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CHARACTERIZATION OF SOME AEROBIC BACTERIAL MICROORGANISM ISOLATED FROM NEWLY HATCHED IMPORTED DUCKLINGS

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ABSTRACT: This study was carried out to determine the occurrence of some important aerobic bacteria in populations of imported newly hatched ducks. Bacterial examination of 2790 samples, collected from 62 flocks of imported duck resulted in isolation of 4 (6.45%) salmonella strains, 7 (11.3%) *E.coli* strains and 15 (24.2%) isolates *Staph.aureus*. Serological identification of the isolated Salmonella strains revealed that they were belonged to S. Derby, S. Newport, S. Togo and S. Ball while, the *E.coli* strains were serogrouped as O15, O169 and untypable. Antimicrobial pattern of isolated bacteria were studied using disc diffusion method. All examined samples were subjected to molecular detection using PCR to record the difference between traditional methods of isolation with the molecular technique. As well as P.M lesions were observed in some examined flocks. This data focusing on the role of imported ducks in introducing the risk of infectious agents as different serovars of Salmonella, E.coli and S.aureus to the country.

Key Words: Ducks, Salmonella, E.Coli , S.Aureus , Sensitivity Test, PCR Detection.

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INTRODUCTION

Duck industry in Egypt and other countries in the world are exposed to many diseases affecting growth rates and production particularly bacterial infections [1]. Salmonellosis is one of the most wide spread food-borne zoonoses in industrialized as well as developing countries [2]. Although ducks are very resistant to systemic infection caused by Salmonella, they are potential reservoirs of this organism and may shed it in the feces, contaminating the environment [3]. Infections with Salmonella are responsible for a variety of acute and chronic diseases in poultry [4]. Outbreaks of human salmonellosis caused by contact with ducks have been reported in some countries, such as Australia, United States, United Kingdom and Denmark [5, 6, 7, 8].

Escherichia coli is one of the most common microorganisms, which affect both animals and humans worldwide by a wide spectrum of diseases ranging from opportunistic wound infection to severe systemic infections. The zoonotic potential, complicated antigenic structure and toxins give importance to E. coli in prophylaxis and treatment regimens [9]. Epidemiological tracing of E. coli strains is of considerable importance in veterinary microbiology. The data can be used to monitor trends in the occurrence of pathogenic strains or to identify possible source of infection. Autologous bacterins provide limited serotype-specific protection, because multiple serogroups are associated with disease [10].

Staphylococcus aureus is an important bacterial cause of disease in poultry. It can be involved in a wide range of clinical conditions such as septicemia, bone and joint infections, abscesses and dermatitis [11]. Although the mechanism of spread of S. aureus infection through poultry flocks is not fully understood, the hatchery played an important role in the spread of infection to rearing farms [12]. The developed resistance to most classes of antimicrobial agents at S.aureus was recorded for example Penicillin was the first choice of antibiotic for treatment staphylococcal infection in 1944, by destroying penicillin by penicillinase, S.aureus become resistant [13]. Also, the effectiveness of currently available antibiotics is decreasing due to the increasing number of resistant strains causing infections [14].

In particular, culture could recognize viable organisms only, while amplification tests are not dependent on viable or structurally intact cells and the presence of DNA was sufficient to yield a positive result. Thus, the potential for detecting non-viable microorganisms explained the discrepancies between PCR and culture results following antibiotic therapy [15]. The use of PCR in routine testing is reduces the time required to obtain results [16]. The phoA housekeeping gene, which is present in all E. coli strains, was used to detect E. coli in different samples by PCR [17, 18, 19]. The invA gene was used as a necessary gene for the invasion to the host cell [20, 21]. Although the use of invA primer due to its accuracy and uniform Distribution among Salmonella, which increase sensitivity of the test and detected Salmonella within maximum time 12h [22, 23, 24]. The S. aureus specific clfA gene, encoding a surface associated fibrinogen binding protein [25].

