MULTIDRUG RESISTANT BACTERIAL PATHOGENS IN EGGS COLLECTED FROM BACKYARD CHICKENS

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ABSTRACT

A total of two hundred eggs collected from backyard balady chickens were examined for the presence of Salmonella spp., E. *coli*, and Coagulase Positive Staphylococci. Out of the total examined samples, no Salmonella was detected. A total of 36 E. *coli* strains were isolated with an overall isolation rate of 18%. Eighty (40%) Coagulase Positive Staphylococci isolates were detected. Serotyping of E.*coli* isolates revealed the predominance of O 27 at a rate of 22% among the 13 identified serotypes. However, 5 (14%) of the E. *coli* isolates were untypeable. The antibiotic susceptibility pattern of Coagulase Positive Staphylococci was studied against 15 different chemotherapeutic agents. The highest resistance rates were detected against oxytetracyclin, oxacillin and trimethoprim sulphamethazole with resistance rates of 90%, 86.7% and 86.7%, respectively. The highest sensitivity rate was detected towards ampicillin with a percentage of 87%. E. *coli* isolates were tested for its susceptibility against 14 different antibiotics, the highest resistance rates were recorded against trimethoprim -sulphamethazole, doxycycline, tetracycline and amoxycillin with resistance rates 94.1%, 93.2%, 92.9%, and 92.3%, respectively. While the highest sensitivity rates were detected for colistin, cefotaxim, gentamycin and ciprofloxacin with sensitivity rates of 94.4%, 76.5% 59.8% and 55.6%, respectively. The antibiotic susceptibility results were judged by PCR, for E. *coli* strains, the bla_{TEM}, *sul*1 and *tet*A(A) genes were tested for the ß-lactams, trimethoprim sulphamethazone and tetracyclines, respectively. While the *mec*A, *bla*Z and *tet*K genes were tested for the evaluation of the susceptibility of Coagulase Positive Staphylococci against oxacillin, ß-lactams, and tetracyclines, respectively. E. *coli* virulence was tested by PCR through the testing of *iss*, *eae*A, *stx*1, *stx*2, *tsh* and *pap*C genes. While the virulence of Coagulase Positive Staphylococci was estimated through testing of the enterotoxins genes in addition to coagulase, *spa* and *hlg* genes.

***Key words**: MDR bacteria, E.coli, PCR, balady chicken eggs.*

INTRODUCTION

Table eggs are consumed worldwide and are considered the most nutritious inexpensive source of protein that can be part of a healthy diet. However poultry may carry bacteria that can cause illness, infected birds do not usually appear sick and even unbroken clean fresh shell eggs may contain harmful bacteria. (Barbara and RON, 2010).

There is now considerable evidence that transfer of antimicrobial resistance from food-producing animals to humans directly via the food chain is a likely route of spread, transmission by direct handling or close contact between infected animals and humans, transmission via contaminated animal products, particularly but not exclusively food products. The World Organisation for Animal Health **(OIE)** has developed a list of antibiotics categorized by the need for their use in animal treatment; The category 'veterinary critically important antimicrobials' includes fluoroquinolones, cephalosporins and macrolides , as well as a number of other families of antibiotics. Thus these antibiotics may all affect bacteria in both animal and human treatment settings (Wooldridge, 2012).

Escherichia *coli* is one of the common microbial flora of gastrointestinal tract of poultry and human, resistant E. *coli* strains from the gut often cause contamination of eggs during lay with multi resistant E. *coli* (Turtura *et al*., 1990).

Egg-associated Salmonellosis is a public health problem, the use of antibiotics in animals disrupts normal flora of intestine, resulting in emergence of antibiotic-resistant Salmonellae and their prolonged

fecal shedding into the environment (Ahmed *et al.*, 2011).

Staphylococcus is considered to be a normal flora of chickens, isolated from the skin and feathers as well as in the respiratory and intestinal tracts (Casey *et al.*, 2007). However, some of the common forms of Staphylococci are associated with poultry infections. Increasing attention has been given to the role of poultry and poultry products, including eggs, as a potential source of infections in humans induced by antibiotic-resistant Staphylococcus strains (Abulreesh and Organji 2011).

