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Bacterial Causes of Embryonic Death in Ostrich Egg

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Abstract: *A total of 141 egg samples (5 non-fertile eggs, 41 dead in shell embryos at 39 days of age , 4 eggs contain dead embryos at 28 days of age) was subjected to bacteriological examination in a trial to detect the actual bacterial causes of embryonic death in ostrich eggs in Ismailia Governorate. 61.7 % of the examined egg samples were positive for bacterial isolation. The result of bacterial isolation revealed that Klebsiella spp. was the most prevalent organism isolated from dead-in-shell embryos and infertile eggs with rates of 23.57 % and 20 %, respectively. Proteus spp was isolated from dead-in-shell embryos with a rate of 18.69 %. The only organism isolated from infertile eggs was Klebsiella spp. with a rate of 20 %. Enterococcus spp. was isolated from dead-in-shell embryos with a rate of 15.44%. The occurrence of Escherichia coli in dead-in-shell embryos was (8.94 %) and Providencia spp. was (3.25%). The lowest incidence of the recovered bacterial species from dead-i- shell embryos was Salmonella spp. and Serratia marcescens with the same isolation rate (2.43%) . From six isolates of enterococci examined using polymerase chain reaction technique, one isolate was mixed and contained both E. faecalis and E. faecium, three isolate of E. faecium and two isolate of E. faecalis.*

Keywords: *Ostrich, embryonic death, bacterial causes, aerobic bacteria*

1. INTRODUCTION

The domestic ostrich (*Struthio camelus domesticus*) is the result of more than 100 years of selective breeding in the arid regions of South Africa for improved reproductive traits (eggs produced per breeding season), feather quality and ostrich meat. Scientific knowledge of ostrich diseases, particularly the microbial agents involved in such diseases is scarce, with specific details on technical aspects of diagnostic and/or screening tests completely absent in most cases. Embryonic mortality is one of the major problems that is facing the ostrich industry and causes great economic losses; it results from environmental factors as well as infections due to bacterial agents [1,2]**.**

Various bacterial pathogens have been associated with ostrich egg contaminations including *Escherichia coli*, *Aeromonas* spp, *Enterobacter* spp, *Acinetobacter* spp, *Citrobacter* spp, *Enterococcus faecalis*, *Klebsiella* spp, *Staphylococcus* spp, *Bacillus licheniformis* and *Achromobacter* spp. [2-4]. Microbial contamination of ostrich eggs is incriminated in embryonic mortality and considered a significant problem and a major health concern of ostrich production [5]

The aim of the present study is to shed light on the bacterial agents that might be responsible for embryonic mortalities in eggs of ostrich through isolation and identification of different bacterial pathogens from egg shells and dead embryos.

2. MATERIALS AND METHODS

2.1. Sampling

A total of 50 ostrich eggs with reproductive disorders was collected from ostrich hatcheries in Ismailia Governorate in Eastern Egypt and transported to the laboratory for bacteriological examination. Eggs have been collected during the period from March 2012 to September 2013 and brought back to the laboratory in an ice box. Samples were collected from yolk fluid and albumen contents of non-fertile eggs, yolk sac, yolk fluid and internal organs of the dead embryos under complete aseptic conditions.

In addition, samples were collected from yolk and albumen contents of four ostrich eggs contained dead embryos at 28 days of age All eggs presented poor quality of the eggshells with deformities and high porosity.

2.2. Enterococci Reference Strains

The reference strains *E. faecalis* (ATCC 29212 isolate) and *E. faecium* (ATCC19434) were obtained from the American Type Culture Collection.