The aim of the present study was to report the occurrence of Salmonella, E.coli and S. aureus in one day old ducklings imported by Egyptian companies during 2013-2014.

MATERIALS AND METHODS

Bacterial Isolation

Examination of 2790 duckling samples, which collected from 62 imported duck flocks, per each flock examined 15 ducklings pooled in three different samples (internal organs "liver, heart and lung, yolk

sac and paper lining duckling box). Ducklings which were submitted to reference laboratory for veterinary quality control on poultry production from 2013-2014. All samples were examined bacteriologically for presence of Salmonella, E .coli and S.aureus. Isolation and Identification of Salmonella, E .coli and coagulase positive Staphylococci were done according to standard methods ISO 6579 [26]; Lee and Arp [27] and ISO 6888-1 [28] respectively.

Serological identification of Salmonella was done according to Popoff [29] and serological typing of E.coli was carried out according to Lee et al. [30] by using Known antisera for each organism (Denka Seiken)

Antimicrobial sensitivity test

The antibiogram of Bacterial Isolates were done by disc-diffusion test according to Koneman et al. [31] for isolates of Salmonella and E.coli against 10 antibiotics, while S. aureus strains were tested against 12 antibiotics (Oxoid) interpretation according to the Clinical and Laboratory Standards Institute/ Formerly National Committee for Clinical Laboratory Standard CLSI/NCCLS, [32].

Conventional PCR technique:

Extraction:

DNA of enriched samples was extracted using commercially available kit, QIAamp DNA Mini Kit, Catalogue no.51304.

PCR amplification:

phoA, invA and clfA genes were amplified according to refernces mentioned in Table (1). Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of template. The reactions were performed in a Biometra T3 thermal cycler.

Analysis of the PCR Products:

The products of PCR were separated by electrophoresis on 1 % agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the PCR products were loaded in each gel slot. A 100 bp and 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

RESULTS

Four Salmonella isolates were detected in internal organs with percentage 6.45%. Seven E. coli strains were isolated from internal organs and yolk sac with percentage 11.3%. Also, 15 coagulase positive Staphylococcus (S.aureus) isolates (24.2%) isolated from both yolk sac and internal organs.

Serotyping of Salmonella isolates revealed the isolation of S.Derby (4,12; f,g; —), S.Newport (6,8,20; e,h; 1,2), S.Togo (4,12; l,w; 1,6) and S.Ball (4, 12, 27; y; e,n,x) were identified according to their serotyping formula as shown.

Serotyping of E. coli showed two isolates O15, one isolate O169 while 4 isolates were untypable.

Antimicrobial sensitivity test

As shown in Table No. (2) Four Salmonella isolates illustrated that the isolates were highly resistance to ciprofloxacin and nalidixic acid with percentage 75% for each. But, highly sensitive to Gentamycin, Amoxicillin + Clavulanic acid, Norfloxacin, Chloramephenicol, Tetracycline and Trimethoprim-sulfamethoxazole with percentage 100%.

Seven E.coli isolates illustrated that the isolates were highly resistance to Tetracycline and Trimethoprim-sulfamethoxazole and nalidixic acid with percentage 57% then ciprofloxacin and Nalidixic acid with percentage 43%. While, Amoxicillin + Clavulanic acid showed highly sensitivity 100% then Gentamycin, Chloramephenicol, Streptomycin, Norfloxacin, Ciprofloxacin, Nitrofurantoin,

Trimethoprim-sulfamethoxazole and Nalidixic acid with percentage 86, 86, 71, 57, 57, 57, 43 and 43% respectively. antibiotic testing of the Fifteen S.aureus isolates illustrated that the isolates were highly resistance to Oxacillin with percentage 100% then Erythromycin (85.7%), penicillin (73.3%), Trimethoprim-sulfamethoxazole (53.3%), Tetracycline (46.7%). While, highly sensitive Amoxicillin + Clavulanic acid 100%, then Amikacin, Norfloxacin, Gentamycin, Ciprofloxacin, Doxycyclin, Ofloxacin and Tetracycline with percentage 73.3, 73.3, 60, 60, 60, 46.7 and 46.7%, respectively. While, Penicillin showing intermediate resistance with percentage 33.3% followed by Gentamycin and Amikacin (20%) for each but the last resistance were detected against Doxycycline (6.7%).