Backyards flocks are reared under limited or no veterinary supervision. In such production systems, antimicrobials are freely used as feed or water additives (Otalu *et al.,* 2011). These practices can facilitate the emergence and spread of antibiotic resistant pathogens among birds with possible transmission to humans. Backyard chickens are extensively reared in close proximity to human dwellings and therefore play an important role in environmental contamination, in addition to serving as significant vehicles for the transfer of pathogens to humans by way of handling of live birds or consumption of contaminated meat and other poultry products. (Suleiman *et al.,* 2013).

Thus the present study aimed to investigate the prevalence of multidrug resistant bacteria in egg produced from backyard chicken.

MATERIAL and METHODS

SAMPLING:
A total of two hundred eggs were collected from house hold backyard balady chicken from several villages in Sharkia governorate during summer 2014. Each egg was uniquely identified, and transported in a sterile plastic bag to the reference laboratory for veterinary quality control on poultry production, Sharkia branch, kept in refrigerator at 2-5 ˚C till examined.

Sample preparation:
From each egg, the contents were separated from the shell; Each of the contents and the shell was collected in a separate sterile flask. Non selective pre-enrichment was performed by adding BPW in 1:10 dilution rate, samples were well mixed and incubated at 37˚C±1 for 18 hours ±2.

Isolation and Identification:
Samples were examined for the detection of Salmonella Spp., Coagulase Positive Staphylococci and E.*coli* according to **ISO 6579:2002-COR 2004, ISO 6888-1:1999-AM:2003** and (Kreig *et al.,* 1984), respectively.

Serotyping: E. *coli* isolates were serotyped in Reference Laboratory for Veterinary Quality Control on Poultry Production using commercially available kits (Test Sera Enteroclon, Anti –Coli, SIFIN Berlin, Germany).

Antibiogram: Antibiotic sensitivity was performed using Mueller Hinton Agar plates (HIMEDIA) using antibiotic discs of 14 commonly used chemotherapeutic agents, for E.*coli* isolates, and 15 commonly used chemotherapeutic agents for Coagulase Positive Staphylococci isolates according to (Bauer *et al.,* 1966). Interpretation of the results based on the diameter of the inhibition zones produced was done according to (CLSI, 2011).

DNA extraction:
DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with few modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample was added to 20 µl of proteinase K and 200 µl of AL lysis buffer and incubated at 56^{o}C for 10 min in a Biometra Tsc thermal block. After incubation, 200 µl of 100% ethanol was added to the lysate and vortexed. The sample was then washed twice and centrifuged according to the manufacturer's instructions. DNA was eluted with 100 µl of elution buffer supplied in the kit.

Oligonucleotide Primers:
Different primers used in PCR were supplied from Metabion (Germany) and Biobasic (Canada) and are listed in Table (1) and Table (2).

PCR amplification:
A 25- µl master mix reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of 20 pmol conc. of each primer, 4.5 µl of water, and 6 µl of template DNA. The reactions were performed in Applied biosystem 2720 thermal cyclers.

Analysis of the PCR Products:
Fifteen microliters of each PCR product were loaded in each gel lane of 1.5% agarose gel (Applichem). Electrophoresis was done in 1x TBE buffer using 5V/cm gradients. A 100 bp DNA Ladder (Fermentas) was used to determine the fragment sizes. The PCR photos were photographed and analyzed by using a gel documentation system (Alpha Innotech, Biometra, Germany) through its computer software.

RESULTS

Prevalence of bacterial isolates:
Out of the total examined 200 eggs, no Salmonella was detected neither from on the shell nor from the contents. Regarding E.*coli* a total of 36 isolates were detected with an overall isolation rate of 18%. Out of total E.*coli* isolates, 21(10.5%) isolates were isolated from on the shell, a total of 9(4.5%) from the contents, and 6 (3%) from both shell and contents, respectively. Concerning the isolation rates of Coagulase Positive Staphylococci, a total of 80 (40%) isolates were detected; Of which, 29 (14.5%)

isolates were detected from the shell,15 (7.5 %) isolates from contents, and 36 (18%) isolates from both shell and contents. Out of total examined 200 eggs, 15 (7.5%) harbor both Coagulase Positive Staphylococci and E.*coli.*, as shown in "Fig.1",and "Table 3".