2.3. Material used for DNA Extraction, PCR, and Agarose Gel Electrophoresis

All chemicals and reagents were molecular biology grade **3.**

Buffers and Solutions used for Molecular Identification:

a. Tris Acetate EDTA (TAE) Electrophoresis in Buffer (50x) Stock Solution Ph 8.0.

b. Taq DNA Polymerase. (Qiagen)

The enzyme was obtained from thermos aquaticus strain YT1 (Thermophilic bacteria) at a concentration of 5 units buffer (20mM Tris-HCL, pH 8.0,1 mM DTT, 1m M EDTA , 100mM KCL, 0.5% Nonidet p40, 0.5Tween 20 and 50% glycerol).

c. Mgcl2: prepared as 2.5m M (Applied biosystems PCR mix , USA, catalogue No.c07954).

d. Emerald Amp Gt Pcr Master Mix: (Takara's Emerald Amp Gt Pcr Master Mix). This master mix includes an optimized buffer, PCR enzyme dNTPmixture , gel loading dye (green) and a density reagent in 2x premix .

e. Ethidium Bromide (1000 X) : (Sigma)

f. DNA Ladder: (Jena Bioscience Germany): mixture of DNA fragment of known length 100bp.

g. Agrose Gel (Sigma).

h. Oligonucleotide Primers.

Three specific primers were used for identification of *Enterococcus* species and one was used for identification of Cy1A virulence gene [6,7].

***Enterococcus* specific**		5′-TACTGACAAACCATTCATGATG -3	112bp
ddlE.faecalis	dd1E1-	ATCAAGTACAGTTAGTCTTTATTA	941bp
	dd1E2-	ACGATTCAAAGCTAACTGAATCAGT	
ddlE.faecium	dd1F1-	TTGAGGCAGACCAGATTGACG	658bp
	dd1F2-	TATGACAGCGACTCCGATTCC	
CylA virulence gene	CYTI-	ACTCGGGGATTGATAGGC	688bp
	CYTIIb-	GCTGCTAAAGCTGCGCTT	

2.4. Isolation and Identification of Bacterial Pathogens

All swabs were directly streaked on mannitol salt agar, MacConkey's agar and defibrinated sheep blood agar media (Oxoid, Ltd., Hampshire, UK). For isolation of salmonella*e,* swabs were inoculated in Rappaport Vassiliadis broth and incubated at 41°C, over night before streaking on MacConkey's agar (Oxoid, Ltd., Hampshire, UK). All plates were incubated at 37°C for 24 hours. Identification was carried out by culture characteristics and bacterial films stained with Gram's technique. After then, the cultures were tested for different biochemical tests (Catalase, coagulase, bile esculin agar, TSI, citrate agar, urea hydrolysis test, oxidase). Identification of the recovered isolates was done according to [8,9].

2.5. PCR for Genotyping of Enterococcus Isolates

Five isolates of Enterococcus species were tested by PCR according to [10] Pure colonies of enterococci were grown over night in 5ml brain heart infusion broth at 37°C.-One ml of culture was centrifuged at 5000xg (rcf) for 15minutes.The cell pellet was washed twice with one ml phosphate buffer saline (pH 7.2) then suspended in 50 u1 PBS. The mixture was vortexed, 20 proteinase K were

pipetted into bottom of a 1.5 ml microcentrifuge tube. The mixture was incubated at 56°C for 10 min. Finally the suspension was centrifuged at 13,000xg for 5 minutes. Five µl of supernatant was used as template DNA. Amplification was performed in enterococcus specific primer as follow 12.5 master mix (5 µL 10X buffer,1.5Mgc12 , 4µLdNTPs,1.5µLTaq DNA polymerase) , one µL from each primer, 4 µL of extracted DNA template from different enterococci isolates and to 25µL deionized water. The program included a denaturation step of 94°C for 5 min and then subjected to 30 cycles of amplification (94°C for 30 s , 50°C for 30 s , 72°C C for one min) with a final soak at 4°C. Fragments DNA were determined by comparing the control-positive bands with amplified fragments of wild enterococci . The expected bands of the amplicons were 658 and 941 pb from dd1 gene in *E . faecium* and *E. faecalis*, respectively. Reference strains of Enterococcus was used as controls. PCR products were visualized in a 2% gel at 80 V.

