Bacterial contamination of dead-in-shell embryos in ostrich hatcheries and antimicrobial resistance patterns of isolated *Escherichia coli*

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Abstract:

BACKGROUND: The bacterial contamination of fertile eggs is the most common cause of embryonic death in ostrich hatchery units leading to financial loss in ostrich industry. **OBJECTIVES:** The aim of this research was to investigate the bacterial contamination status, with emphasis on *Escherichia coli*, of ostrich hatcheries and the antimicrobial resistance profile of isolated *Escherichia coli*. **METHODS:** A total of 120 ostrich eggs with dead embryos, at weekly intervals, were collected from three ostrich hatcheries. The dead embryos were sent to laboratory and samples were collected aseptically from different organs. Bacterial detection and identification were performed by using standard bacteriological and biochemical techniques. Antimicrobial susceptibility test was carried out by agar disk-diffusion method against 27 antimicrobial agents. **RESULTS:** Different types of bacteria were isolated from 56 eggs (46.7%). Twenty-four ostrich eggs were shown to carry *E. coli*. In some eggs, in addition to yolk sac, *E. coli* was also isolated from meconium, liver, or heart blood which increased the total number of *E. coli* isolates to 32. All *E. coli* isolates were susceptible to trimethoprim + sulphamethoxazole, danofloxacin, and flumequine, whereas all were resistant to carbenicillin and erythromycin. Resistance to other agents was variable. Multi-drug resistance pattern was found among all *E. coli* isolates and included 2 to 12 drugs. Thirty-two *E. coli* isolates generated 30 different resistance profiles against 27 antimicrobial drugs. **CONCLUSIONS:** This was the first comprehensive report regarding the bacterial, particularly *Escherichia coli*, contamination of dead-in-shell ostrich embryos and antimicrobial resistance status of the *Escherichia coli* isolates from ostrich eggs in Iran.

Introduction

Since mid-1990s when Iran started to import ostriches, the ostrich farming industry has expanded considerably and even is facing a competitive market for its products now. However, it seems that the ostrich industry needs much improvement in different aspects of production. Several factors such as reasonable fertility, hatchability, and chicks'

livability rates need to be improved because these factors have been shown to have considerable influence on the profitability of ostrich farming. Bacterial contaminations of fertile eggs, dead-in-shell embryos, and yolk sac infection have been associated with lower hatchability rate (Deeming, 1996; Mushi et al., 2008; Dzoma, 2010).

Currently, little information is available about the bacterial contaminations of fertile ostrich eggs, dead-in-shell embryos, and yolk sac infections in Iran's ostrich farming industry. This is the first comprehensive study in Iran on the status of bacterial contaminations of ostrich eggs in hatcheries

Materials and Methods

Sampling and Bacteriological Procedures: A total of 120 ostrich eggs with dead-in-shell embryos were collected at weekly intervals from three ostrich hatchery units during a 3-month period. Standard bacteriological techniques were used for the isolation and identification of the bacteria from the samples, which included yolk sac, liver, heart, and pericardium from each dead embryo (Quinne et al., 1994, Waltman et al., 1998). Different types of bacteria were identified in initial screenings but only *E. coli* isolates were included in this study.

Antimicrobial Susceptibility: In this investigation, 32 *E. coli* isolates were recovered from 120 dead-in-shell embryos, their susceptibility to a panel of 27 antimicrobial agents was determined by the agar disk-diffusion method, and the interpretation of results was carried out according to the National Committee for Clinical Laboratory Standards guidelines (CLSI, 2006). The tested antimicrobial agents and their concentrations (µg) were: ciprofloxacin (5), danofloxacin (10), difloxacin (10), enrofloxacin (5), nalidixic acid (30), flumequine (30), cephalothin (30), cefixime (5), ampicillin (10), amoxi-clav (30), carbenicillin (100), erythromycin (15), kanamycin (30), neomycin (30), streptomycin (10), amikacin (30), gentamicin (10), lincospectin (15/200), Fosbac® (fosfomycin + fructose 1.6 diphosphate) (200), fosfomycin (200), chloramphenicol (30), florfenicol (30), furazolidone (100), Colistin (10), tetracycline (30), oxytetracycline (30), and trimethoprim-sulfamethoxazole (1.25/23.75). Fosbac® and fosfomycin disks were provided from Bedson Co. (Buenos Aires, Argentina). All other antibacterial disks were purchased from Padtan Teb Co (Tehran, Iran). The ATCC reference strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa*, ATCC 27853, and *E. coli* ATCC 35218 were used for quality control purposes. The isolates were classified as susceptible, intermediate susceptible, or resistant based on the standard interpretation chart updated according to the current CLSI standard (CLSI, 2006). In this study, the *E. coli* isolates with intermediate susceptibility classification were considered not to be resistant to that drug and multi-resistance was defined as resistance to more than one drug.