In the present study 36 E.*coli* were isolated out of total 200 examined eggs with an overall prevalence rate of 18%. Serotyping of the isolates by slide agglutination technique revealed the distribution of the detected isolates in 13 different serotypes, which belonged to 6 somatic "O" groups "2,3,4,5,6 ,and 8" . Five isolates belonged to Poly 2 were detected, of which 1 strain O91 isolated from on the shell, O 125 (3 isolates were detected, of which 2 isolates from on the shell, and 1 isolate from contents), respectively; And O 166 (1 isolate) from on the shell. Poly 3 ; O 145 (1 isolate) from on the shell. Poly 4 (14 isolates), of which 3 strains were O6 (2,1) from on the shell and contents, respectively; Eight isolates were identified as O27 (2,2, 4) from on the shell, contents, and from both on the shell and contents, respectively; Also 3 strains O 159 (2,1) from on the shell and contents, respectively. Poly 5 (2 isolates) 1 strain O 25, 1 strain O 153 both were isolated from on the shell. In addition to 4 strains identified as Poly 6 of which 1 strain O 115 from on the shell, 3 strains O 169 (2,1) from the contents and from both on the shell and contents, respectively. Finally 5 isolates were identified as Poly 8 of which 4 strains belonged to O 152(2,1,1) from on the shell, contents and from both on the shell and contents, respectively; One strain O 29 from on the shell. Five untypeable isolates were detected (4,1) from on the shell and contents, respectively as shown in Table 3".

Antibiotic susceptibility pattern of E.*coli* isolates was studied using agar disc diffusion technique against 14 commonly field used chemotherapeutic agents. The study detected the prevalence of multidrug resistant MDR E.*coli* ; As 34 (94.1%) of isolates were resistant to more than 5 chemotherapeutic agents, 11 (29.4%) of the isolates were resistant to more than 9 chemotherapeutic agents. The highest resistance rates were recorded against trimethoprim sulphate , doxycyclin, tetracycline, and amoxicillin; 94.1%, 93.2%, 92.9%, and 92.3 %, respectively. While the highest sensitivity rates were detected to colistin, cefotaxim, gentamycin, and ciprofloxacin; 94.4.%, 76.5%, 59.8%, and 55.6, respectively as shown in "Table 4", and "Fig.2".

Antibiotic susceptibility pattern of Coagulase Positive Staphylococci isolates was studied using agar disc diffusion technique against 15 commonly field used chemotherapeutic agents. The study detected the prevalence of multidrug resistant Coagulase Positive Staphylococci; As 72 (90%) of isolates were resistant to 3 and more chemotherapeutic agents, 29 (36.7%) of isolates were resistant against 9 and more chemotherapeutic agents. The most predominant resistance rates were recorded against oxytetracyclin (90 %), followed by trimethoprim-sulphamethazone, and oxacillin 86.7%, each. The least resistance rates were recorded against ampicillin, ciprofloxacin, and gentamycin; 13.1%, 26.7 %, and 30 %, respectively, as shown in "Table 5" and "Fig 3".

(Table 6) showing the relation between the PCR results of the different antibiotic resistance genes and antibiotic resistance profile of Coagulase Positive Staphylococci.

(Table 7) showing the PCR results of the different virulence genes of coagulase positive staphylococci; Positive results were detected for all genes except for *Sea* and *Sed* genes.

(Table 8) showing the relation between the PCR results of the different antibiotic resistance genes and antibiotic resistance profile of E. *coli*.

(Table 9) showing the PCR results of the different virulence genes of E. *coli*. All the genes were positively amplified in some isolates except for *stx*1 gene.