Conventional PCR technique:

The investigating invA, clfA and phoA for different examined organisms by PCR technique was shown in Table 3 and Fig. 1

The necropsy findings have been reported to be included omphalitis, yolk sac infection, enteritis and congestion of liver as shown in Fig 2, 3 and 4 (represented demonstration to some observed P.M lesions).

DISCUSSION

Salmonella enterica is a zoonotic organism which can acquire its resistance in livestock that resulting animal food products were important vectors for the transfer of resistant bacteria from animals to humans [36]. In fact, Salmonella prevalence in hatcheries has been estimated between 20 and 60% for ducks [37]. In the present study Four Salmonella isolates were detected in internal organs with percentage 6.45%. Serotyping of Salmonella isolates showing S.Derby, S.Newport , S.Togo and S.Ball. These results were agreed with [38] who isolated Salmonella from ducks in percentage 6.7% from 143 collected duck samples during 1962- 1991. The obtained results were disagreed with [39] who isolated Salmonella in rate 65% of duckling flocks from 1998- 2003, Also [40, 41] where they isolated Salmonella in rate of 19.3% and 18.5%, respectively. Regarding to the isolated Salmonella serotypes the results were disagree with [41] and partial agree with [39] who isolated S. Saintpaul, S. Kottbus. and S.Newport from duckling flocks from 1998- 2003 in Brazilian.

The emergence of resistance to fluoroquinolones is of particular concern, because this class of antimicrobial agents constitutes the ‘drug of choice’ for treating potentially life threatening Salmonella infections caused by multiple antibiotic-resistant strains [42, 43].

In our study Salmonella isolates were highly resistance to ciprofloxacin and nalidixic acid (quinolone) with percentage 75%. [44] and [45] focused in the decades following the licensing of fluoroquinolones, an increased prevalence of quinolone resistant salmonella has been observed in clinical. our result were supported by [41] who reported that most of Salmonella isolated from imported ducks were resistant to six different antimicrobial groups including fluoroquinolones.

Colibacillosis causes high morbidity and mortality throughout the life span of poultry from an egg to an adult bird and constantly results in huge economic losses [46, 47]. In the past few years, both the incidence and severity of colibacillosis have increased rapidly and current trends indicate that it is prevail continue and has even greater problem in the poultry industry [48, 49].

Bacteriological examination in the present study revealed that 7 E. coli isolates were present in internal organs and others in yolk sac with percentage 11.3%. These results in accordance with [50] who isolated E.coli with a percentage of 11% from duckling have omphalitis. In addition to, [51] and [1] reported the isolation of E.coli with a percentage of 27.3% from duckling in Egypt. The isolation of E.coli

from duckling indicated egg transmission of E.coli.

Serotyping of E. coli showed two isolates O15, one isolate O169 but 4 isolates were untypable. In the present studies the percentage of not typed because of antiserum un availability E.coli strains. This nearly agreed with studies of [52] and [53], who found that the large percentage of untyped because of antiserum un availability E.coli strains was common characteristics of all groups of E.coli recovered from avian colibacillosis regardless of geographic location. Also, [54] reported 20 strains untypable from duck E.coli isolates. Our serotyping was agree with [55] and [56] who recorded common pathogenic E.coli strains in poultry was O78, O1 and O2, and to some extent O15 and O55.

The increasing use of antibiotics for prophylactic, therapeutic and nutritive purposes in veterinary medicine creates a potentially powerful selective pressure for the spread of antibiotic resistance [57]. However, since January 2006, all growth promoters in the feed have been forbidden in the European Union [58].