3. RESULTS

3.1. Isolation and Bacteriological Identification

Out of 50 collected eggs, 35 (70%) were positive for bacterial isolation. Figure 1 summarizes the isolation rates of different bacterial pathogens. The most common isolated bacterial species recovered from egg samples were *Klebsiella pneumoniae* (21.98%), followed by *Proteus vulgaris* (16.31%), *Enterococcus* spp (13.47%), *Escherichia coli* (7.8%), *Providencia* spp. (2.83%), *Salmonella* spp. (1%) and *Serratia marcescens* (1%).

Bacteria isolated from ostrich dead-in-shell embryos are depicted in Table 1, which shows that, single bacteria species were isolated from 31 dead-in-shell embryos (75.61%), while mixed bacteria were recovered from 3 embryos only (7.31%), while no bacteria were isolated from 7 embryos (17.07%). The most common bacteria isolated from ostrich dead-in-shell embryos singly were *Proteus Spp*, which were recovered from 26.83% of examined embryos, followed by *Klebsiella Spp* (21.95%), *Enterococcus Spp*(12.19%), *E. coli* (7.31%), *Providencia Spp* (4.87%) and *Serratia marcescens* (2.43%).

Table1. *Bacteria isolated from ostrich dead-in-shell embryos*

Types of bacteria	**NO**	**%**
Single isolates	31	75.61
Proteus Spp	11	26.83
Klebsiella Spp	9	21.95
Enterococcus Spp	5	12.19
E. coli	3	7.31
Providencia Spp	2	4.87
Serratia marcescens	1	2.43
Mixed isolates	3	7.31
Enterococcus Spp+ Salmonella Spp	1	2.43
Enterococcus Spp + Klebsiella Spp	2	4.87

% calculated to the number of examined embryos (41)

Table2. *Incidence of isolation of different bacterial species recovered from ostrich dead-in-shell embryos collected from different samples*

Type of bacterial species	**Yolk fluid**	**Yolk sac**	**Liver**	**Total positive samples**	**%**
Klebsiella spp.	9	9	10	28	22.76
Proteus spp.	6	9	7	22	17.88
Enterococcus spp.	6	7	6	19	15.44
Escherichia coli	4	4	3	11	8.94
Providencia spp.	-	2	2	4	3.25
Salmonella spp.	1	1	1	3	2.43
Serratia marcescens	1	1	1	3	2.43
Total	27	33	30	90	73.17

%was calculated according to total number of examined samples(41x3=123).

It is evident from Table 2, that the highest isolated bacteria were obtained from the yolk sac, followed by the liver then the yolk fluid. Table 3 indicates that, the liver alone was positive in 2 embryo, yolk sac alone in 3 embryos, yolk fluid alone in none of the embryos. On the other hand, the liver and yolk sac were positive in 5 embryos, the liver and yolk fluid in 2 embryos and the liver, yolk sac and yolk fluid in 21embryos, while the yolk sac and yolk fluid were positive in 4 embryos.

Table3. *No.of positives from different organs*

Types of samples	No.of positive samples
Liver,yolk sac and yolk fluid	21
Liver and yolk sac	5
Yolk sac and yolk fluid	4
Yolk sac only	3
Liver only	2
Liver and yolk fluid	2
Yolk fluid only	0

3.2. *Enterococci* Identification by PCR

From Fig. 1, it is clear that, from six isolates of *enterococci* examined using polymerase chain reaction technique, one isolate was mixed and contained both *E. faecalis* and *E. faecium*, three isolates were confirmed as *E. faecium* and two isolate of *E. faecalis*.