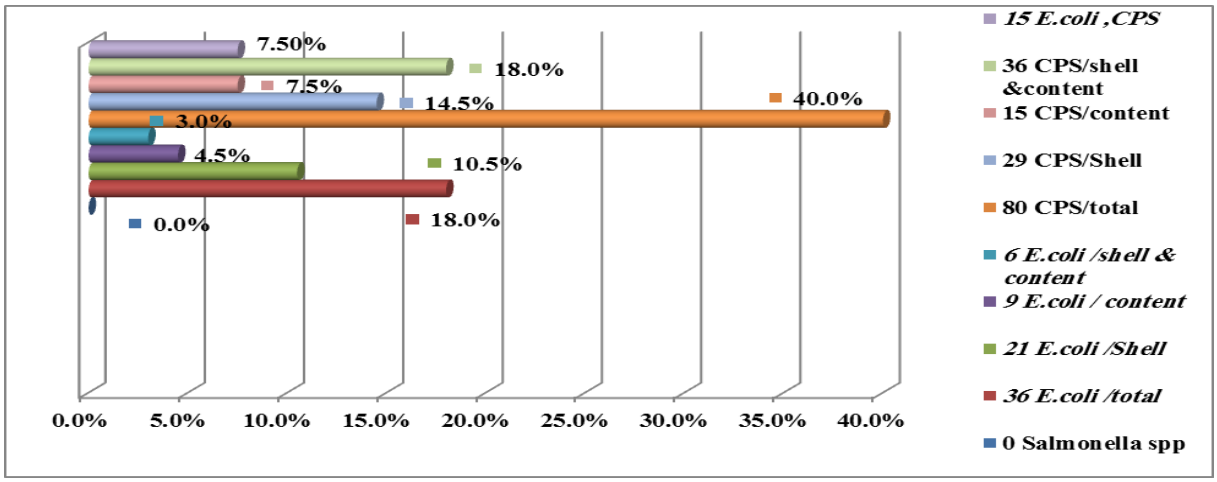

Figure 1: Prevalence rates of isolates.

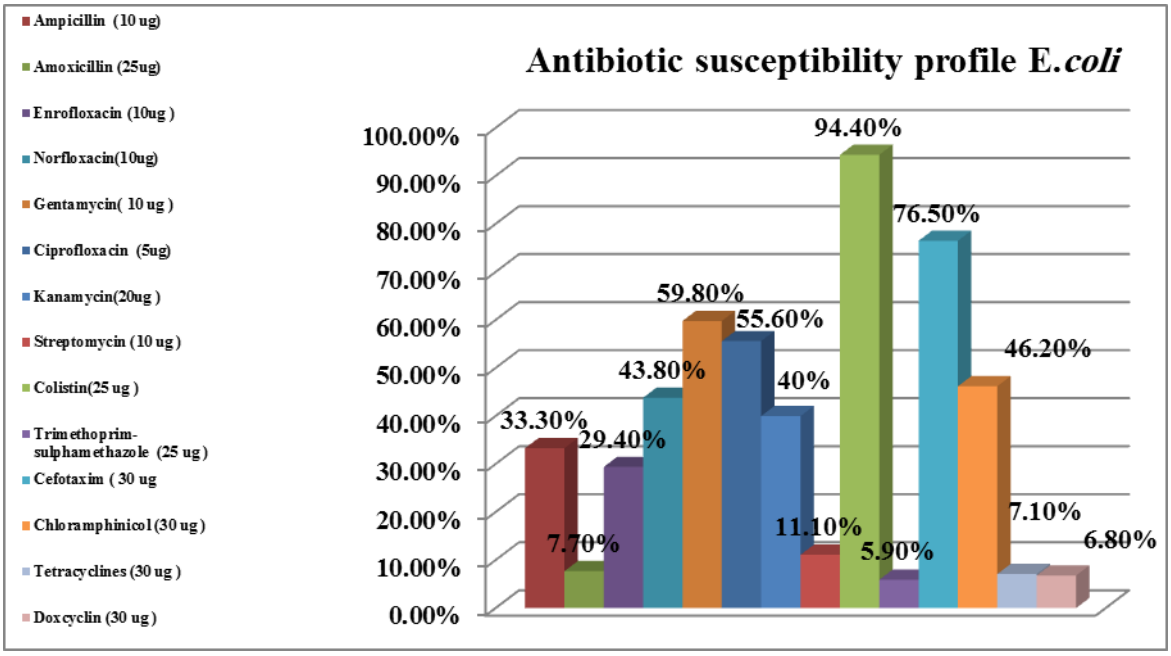

Figure 2: Antibiotic susceptibility profile of E.*coli* isolates.