Our results obtained in the antibiotic sensitivity test revealed variable resistance and sensitivity and this was in accordance with [59] and [51] who mentioned that antimicrobial agents were of considerable importance against E.coli infections. However, resistance has developed to some antimicrobial agents, which stimulated us to study the antibiotic resistance of E.coli strains of avian origin in Egypt. Moreover, [60] mentioned that all the isolated E.coli showed multi resistance when they were tested against 8 antibiotic groups.

S. aureus is a bacterial pathogen in a variety of infectious diseases in both humans and animals [61]. A reliable and rapid identification of S. aureus colonies from samples is a cornerstone in the control of S. aureus infection. Identification of bacterial pathogens still relies mainly on phenotypic criteria [62].

In our study 15 coagulase positive Staphylococcus (S.aureus) isolates (24.2%) were positive mainly from yolk and internal organs together. Confirmed by [63] isolated 18% S.aureus associated with arthritis in duck. In our results S.aureus isolates were highly resistance to Oxacillin with percentage 100% then penicillin (73.3%), Trimethoprim-sulfamethoxazole (53.3%), Tetracycline (46.7%). While, Amoxicillin + Clavulanic acid showed highly sensitivity 100%, then Amikin, Norfloxacin, Gentamycin, Ciprofloxacin, Doxycyclin, Ofloxacin and Tetracycline with percentage of 73.3, 73.3, 60, 60, 60, 46.7 and 46.7%, respectively. This nearly agreed with [64] who examined 20 Omphalitis cases in ducklings caused by S.aureus and reported that the antibogram showed highly sensitive to Ciprofloxacin and Gentamicin. While, moderately sensitive to Ofloxacin but were resistant to Sulphamethizole. Also, [65] showed the susceptibility testing of 15 isolated S. aureus strains which were resistant to erythromycin, tetracycline, and trimethoprim, but all strains were susceptible to chloramphenicol, ciprofloxacin. While, [66] stated that 100% resistance to ciprofloxacin among S. aureus on poultry farms in Malaysia and revealed 100% susceptibility towards clindamycin, erythromycin, gentamicin, trimethoprim-sulfamethoxazole and penicillin. On the other hands, [62] recorded high resistance was among the examined S. aureus isolates to amoxycillin, amoxicillin clavulanic acid and gentamicin (66.7% each). Also, [67] did not find oxacillin-resistant S. aureus.

This variation of antimicrobial resistance in staphylococci of poultry origin indicated the misuse of antibiotics in field which increase its risk on population.

The results of necropsy observed in this study are in conformity with the earlier

reports of [68] and [69], in addition recently with that of [50] and [1].

In vitro amplification of DNA by PCR method is a powerful tool in microbiological diagnostics [70]. Several genes have been used to detect salmonella from different samples as invA gene [71]. In our study the PCR test which done from the enriched samples detected 4/62 positive salmonella 19/62 E.coli and 11/62 S. aureus which revealed after 24 h rather than traditional method of detection 4-7 days. In most laboratories, the accurate assessment of these issues is dependent on the phenotypic characterization of cultured bacteria. However, there are numerous reports describing the use of PCR for the identification and characterization of staphylococcal isolates [72, 73 and 74]. To maximize sensitivity, most protocols focused on amplification of conserved regions of bacterial genes however Many authors as [75], [76], [77] and [78] reported that an enrichment broth accompanied with PCR increase positive samples and dilute substances which inhibits the test and increase the viability of the organism.

CONCLUSION

Occurrence of pathogenic bacteria in apparently health duckling which showed resistance pattern to antibiotic, inducted the increasing transmition probability of this pathogens with its resistance activity from parents to duck through eggs. Small difference between phenotypic and genotypic indicates the high sensitivity of molecular methods.