Results

Bacterial contaminations were detected in 56 (46.7%) out of 120 samples. More than one type of bacteria was detected in 25 (44.6%) of the 56 contaminated eggs. The identified bacterial types included *Pseudomonas* spp., *Escherichia coli*, *Klebsiella* spp., *Bacillus* spp., *Citrobacter* spp., *Staphylococcus* spp., *Proteus* spp., *Aeromonas* spp., and *Enterobacter* spp. No bacterial contamination was detected in 53.3% of 120 samples. Twenty-four ostrich eggs were shown to carry *E. coli*. In some eggs, in addition to yolk sac, *E. coli* was also isolated from meconium, liver, and heart blood, which increased the total number of *E. coli* isolates to 32.

All *E. coli* isolates of this study showed susceptibility to danofloxacin, flumequine, trimethoprim+sulphamethoxazole, whereas all were resistant to carbenicillin and erythromycin (Table 1). High rates of resistance also were detected against colistin, amoxi-clav, and ampicillin. Multi-drug resistance (MDR) was found among all *E. coli* isolates. The MDR pattern was variable and ranged from 2 to 12 drugs (Table 2). Thirty-two *E. coli* isolates generated 30 different patterns of antimicrobial resistance (Table 3). The susceptibility of 32 *E. coli* isolates to six commonly used antibacterials in Iran's poultry industry (enrofloxacin, flumequine, lincospectin, florfenicol, and trimethoprim+sulfa, tetracycline) produced six patterns (Table 4).

Discussion

Early embryonic death and dead-in-shell embryos

Table 1. Resistance of 32 ostrich *Escherichia coli* isolates to 27 antimicrobial drugs.

Drugs	% Resistant
Ciprofloxacin	0
Danofloxacin	0
Difloxacin	6.2
Enrofloxacin	0
Nalidixic Acid	3.1
Flumequine	0
Cephalothin	56.2
Cefixim	21.8
Ampicillin	62.5
Amoxi-clav	75
Carbenicillin	100
Kanamaycin	28.1
Neomycin	15.6
Streptomycin	37.5
Amikacin	0
Gentamicin	3.1
Fosbac®	0
Fosfomycin	3.2
Erythromycin	100
Lincospectin	25
Chloromphenicol	6.2
Florfenicol	3.1
Furazolidone	0
Tetracycline	53.1
Oxytetracycline	21.8
Trimethoprim+sulfa	0
Colistin	93.7

Table 2. Multi-drug resistance among 32 ostrich *Escherichia coli* isolates.

No. (%) of resistant isolates	No. of antimicrobial drugs
32 (100)	1
32 (100)	1 <
32 (100)	2 <
30 (93.75)	3 <
29 (90.6)	4 <
25 (78.1)	5 <
18 (56.25)	6 <
12 (37.5)	7 <
8 (25)	8 <
4 (12.5)	9 <
3 (9.37)	10 <
3 (9.37)	11 <
2 (6.25)	12 <
0 (0)	13 <

have been considered as the main causes of poor hatchability in ostrich farming (Cooper, 2001; Mushi et al., 2008; Dzoma, 2010). Yolk sac retention and yolk sac infection are also known as important causes of early chick mortality in different species of birds (Khan, 2004). Despite diverse bacterial species that are involved in yolk sac infection, *E. coli* has been the predominant pathogen that is isolated in avian species (Khan, 2004).

There have been some studies on embryonic death and early chick mortality in ostriches (Deeming, 1996; Dzoma and Dorrestein, 2001; Cabassi et al., 2004; Mushi et al., 2004; Jahantigh, 2010) and their impact on hatchability rate (Deeming, 1995; Mushi et al., 2008; Dzoma, 2010). As it is the case in other avian species, *E. coli* has been frequently the main bacterium involved in the contamination of ostrich eggs (Deeming, 1996; Dzoma and Dorrestein, 2001; Cabassi et al., 2004). In the present study, after pseudomonas, *E. coli* was the most frequent bacterium isolated from the ostrich eggs. In this study, 46.6% of samples showed bacterial contamination and *E. coli* was isolated from 42.8% of contaminated eggs.

