicken embryo from local hatcheries in Zambia , also agree with ***(17)*** who recover *A.hydrophila* from dead-in –shell chicken embryo with percentage of 3.30% in Kartoum. Other studies recorded isolation *A.hydrophila* but in dead-in –shell ostrich embryo and non hatched ostrich eggs ***(27,28)***, while ***(29)*** isolated 6 *A.hydrophila* isolates (16.6%) from unhatched ostrich eggs . Out of the 30 isolates 14 (7%) were isolated from the mechanical hatchery, and 16(8 %) isolates were from the balady hatchery this little difference may due to poor levels of hygiene existing in balady hatchery, also contamination of eggs with faecal material or soil due to laying and putting

eggs on the floor with inadequate disinfection, *(4)*. In the present work, cellular and colonial morphology, biochemical characteristic of *A.hydrophila* were studied. It was Gram negative , straight rods, the isolated strain gave translucent pink colonies with darker centers onto the surface of Aeromonas agar , while on MacConkey -mannitol agar plus ampicillin, were pale yellow ,non lactose fermenter colonies. This conclusion had been also recorded by *(24,30)*. Our results showed that, *A. hydrophila* , kill the chicken embryos. There was a correlation between the route of infection and mortality rate. Inoculation via Yolk sac revealed mortalitiy 100% of the embryos within 3days post inoculation in group 1. While the dipping of ECE in media containing *A.hydrophila* revealed mortalities 25% after 7 days post dipping in group 2. as compared with 0% in non-infected control groups. This finding assumed the possibility of transmission of *A. hydrophila* via egg shell penetration This finding supported the results of *(19,20)*.The differences in mortality percentage could be attributed to the route of infection with *Aeromenas*. It was found that the yolk sac inoculation route was more effective than the dipping method this also confirmed by the results of *(31)* who found that *A.hydrophila* induced acute death within 24 hrs of the inoculated chicks with 100% mortality rate after yolk sac inoculation and 86.6% after subcutaneous inoculation. As the inoculation rate results in insert of high level of microorganisms inside the ECE and result in 100% mortality while dipping of ECE in the *Aeromonas* suspension results in 25% mortality in 5-days old ECE in 7days post infection and , 30% mortality of ECE 17-days old after 4days post infection . From the previous results we can expect the effect of *Aeromonas* on ECE from infected females may be due to contamination of egg shell by contaminated dropping will be less than infection from the ovary these results completely agreed with *(20)* who mentioned that inoculation of *A.hydrophila, A.caviae and A.sobria* via Yolk sac revealed mortality 100% of the embryos chicken eggs within 3 days post inoculation in group A while the dipping of ECE in media containing (*A.hydrophila , A.caviae and A.sobria*) revealed mortalities, 25% ,33.4% and 17% respectively after 11days post dipping ,and partially agreed with *(18)* who recorded the pathogenic effect of *A.hydrophila* on ECE aged 5and 17 days by dipping and resulted in 36.7% and 30% embryonic mortality, and *(19)* who recorded 8.3% embryonic mortality after dipping of 18- day- old ECE in broth culture containing *A.hydrophila* .Increased humidity and temperature as well as poor hygienic hatchery conditions are incriminated in provoking *A. hydrophila* infection via egg shell penetration, as mentioned by. *(32)*, who isolated *A.hydrophila* and other enterobacteria from the egg shell of chickens. *A.hydrophila* affected the percentage of hatchability of ECE as shown on Table(2,3),the inoculation of *A.hydrophila* in the ECE of group2 ,cause mortality of 100% of the chicken embryos, so the hatchability percentage was 0% while in the group 4 which dipped in *Aeromonas* suspension showed hatchability percentage of 70% these results completely agreed with *(18)* who recorded hatchability percentage of 63.3% in infected 5- days ECE and 70% in 17 -days ECE by dipping, and with *(20)* who observed similar results in the ECE inoculated via yolk sac(0%), but 75% hatchability in dipped group. Also with *(19)* who recorded, 76.7% hatchability percentage in 18- day- old ECE. The reduction in the hatchability mainly due to mortality of the weak embryos these results agreed with that obtained by *(33)*.The post mortem lesions in dead embryos showed severe congestion of the liver, myocardium and yolk sac. Moreover, hatched sacrificed chicks showed moderate congestion of liver, heart and yolk sac, severe enteritis with precipitation of urates in the ureters ,similar results were cited by *(13,18 ,19 ,25)* ,the rate of re- isolation of *A.hydrophila* from sacrificed hatched chicks internal organs was 93.1, 68.9, 17.2 and 51.7% from intestine, liver, heart, and yolk sac ,respectively, this come in accordance with *(7,19 ,25)* who isolated *A. hydrophila* from the yolk sac, heart blood, lung, brain and cloacal swabs of experimentally infected chicks. Recently,