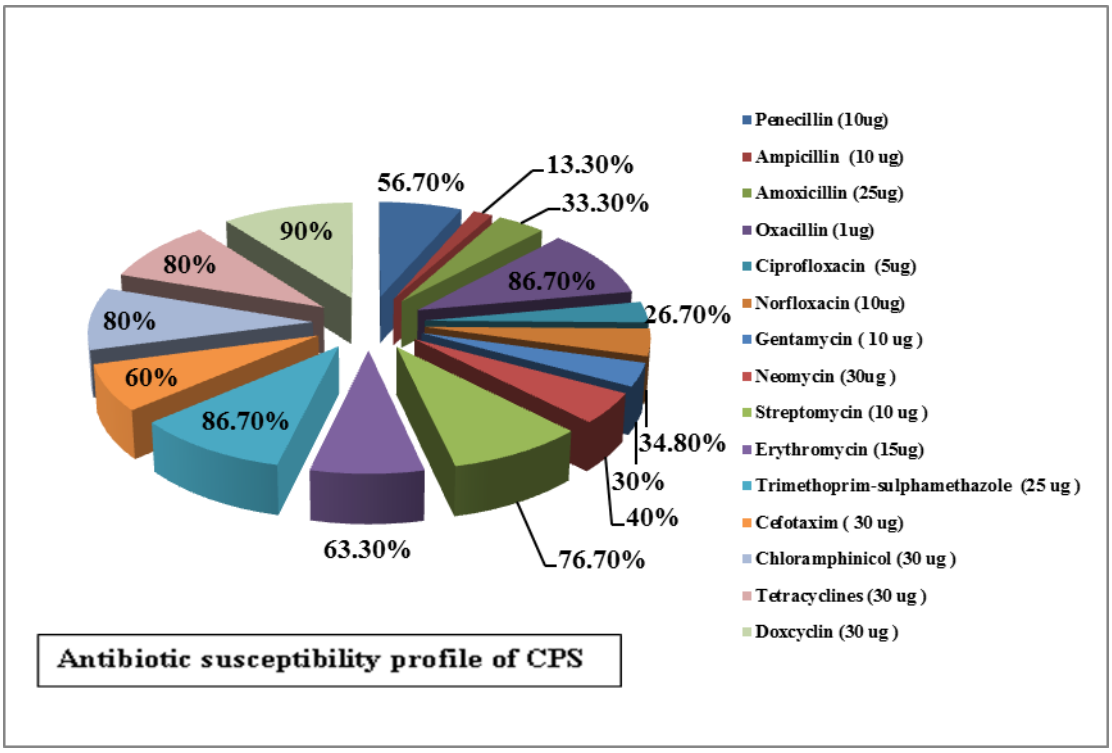

Figure 3: Antibiotic susceptibility pattern of Coagulase Positive Staphylococci isolates.

Table 1: Sequences and cycling conditions of the different PCR primers used for amplification of different virulence genes of E. *coli* and CPS.

Gene	Disease agent	Primers sequences	Amplified segment	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
***eae*A**	*E. coli*	GACCCGGCACAA GCATAAGC CCACCTGCAGCA ACAAGAGG	384 bp	94˚C 5 min.	94˚C 45 sec.	54˚C 45 sec	72˚C 45 sec	72˚C 7 min.	**(Wen-jie *et al.*, 2008)**
***pap*C**		TGATATCACGCA GTCAGTAGC CCGGCCATATTCA CACATAA	501bp	94˚C 10 min.	94˚C 45 sec.	59˚C 45 sec	72˚C 45 sec	72˚C 10 min.	
iss		ATGTTATTTTCTG CCGCTCTG CTATTGTGAGCA ATATACCC	266 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**(Yaguchi *et al.*, 2007)**
***Stx*1**		ACACTGGATGAT CTCAGTGG CTGAATCCCCCTC CATTATG	614 bp	94˚C 10 min.	94˚C 1 min.	58˚C 1 min.	72˚C 1 min.	72˚C 10 min.	**(Dipineto *et al.*, 2006)**
***Stx*2**		CCATGACAACGG ACAGCAGTT CCTGTCAACTGAGC AGCACTTTG	779 bp	94˚C 10 min.	94˚C 1 min.	58˚C 1 min.	72˚C 1 min.	72˚C 10 min.	
tsh		GGTGGTGCACTG GAGTGG AGTCCAGCG TGATAG TGG	620 bp	94˚C 10 min.	94˚C 45 sec.	54˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**(Delicato *et al.*, 2003)**
hlg	**Coagulase positive Staphylococci**	GCCAATCCGTTATT AGAAAATGC CCATAGACGTAG CAACGGAT	937 bp	94˚C 15 min.	94˚C 1.5 min.	55˚C 1.5 min.	72˚C 1.5 min.	72˚C 10 min.	**(Kumar *et al.*, 2009)**
Coa		ATAGAGATGCTG GTACAGG GCTTCCGATTGTT CGATGC	Four different types of bands may be detected 350 bp or 430 bp or 570 bp or 630 bp	94˚C 10 min.	94˚C 45 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**(Iyer and Kumosani, 2011)**
spa		TCAACAAAGAACAA CAAAATGC GCTTTCGGTGCTT GAGATTC	226 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**(Wada *et al.*, 2010)**
Sea		GGTTATCAATGTG CGGGTGG CGGCACTTTTTTC TCTTCGG	102 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**(Mehrotra *et al.*, 2000)**
Seb		GTATGGTGGTGT AACTGAGC CCAAATAGTGAC GAGTTAGG	164 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	
Sec		AGATGAAGTAGT TGATGTGTATGG CACACTTTTAGAA TCAACCG	451 bp	94˚C 10 min.	94˚C 45 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	
Sed		CCAATAATAGGA GAAAATAAAAG ATTGGTATTTTTT TTCGTTC	278 bp	94˚C 5 min.	94˚C 30 sec.	48˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	
See		AGGTTTTTTCACA GGTCATCC CTTTTTTTTCTTC GGTCAATC	209 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	