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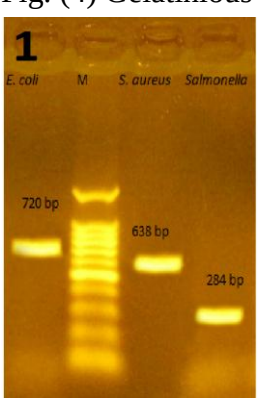
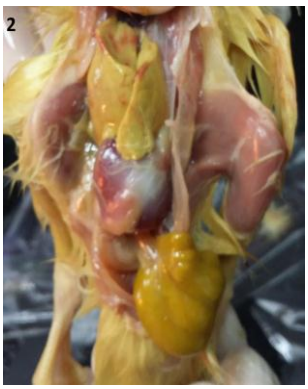
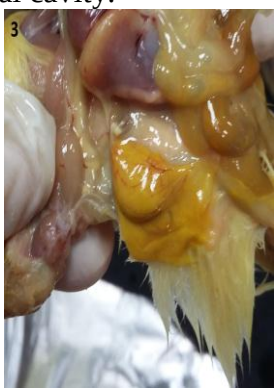
Table (1): Sequences of primers and the size of amplified products required for detecting the tested genes.

Target gene	Primer sequence (5'-3')	Amplicon (bp)	References
E. coli phoA	F: CGATTCTGGAAATGGCAAAAG R: CGTGATCAGCGGTGACTATGAC	720	[33]
S. aureus clfA	F: CAAAATCCAGCACAACAGGAAACGA R: CTTGATCTCCAGCCATAATTGGTGG	638	[34]
Salmonella invA	F: GTGAAATTATCGCCACGTTCGGGCAA R: TCATCGCACCGTCAAAGGAACC	284	[35]

Fig. (1): Represented positive amplification bands for the examined organisms by conventional PCR test.
Fig. (2) Unabsorbed yolk sac with congested liver.
Fig. (3) Omplilitis (congestion in yolk sac).
Fig. (4) Gelatinious material in the abdominal cavity.







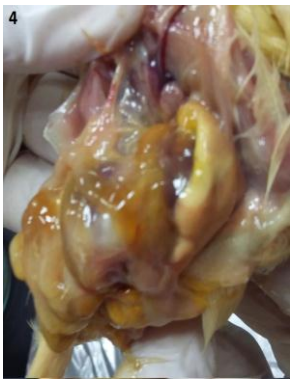


Table (2): Results of antimicrobial sensitivity tests.