The microbiologic investigation of ostrich eggs from farms with embryo mortality in Italy showed 19.3% bacterial contamination and *E. coli* was the most common bacterial species isolated from 19% of contaminated eggs (Cabassi, 2004). Investigation of dead-in-shell embryos in one farm in Iran with infertility problem and post-hatching yolk sac infection showed 100% microbial contamination with 45% *Bacillus* spp. as the most frequent bacterium (Jahantigh, 2010).

Unlike Cabassi et al. (2004) who did not report the isolation of multiple bacterial species form ostrich eggs, we isolated multiple bacterial species from 44.6% of the contaminated eggs. Our findings were compatible with those of a previous study in Iran (Jahantigh, 2010).

Salmonella is an important zoonotic pathogen and is a frequent cause of embryonic death and early chick mortality in poultry. Unlike Al-Nakhil et al. (2004) who reported the isolation of Salmonella from ostrich chicks in Saudi Arabia, our bacteriologic cultures from ostrich eggs did not result in Salmonella isolation, which corroborated the findings of Cabassi et al. (2004) and De Freitas Net et al. (2009).

Microbial contamination of fertile eggs is associated with the death of embryos and decreased hatchability rate (Deeming, 1996; Cooper, 2001). Fecal contamination of eggs and poor hygienic conditions of hatcheries are among the main sources of infection of fertile eggs (Khan, 2004). In the open system of ostrich breeding, the eggs are exposed to

Table 3. Drug resistance patterns among 32 ostrich *Escherichia coli* isolates. CB = Carbenicillin, E= Erythromycin, Cl = Colistin, AMC = Amoxicillin-clavulanate, TE = Tetracycline, T = Oxytetracycline, AM = Ampicillin, K = Kanamycin, S = Streptomycin, N = Neomycin, CF = Cephalothin, CFM = Cefixim, LP = Lincospectin, DF = Difloxacin, NA = Nalidixic acid, C = Chloramphenicol, FF = Florfenicol, GM = Gentamicin, FOSFO = Fosfomycin, SXT= Trimethoprim-Sulphamethoxazole.

Pattern (#)	Resistance to	No. of isolates (%)
1	CB, E, CL, AM, AMC	2 (6.2)
2	CB, E, CL, CF, AM, AMC	2 (6.2)
3	CB, E, CL	
4	CB, E, AM	
5	CB, E, CL, AMC	
6	CB, E, CL, AMC, TE	
7	CB, E, CL, AMC, TE, T	
8	CB,E,CL,AM,AMC,S	
9	CB,E,CL,AM,AMC,TE,T	
10	CB,E,CL,AM, AMC,K,S,TE, T	
11	CB,E,CL,S,TE	
12	CB,E,CL,CF,AM,AMC,N,TE	
13	CB,E,CL,CF,AM,AMC,K,TE	
14	CB,E,CL,CF,AM,AMC,K,S,TE	
15	CB,E,CL,CF,AMC,TE	
16	CB,E,CL,CF,AMC,K,TE	Each pattern included only one isolate
17	CB,E,CL,CF,CFM,AM,AMC	(3.1)
18	CB,E,CL,CF,CFM,AM,AMC,TE	
19	CB,E,CL,CF,CFM,AM,AMC, TE,T	
20	CB,E,CL,CF,CFM,AM,AMC,K,S,TE	
21	CB,E,CL,CF,CFM,AM,AMC,K,N,S,LP,TE	
22	CB,E,CL,CF,CFM, S,LP,TE,T	
23	CB,E,CL, CF, AM,N,S,LP	
24	CB,E,CL,K,S, LP	
25	CB,E,CL,N,S,LP	
26	CB,E,CL,K,N,S,LP	
27	CB,E,CL,DF,CF,AM,AMC	
28	CB,E,CL,DF,CF,CFM,AM,AMC	
29	CB,E,CL,NA,CF,AMC,K,S,LP,C,FF,TE,T,	
30	CB,E,CL,CF, AM,AMC,GM,FOSFO,LP,C, TE,T,SXT	

Table 4. Drug resistance patterns among 32 ostrich *Escherichia coli* isolates to six commonly used antibacterials in Iranian poultry industry. (a)For abbreviation of drugs refer to table 3.

Pattern (#)	Resistance to(a)	No. of isolates (%)
1	None of drugs tested	11 (34.5)
1	TE	13 (40.6)
2	LP	4 (12.5)
3	LP, TE	2 (6.2)
4	LP,FF,TE	1 (3.1)
5	LP, TE, SXT	(۱.٤) ۱

contamination after oviposition; therefore, strict hygienic measures should be taken from nests to hatcheries.