Table 2: Sequences and cycling conditions of the different PCR primers used for amplification of different antibiotic resistance genes of *E. coli*, and CPS.

Gene	Disease agent	Primers sequences	Amplified segment	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
***bla*TEM**	*E. coli*	ATCAGCAATAAA CCAGC	516 bp	94˚C 10 min.	94˚C 45 sec.	54˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Colom *et al*., 2003**
		CCCCGAAGAACG TTTTC							
Sul1		CGG CGT GGG CTA CCT GAA CG	433 bp	94˚C 10 min.	94˚C 45 sec.	60˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**(Ibekwe *et al*., 2011)**
		GCC GAT CGC GTG AAG TTC CG							
***Tet*A(A)**		GGTTCACTCGAAC GACGTCA	576 bp	94˚C 10 min.	94˚C 45 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**(Randall *et al.*, 2004)**
		CTGTCCGACAAGT TGCATGA							
***mec*A**	**Coagulase Positive Staphylococci**	GTAGAAATGACT GAACGTCCGATA A	310 bp	94˚C 10 min.	94˚C 45 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**(McClure *et al*., 2006)**
		CCAATTCCACATT GTTTCGGTCTAA							
***tet*K**		GTAGCGACAATA GGTAATAGT	360 bp	94˚C 10 min.	94˚C 45 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**(Duran *et al*., 2012)**
		GTAGTGACAATA AACCTCCTA							
blaZ		ACTTCAACACCTG CTGCTTTC	173 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	
		TGACCACTTTTAT CAGCAACC							

Table 3: Isolation rates of detected E.*coli* serotypes:

Serotype	Site of isolation			Number of isolates	Isolation rate
	On the shell	content	On the shell and content		
Poly 2 O91	**1**	**0**	**0**	**1**	**3%**
Poly 2 O125	**2**	**1**	**0**	**3**	**8%**
Poly 2 O166	**1**	**0**	**0**	**1**	**3%**
Poly 3 O145	**1**	**0**	**0**	**1**	**3%**
Poly 4 O6	**2**	**1**	**0**	**3**	**8%**
Poly 4 O27	**2**	**2**	**4**	**8**	**22%**
Poly 4 O159	**2**	**1**	**0**	**3**	**8%**
Poly 5 O25	**1**	**0**	**0**	**1**	**3%**
Poly 5 O153	**1**	**0**	**0**	**1**	**3%**
Poly 6 O115	**1**	**0**	**0**	**1**	**3%**
Poly 6 O169	**0**	**2**	**1**	**3**	**8%**
Poly 8 O152	**2**	**1**	**1**	**4**	**11%**
poly 8 O29	**1**	**0**	**0**	**1**	**3%**
Untypeable	**4**	**1**	**0**	**5**	**14%**
Total	**21**	**9**	**6**	**36**	**100%**