A.hydrophila was isolated in pure culture from intestine, liver, lungs and trachea of adult ostriches died with severe necrotizing enteritis and septicaemia *(34)* . The isolation of *A. hydrophila* from the intestine of infected birds indicates intestinal colonization, *(35).* Isolation of the organism from liver, heart can be explained by infection via the blood stream (bacteriaemia) *(6). A. hydrophila* which adhere to epithelial cells are believed to colonize, produce toxins lead to pathological changes in the internal organs. Liver lesions in the form of congestion in the central vein in addition to vacuolar degeneration and hepatocellur necrosis with hemorrhages, similar results were recorded by *(7,17,19 ,20).*The heart showed vacuolar degeneration and ,myocardial congestion *(19).* The intestinal lesions varied from sloughing of the epithelial cells in the intestinal lumen to hemorrhagic enteritis, these results agreed with *(19,30)* ,but incomplete agreed with *(20)* who found excessive mucous secretion in intestine as a result of hyperactivity of mucous gland, and sever hyperplasia in the intestinal epithelium.

Conclusion: our results are indicating that *A. hydrophila* is prevlant in chicken hatching eggs and highly pathogenic for chicken embryo and causing embryo mortalities and decrease of the hatchability ,so control measures and biosecurity in hatcheries should be considered as regular monitoring of hygiene in the incubators and assessing effectiveness of disinfection and using clean ,disinfected egg for hatching.

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الملخص العربى

أهمية ومدى تواجد ميكروب الايروموناس هيدروفيلا فى بيض الدجاج الغير فاقس

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أجريت هذة الدراسة لمعرفة مدى انتشار ميكروب الايروموناس هيدروفيلا فى بيض مفرخات الدجاج الميكانيكية والبلدية فى محافظة أسيوط و كذلك دراسة تأثير الميكروب على معدلات الفقس اضافه الى دراسة التأثير الباثولوجى على الأجنة و الكتاكيت الفاقسة حيث انه تم جمع ٤٠٠ عينة بيض غير فاقس تشمل٢٠٠ بيضة من المفرخ الميكانيكى و ٢٠٠ بيضة من مفرخ البلدى .وقد تم اخذ العينات من الكبد ،القلب، كيس المح من الاجنة الغير فاقسة كذلك أخذ مسحات من قشرة هذا البيض كذلك أخذ مسحات من المفرخات وقد تم التعرف على عدد ٣٠ معزولة بنسبة(٧،٥%) وذلك طبقا للاختبارات البكتريولوجية والبيوكيميائية للعزل والتعرف على هذا المكروب ،منهم ١٤ معزولة (٧%) من بيض المفرخ الميكانيكى، ١٦(٨%) من المفرخ البلدى. وبأ جراء العدوى الصناعية فى البيض المخصب عمر ٥ أيام ،١٧ يوم لدراسة ضراوة عترات ميكروب الايروموناس هيدروفيلا فى بيض التفريخ كذلك تأثيرة على الكتاكيت الفاقسة.وذلك عن طريق عمل مجموعتين من البيض المخصب عمر٥ أيام الاولى تم حقن كيس المح فيها ١،٥×$١٠^{٧}$والمجموعه الاخرى تم تغطيسها فى محلول مبرد يحتوى على٦,١×$١٠^{٩}$ من الميكروب وكذلك تم التغطيس لمجموعة ثالثة عمر ١٧ يوم فى محلول مبرد يحتوى على٦,١×$١٠^{٩}$ من الميكروب وتم وضع البيض فى الحضانة مع التقليب اليومى والكشف عن البيض الذى يحتوى على اجنة ميتة واستبعاده حتى الفقس.وقد نتج عن هذة العدوى نفوق جميع الاجنة (١٠٠%)على مدى ٣ أيام فى المجموعة الاولى بعد الحقن فى كيس المح و نفوق (٢٥%) من الاجنة فى المجموعة الثانية عمر ٥ أيام التى تم لها التغطيس وذلك فى خلال ٧ أيام من العدوى ولكن نسبة النفوق فى المجموعة الاخرى عمر ١٧ يوم كانت (٣٠%) على مدى ٤أيام من العدوى وكانت نسبة الفقس فى المجموعة الثانية والثالثة (٧٥% و ٧٠%)على التوالى.وقد تم اعاده عزل الميكروب من الاعضاء الداخليه للاجنة الميتة والكتاكيت الفاقسة .وقد اظهرت نتائج الفحص النسيجى وجود تغيرات فى انسجة الكبد، القلب والامعاء،تمثلت فى حدوث احتقان فى الاوردة الكبدية وظهور هالات تحللية فجوية وتنكرز فى خلايا الكبد مع وجود انزفة وايضا وجود احتقان فى عضلة القلب كذلك حدوث التهاب بالامعاء وجود انزفة وتنكرز وارتشاحات خلوية ليمفاوية.