Antimicrobial resistance among bacteria isolated from food animals is a matter of concern to public health and animals as well. Investigations on *E. coli* isolates from poultry have shown increased resistance to antimicrobials (Khoshkhoo and Peighambari, 2005; Li et al., 2010). Johnson et al. (2007) demonstrated a close similarity between resistant human and poultry *E. coli* isolates. They postulated that resistant human isolates have been derived from poultry isolates, whereas resistant poultry isolates have been originated from suscept-ible poultry isolates.

High rates of antimicrobial resistance among *E. coli* isolates from poultry origin have been reported in recent years in Iran (Khoshkhoo and Peighambari, 2005; Moniri and Dastehgoli, 2007). In this study, a high percentage of isolates were resistant to ampicillin (62.5%), amoxi-clav (75%), carbenicillin (100%), erythromycin (100%), and colistin (93.7%), which verifies the findings of *E. coli* isolates from poultry in Iran (Khoshkhoo and Peighambari, 2005; Moniri and Dastehgoli, 2007). The use of ampicillin, amoxi-clav, and carbenicillin is uncommon in Iranian poultry industry; however, erythromycin and colistin are relatively used. Interestingly, unlike the high resistance rate that has been observed in commonly used antimicrobials such as enrofloxacin, flumequine, lincospectin, florfenicol, and trimethoprim+ sulfa among poultry *E. coli* isolates (Khoshkhoo and

Peighambari, 2005; Moniri and Dastehgoli, 2007), our *E. coli* isolates from ostriches demonstrated a high rate of susceptibility to these antimicrobials. The high frequency of resistance to ampicillin, amoxi-clav, and oxytetracycline among *E. coli* isolates of ostrich origin has been reported previously (Cabassi et al., 2004). Almost half of Cabassi et al.'s isolates were resistant against enrofloxacin, amoxi-clav, and oxytetracycline. However, in the current study, all *E. coli* isolates were susceptible to enrofloxacin, and 21.8% and 75% were resistant against oxytetracycline and amoxi-clav, respectively. In another study, Carneiro et al. (2010) found 16.6% resistance to ampicillin and 1.8% resistance to amoxicillin-clav among ostrich fecal *E. coli* isolates.

Drug resistance patterns may differ from one place to another and from time to time due to different prophylactic and/or therapeutic usage of antimicrobial agents. However, they may be useful markers among bacterial isolates from the same environment. The resistance pattern may reflect the extensive use of drugs in the poultry industry and the selection of resistant isolates over time. It was not surprising that a significant number of ostrich *E. coli* isolates were resistant to penicillin, amoxi-clav, carbenicillin, erythromycin, colistin, and tetracycline. The first five agents are generally ineffective against *E. coli*, and tetracycline resistance among *E. coli* is now widespread (Peighambari et al., 1995; Ley et al., 2001; Khoshkhoo and Peighambari, 2005). Resistance to these agents may be an indicator of long-term use in the ostrich industry or of reasons other than drug use. The latter could occur if genes for drug resistance become associated with genes for some other property, which confers an advantage to the strain.

Susceptibility of 32 *E. coli* isolates from ostriches to 27 antimicrobial agents provided 30 patterns. However, when the susceptibility of 32 *E. coli* isolates to six commonly used antibacterials in Iranian poultry industry (enrofloxacin, flumequine, lincospectin, florfenicol, and trimethoprim+sulfa, tetracycline) was analyzed; only six patterns were observed. Cabassi et al. (2004) detected 15 different resistance patterns among ostrich *E. coli* isolates. They attributed the presence of different patterns, among the same bacterial species and the same herd, to the different sources of the birds.

Based on OIE list of veterinary critically important antimicrobials (VCIA), kanamycin and neomycin usage is restricted in veterinary medicine, and in this research only %6.2 of isolates were susceptible to each of kanamycin and neomycin.