Table 4: Antibiotic susceptibility pattern of E.*coli* isolates

Antibiotic group	SN	Chemotherapeutic agent	Susceptible S	Intermediate I	Resistant R
penicillins	1	Ampicillin (10 ug)	33.3%	0	66.7%
	2	Amoxicillin (25ug)	7.7%	0	92.3%
Quinolones	3	Ciprofloxacin (5ug)	55.6%	0	44.4%
	4	Enrofloxacin (10ug)	29.4%	2%	68.6%
	5	Norfloxacin (10ug)	43.8%	1.2 %	55 %
Aminoglycosedes	6	Gentamycin (10 ug)	59.8%	0	40.2%
	7	Kanamycin(20ug)	40%	2%	58%
	8	Streptomycin (10 ug)	11.1%	1.9%	87%
Polymyxins	9	Colistin(25 ug)	94.4%	0	0.6%
Potentiated sulphonamides	10	Trimethoprim-sulphamethazole (25 ug)	5.9%	0	94.1%
Cephalosporins	11	Cefotaxim (30 ug)	76.5%	1.5%	22%
Phenicols	12	Chloramphinicol (30 ug)	46.2%	2%	51.8%
Tetracyclines	13	Tetracycline (30 ug)	7.1%	0	92.9%
	14	Doxycyclin (30 ug)	6.8%	0	93.2%

Table 5: Antibiotic susceptibility pattern of Coagulase Positive Staphylococci isolates:

Antibiotic group	SN	Chemotherapeutic agent	Susceptible I	Intermediate I	Resistant R
Penicillins	1	Penecillin (10ug)	42.3%	1%	56.7%
	2	Ampicillin (10 ug)	87%	0	13.3%
	3	Amoxicillin (25ug)	66.7%	0	33.3%
Anti-staphylococcal β-lactams	4	Oxacillin (1ug)	13.3%	0	86.7%
Quinolones	5	Ciprofloxacin (5ug)	73.3%	0	26.7%
	6	Norfloxacin (10ug)	63.2%	2%	34.8%
Aminoglycosides	7	Gentamycin (10ug)	70%	0	30%
	8	Neomycin (30ug)	60%	0	40%
	9	Streptomycin (10 ug)	23.3%	0	76.7%
Macrolydes	10	Erythromycin (15ug)	36.7%	0	63.3%
Folate pathway inhibitors	11	Trimethoprim-sulphamethazole (25 ug)	13.3%	0	86.7%
Cephalosporins	12	Cefotaxim (30 ug)	38%	2%	60%
Phenicols	13	Chloramphinicol (30 ug)	20%	0	80%
Tetracyclines	14	Tetracyclines (30 ug)	20%	0	80%
	15	Oxytetracyclin (30 ug)	10%	0	90%

Table 6: PCR results of the different antibiotic resistance genes and antibiotic resistance profile of Coagulase Positive Staphylococci.

CPS						
Isolate code	***bla*Z**	**Penicillin**	***mec*A**	**Oxacillin**	***tet*K**	**Tetracycline**
Z13	-	**R**	-	**R**	+	**R**
33	-	**R**	-	**R**	+	**R**
E85	+	**R**	+	**R**	+	**R**
31	+	**R**	+	**R**	+	**R**
23	-	**S**	-	**S**	+	**S**
Z6	-	**R**	-	**R**	-	**R**
H4	-	**S**	-	**R**	-	**R**
H34	+	**R**	+	**R**	+	**R**
E58	+	**R**	+	**S**	+	**R**
E103	+	**R**	-	**R**	-	**R**
88	+	**R**	-	**R**	+	**R**
Pos PCR % or antibiotic resistance %	**54.54**	**81.81**	**36.36**	**81.81**	**72.72**	**90.9**

Table 7: PCR results of the different virulence genes of the CPS isolates.