Antimicrobial Discs	interpretation	Salmonella N=4			E.coli N=7			S.aureus N=15		
	Zone diameter (mm)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Amoxicillin + Clavulinic acid. Am+CL 20-10	13,(14-17),18 [a] 19,(—), 20 [b]	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	7 (100)	0 (0)	0 (0)	15 (100)
Ciprofloxacin. CF5	15,(16-20),21 [a,b]	3 (75)	0 (0)	1 (25)	3 (43)	0 (0)	4 (57)	0 (0)	6 (40)	9 (60)
Norfloxacin. NX10	12,(13-16),17 [a,b]	0 (0)	0 (0)	4 (100)	3 (43)	0 (0)	4 (57)	0 (0)	4 (26.7)	11 (73.3)
Gentamicin. G10	12,(13-14),15 [a,b]	0 (0)	0 (0)	4 (100)	1 (14)	0 (0)	6 (86)	3 (20)	3 (20)	9 (60)
Tetracycline. T30	11,(12-14),15 [a] 14,(15-18),19 [b]	0 (0)	0 (0)	4 (100)	4 (57)	3 (43)	0 (0)	7 (46.7)	1 (6.6)	7 (46.7)
Trimethoprim-sulfamethoxazole. SXT1.25-23.75	10,(11-15),16 [a,b]	0 (0)	0 (0)	4 (100)	4 (57)	0 (0)	3 (43)	8 (53.3)	4 (26.7)	3 (20)
Chloramphenicol. C30	12,(13-17),18 [a]	0 (0)	0 (0)	4 (100)	1 (14)	0 (0)	6 (86)	-	-	-
Nalidixic acid. NA30	13,(14-18),19 [a]	3 (75)	0 (0)	1 (25)	4 (57)	0 (0)	3 (43)	-	-	-
Nitrofurantoin. F300	14,(15-16),17 [a]	0 (0)	1 (25)	3 (75)	2 (29)	1 (14)	4 (57)	-	-	-
Streptomycin. S10	11,(12-14),15 [a]	1 (25)	0 (0)	3 (75)	1 (14)	1 (14)	5 (71)	-	-	-
Penicillin. P10	28,(—),29 [b]	-	-	-	-	-	-	11 (73.3)	0 (0)	4 (26.7)
Doxycycline. DO30	12,(13-15),16 [b]	-	-	-	-	-	-	1 (6.7)	5 (33.3)	9 (60)
Erythromycin. E15	13,(14-22),23 [b]	-	-	-	-	-	-	6 (85.7)	0 (0)	1 (14.3)
Ofloxacin. Of 5	14,(15-17),18 [b]	-	-	-	-	-	-	5 (33.3)	3 (20)	7 (46.7)
Oxacillin. O1	10,(11-12),13 [b]	-	-	-	-	-	-	15 (100)	0 (0)	0 (0)
Amikacin. Ak30	14,(15-16)17 [b]	-	-	-	-	-	-	3 (20)	1 (6.7)	11 (73.3)

a: Enterobacteriacae (Salmonella and E.coli), b: Staphylococci , R:resistant, I: Intermediate, S: Sensitive.

Table (3): The result of frequency of isolation and PCR for the examined samples.

Organism	Frequency of isolation	PCR
Salmonella	4/62 (6.45%)	4/62 (6.45) %
E.coli	7/62(11.3%)	19/62 (30.6) %
S. aureus	15 /62 (24.2%) Coagulase positive staphylococci	11/62 (17.7)%

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الملخص العربى

توصيف بعض الميكروبات الهوائية المعزولة من كتاكيت البط المستورد حديثة الفقس

هبه بدر ,منى على عبد الرحيم, ايمان محمد فرغلى, ازهار جابر, هبه رشدى و سعاد عبد العزيز ناصف

هذه الدراسة قد أجريت لتحديد تواجد بعض انواع البكتيريا الهوائية المهمة في البط المستورد حديثة الفقس. أسفر الفحص البكتيري من ٢٧٩٠ عينة تم جمعها من ٦٢ مجموعة من البط المستوردة في عزل ٤ (٦,٤٥٪) ميكروب السالمونيلا، ٧ (١١,٣٪) لميكروب الايشريشيا كولاى و ١٥ (٢٤,٢٪) معزولة من ميكروب الاستاف اورويس . تم توصيف المعزولات سيرولوجيا كشف من السالمونيلا S.Derby، S.Newport، S.Togo، S.Ball بينما كانت سلالات ، ميكروب الايشريشيا كولاى O15، O169 وسلالات غير مصنفة. تم دراسة حساسية الميكروبات المعزولة للمضادات الحيوية المختلفة باستخدام طريقة الانتشار القرصي. تم إخضاع جميع العينات المفحوصة إلى الكشف الجزيئي باستخدام اختبار تفاعل البلمرة المتسلسل لتسجيل الفرق بين الطرق التقليدية في عزلة مع التقنية الجزيئية. فضلا عن ان الفحص الداخلى للكتاكيت اظهرت صفات مميزة في بعض القطعان التي تم فحصها. هذه البيانات مع التركيز على البط المستوردة تبين انها تمثل مخاطر فى إدخال العوامل المعدية إلى البلاد، بما في ذلك سلالات مختلفة من السالمونيلا، ميكروب الايشريشيا كولاى وميكروب الاستاف اورويس.