Increased MDR has been reported in *E. coli* isolates in many countries including Iran (Cabassi et al., 2004; Khoshkhoo and Peighambari, 2005; Ozawa et al., 2008; Carneiro et al., 2010; Li et al., 2010). This situation in avian origin *E. coli* emphasized the importance of antimicrobial susceptibility tests to select an efficient antimicrobial agent against this important pathogen. In our study, all *E. coli* isolates demonstrated the MDR pattern and the number of antibacterial agents varied between 2 to 12 among MDR types, which was similar to our previous work on poultry *E. coli* (Khoshkhoo and Peighambari, 2005). The wild birds are able to act as a carrier of MDR types of *E. coli* isolates and spread these bacteria via feces. Guenther et al. (2010) studied 187 *E. coli* isolates from the feces and internal organs of wild birds, and it was revealed that 8% of isolates were multi-drug resistant. The ostrich breeding farm is an open system, so the contact with wild birds and their feces, in addition to the increased risk of diseases transmission, could act as a potential source of antimicrobial resistant isolates.

MDR bacterial isolates of animal origin may spread into human population by direct contacts or through animal-origin foods (Soulsby, 2008). These resistant bacteria may be colonized in the human intestinal tract, and the genes coding for antibiotic resistance can be transferred to the bacteria of natural micro flora or pathogenic bacteria. The resistance bacteria that are thrown in the environment may infect animals and then through food chain return to humans (Hawkey, 2008).

In conclusion, this study demonstrated the bacterial, particularly *Escherichia coli*, contamination of dead-in-shell ostrich embryos and antimicrobial resistance status of the *Escherichia coli* isolates from ostrich eggs in Iran. It appears that to reduce the bacterial contamination of ostrich fertile eggs, the breeders should pay sufficient attention to the sanitary conditions in ostrich breeder flocks and the related hatchery facilities. The findings of the present study are signifacant for Iranian ostrich industry.

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آلودگی باکتریائی تلفات جنینی در جوجه کشی های شترمرغ و الگوی مقاومت ضد میکروبی اشریشیا کلی جدا شده

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چکیده

زمینه مطالعه: رایج ترین عامل تلفات جنینی در جوجه کشی های شترمرغ آلودگی باکتریایی تخم های نطفه دار می باشد که به زیان اقتصادی این صنعت منجر می شود. **هدف:** هدف از این مطالعه بررسی وضعیت آلودگی باکتریایی، علی الخصوص آلودگی به اشریشیا کلی، در جوجه کشی های شترمرغ و تعیین الگوی مقاومت جدایه های آن نسبت به ترکیبات ضد میکروبی بود. **روش کار:** بدین منظور تعداد ۱۲۰ تخم شترمرغ بارور با جنین های تلف شده، به صورت هفتگی از سه مرکز جوجه کشی جمع آوری شدند. بعد از انتقال به آزمایشگاه و در شرایط استریل از اندام های مختلف جنین ها نمونه برداری شد. جداسازی و شناسایی جدایه های باکتری بر اساس روش های استاندارد باکتری شناسی و بیوشیمیایی صورت گرفت. آزمون تعیین حساسیت بر علیه ترکیبات ضد میکروبی به روش دیسک دیفوزیون در برابر ۲۷ ترکیب دارویی صورت گرفت. **نتایج:** از ۵۶ (۴۶/۷٪) مورد بیش از یک جنس باکتری جداسازی شد. تعداد ۲۴ عدد از تخم های شترمرغ به اشریشیا کلی آلوده بودند که در برخی از نمونه ها علاوه بر کیسه زرده از مکونیوم، کبد یا خون قلب جنین تلف شده نیز جداسازی شد که تعداد مجموع جدایه های اشریشیا کلی را به ۳۲ افزایش داد. تمامی جدایه های اشریشیا کلی به دانوفلوکساسین، فلومکوئین، تریمتوپریم +سولفامتوکسازول حساس بودند در حالیکه همگی به کربنی سیلین و اریترومایسین مقاوم بودند. وضعیت مقاومت جدایه ها در برابر سایر ترکیبات متفاوت بود. مقاومت چندگانه در تمامی جدایه ها مشاهده شد که شامل ۲ تا ۱۲ ترکیب دارویی بود. در مجموع در بین ۳۲ جدایه باکتری، ۳۰ الگوی مقاومت دارویی متفاوت بر علیه ۲۷ ترکیب مشاهده شد. **نتیجه گیری نهایی:** تحقیق موجود نخستین بررسی جامع در مورد وضعیت آلودگی باکتریایی جنین های تلف شده در جوجه کشی های شترمرغ و وضعیت مقاومت جدایه های اشریشیا کلی بدست آمده در برابر ترکیبات ضد میکروبی در ایران بود.

واژه های کلیدی: اشریشیا کلی، شترمرغ، مقاومت ضد میکروبی، جوجه کشی، تلفات جنینی

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