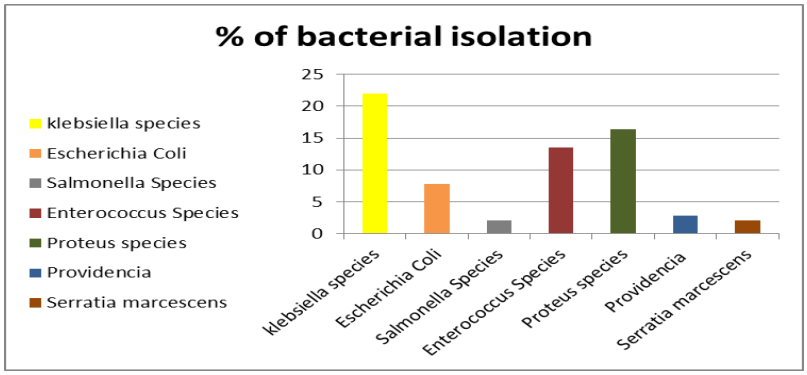

Figure1. *Incidence of isolation of different bacterial species recovered from egg samples collected from Ismailia governorate.*

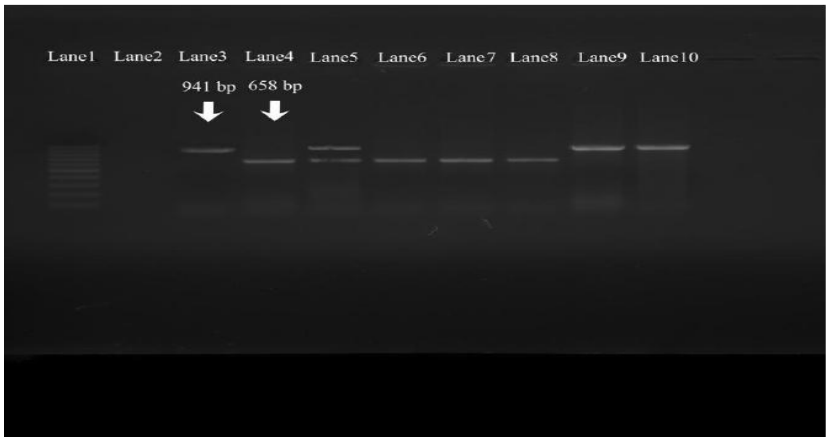

Figure2. *Electrophoretic profile of PCR products of Enterococcus isolates.* ***(Lane 1)*** *marker 100bp,* ***(Lane2)*** *negative control,* ***(Lane3)*** *941bp positive control of E. faecalis ATCC No(29212),* ***(lane4)****658bp positive control of E. faecium ATCC No (55593),* ***(Lane5)****mixed isolate contain both E. faecium and E. faecalis,* ***(Lane 6,7,8)*** *isolate of E. faecium,* ***(Lane 9, 10)*** *isolate of E. faecalis.*

4. Discussion

Ostrich farms are considered to be among the most profitable agricultural projects because of the large variety of possible products and hence their profit potential. Ostrich meat is far better from the health point of view as it is high in protein and contains far less fats, and particularly less cholesterol, than other types of meat, so the demand for ostrich meat in the international markets has been growing [11].

Embryonic mortality is one of the major problems facing the ostrich industry and cause great economical losses, it results from environmental factors as well as nutritional causes (Deeming, **1997).** Various microorganisms have been associated with ostrich egg contaminations, which may result in embryonic death [12].

In the present study, the highest incidence of isolation was obtained from samples collected from dead-in-shell embryos (69.1%), followed by infertile eggs (20%). The most common isolated bacterial species recovered from egg samples were *Klebsiella* species (21.98%), followed by *Proteus* species (16.31%), *Enterococcus* species (13.47%) *Escherichia coli* species (7.8%), *Providencia* species (2.83%). *Salmonella* species and *Serratia marcescens* were isolated with the same incidence rate (2.12%). This result came parallel to results obtained by [12], who isolated *Klebsiella* species from surface of ostrich embryo and from yolk sac and the embryo content with mean prevalence of 15%. The obtained results, however, disagree with [4], who recorded a percentage of 1.9 % for the isolation of *Klebisella* spp. In the present investigation *Proteus* species was isolated from yolk fluid, yolk sac and liver of dead-in-shell embryos with an incidence of 16.31%. This result agreed with [12], who recorded it from egg in percentage of 12.5%. Also the low isolation rate of *Proteus* species has been reported by [4]. They recovered one isolate of *Proteus mirabilis* from 543 dead and dead-in-shell ostrich embryo in 44 farms from Northern and central Italy. The results of bacterial isolation revealed that *Enterococcus* species were isolated from yolk fluid (6), yolk sac (7), liver (6) of dead-in-shell embryos at rate of 13.47 %. This result is higher than that reported by [13], who isolated (4) isolates of *Enterococci* from 320 ostrich eggs, which were collected from nine commercial farming operations in Zimbabwe and [4], who recovered one isolate of *Enterococcus aerogenes* from 543 ostrich eggs belonging to 44 farms from Northern and central Italy. The obtained results showed also that *Escherichia coli* species was isolated from yolk fluid (4) , yolk sac(4) , liver (3) of dead-in-shell embryos at rate of 7.8%.This result agreed with [12], who recorded a percentage of 10% .

This result goes parallel with the work of [14]**,** who isolated two strains of *E. coli* serotype 078 (2.7%), (5) isolates of *E. coli* serotype 01 (6.8%), (2) isolates of *E. coli* serotype 0128 (2.7%), (6) Isolates of *E. coli* serotype 0166 (8.2 %). On the other hand, this result is lower than that obtained by [2,12], who recorded a percentage of 12.5% and 27.7 %, respectively. While in the work of [5], non-haemolytic *Escherichia coli* was the commonest bacterium isolated from nine commercial farming operation in Zimbabwe.

In the present work, *Providencia* species were isolated from yolk sac (2) , liver (2) and not isolated from yolk fluid of dead-in-shell embryos at rate of 2.83 % .According to the available literature ,it is the first time to isolate this organism from ostrich egg content.

Three isolates of both of *Salmonella* species and *Serratia marcescens* species were isolated from yolk fluid , yolk sac and liver of dead-in-shell ostrich embryos at rate of 2.12 %. This result agreed with [4], who isolated one isolate of *Seratia marcescens* from 543 ostrich eggs in 44 farms from northern and central Italy. This result is lower than that reported by [15], **Knobl (2012)** who isolated *Serratia* species in rate of 20%.

The interesting finding in the present study is the isolation of the Gram positive *Enterococcus* species from 5 dead-in-shell embryos. These organisms have no flagellae that help their penetration through the shell pores. However, *Enterococcus faecalis* have the capability to make surface pili which can lead to the formation of a biofilm on the shell surface [16].

These organisms were selected in the present study for molecular characterization. The results of PCR of 6 isolates indicated that 3 isolates were *Enterococcus faecium*, 2 isolates were *Enterococcus faecalis* and one isolate was mixed containing both *Enterococcus faecium* and *Enterococcus faecalis.* The molecular identification of these isolates was achieved using the primers ddlE1 and ddlE2- for *Enterococcus faecalis* and ddlF1 and ddlF2 for *Enterococcus faecium* as recommended by [6,7].

It is known that Enterococcus spp. colonize the gastrointestinal tract of humans and many animals, and are also commonly found in soil and sediments, beach sand, plants, food and waters [17-24]. Furthermore, they are also important cause of nosocomial opportunistic infections in humans and have been reported in sporadic infections in animals [19].

Over the last decades, antibiotic multiresistance has increased in enterococci; this can be explained by the massive use of antibiotics both in the human health care system and in agriculture [25-29]. In fact,

enterococci might act as a reservoir of antimicrobial resistance genes that could be transmitted to other pathogenic bacteria through the exchange of plasmids and conjugative transposons, and for this reason might represent a worldwide problem in public health [25-27]. Enterococci are intrinsically resistant to a number of antimicrobial agents, but they can also acquire resistance to other antimicrobial agents, such as quinolones, macrolides, tetracycline, streptogramines and glycopeptides [19, 28, and 30].

In conclusion , application of hygienic measurement , adequate egg turning , sufficient incubator humidity , periodic weighing of eggs for water loss in the incubators and hatcheries and proper egg handling are recommended in ostrich farms to reduce bacterial contamination and obtain significant and successful hatching results .

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