CPS isolate	***Sea***	***Seb***	***Sec***	***Sed***	***See***	***spa***	***Coa***	***hlg***
Z13	-	-	-	-	-	+	+	-
33	-		+	-	+	+	+	+
E85	-	+	-	-	-	+	+	+
31	-	+	+	-	-	+	+	+
23	-	+	+	-	-	+	+	-
Z6	-	-	-	-	-	-	+	-
H4	-	-	+	-	-	-	+	-
H34	-	-	+	-	+	+	+	+
E58	-	+	-	-	+	+	+	+
E103	-	-	-	-	-	+	+	-
88	-	+	-	-	-	+	+	-
Pos %	**0**	**45.45**	**45.45**	**0**	**27.27**	**81.81**	**100**	**45.45**

Table 8: PCR results of the different antibiotic resistance genes and antibiotic resistance profile of *E. coli*.

E. *coli*						
Isolate code	***bla*TEM**	**Amoxicillin**	***Sul*1**	**Trimethoprim-sulphamethazole**	***tet*A(A)**	**Tetracycline**
E49	+	**R**	-	**S**	+	**R**
E4	+	**S**	+	**S**	+	**S**
T2	+	**R**	+	**R**	+	**R**
E30	+	**R**	+	**R**	+	**R**
E22	+	**R**	-	**R**	+	**R**
Z9	+	**R**	-	**R**	+	**R**
Z18	+	**R**	+	**R**	+	**R**
Z13	+	**R**	+	**R**	+	**R**
H3	+	**R**	-	**R**	+	**R**
E31	+	**R**	+	**R**	+	**R**
E21	+	**R**	+	**R**	+	**R**
E23	-	**R**	-	**R**	+	**R**
E47	+	**R**	+	**R**	+	**R**
Pos PCR % or antibiotic resistance %	**92.3**	**92.3**	**61.53**	**84.61**	**100**	**92.3**

Table 9: PCR results of the different virulence genes of the *E. coli* isolates.

E. *coli* isolate	***tsh***	***iss***	***Stx*1**	***Stx*2**	***eae*A**	***pap*C**
E49	+	+	-	-	+	-
E4	-	+	-	+	+	-
T2	-	+	-	+	+	-
E30	+	+	-	+	+	-
E22	-	+	-	-	+	+
Z9	+	+	-	-	+	+
Z18	+	-	-	-	+	-
Z13	-	+	-	-	+	-
H3	-	+	-	+	+	-
E31	-	+	-	-	+	-
E21	-	-	-	-	+	+
E23	-	-	-	-	+	-
E47	-	+	-	+	+	-
Pos %	**30.76**	**76.92**	**0**	**38.46**	**100**	**23.07**

DISCUSSION

Eggs produced from backyard house hold chickens in Egyptian villages are commonly used for own consumption or to be sold in local markets,(most commonly used unwashed).

Special attention has been paid for raw or undercooked eggs because the hens act as natural reservoirs of a variety of pathogens. The contamination occurs through the shell; But humidity, temperature and storage time are critical for migration of bacteria from the surface of the shell to the inner structures of the egg (Evêncio *et al.,* 2012).

In the present study 200 eggs produced from backyard chickens were examined for the presence of Salmonella spp., E.*coli*, and Coagulase Positive Staphylococci.

The study detected no Salmonella neither from on the shell nor from the egg contents. Although many researchers have reported similar results as (Chousalkar *et al.,* 2010), higher isolation rates of Salmonella spp. were reported by other researcher as (Mona *et al.,* 2014) who detected Salmonella in eggs at a rate of (1.5%). However, (Camilleri, 1992) stated that failure to detect salmonella spp. from eggs does not imply that local flocks are not infected by salmonella.

The study declared a total of 36 E.*coli* isolates with an overall prevalence rates of 18%. Among the total isolates, 21 (10.5%) isolates, 9 (4.5 %) isolates, and 6(3.0%) isolates were detected from over the shell, egg contents, and both shell and contents, respectively. Almost similar results were reported by (Arathy *et al.,* 2011) they could detect an overall isolation rate of 12.2%, while 8% of the isolates were detected from shell and 5% from yolk samples, respectively. Higher isolation rate was reported by (Adesiyun *et al.,* 2005) who recorded (37.0%) as an overall isolation rate, (28.3%) as an isolation rate from egg shell, while they recorded almost similar isolation rate from egg content samples (3.8%). Lower isolation rates were recorded by (Saitanu *et al.,*1994) who isolated E.*coli* from egg shells and in egg contents with a rate of 3.5% and 1.2%, respectively.

Coagulase Positive Staphylococci including Staphylococcus *aureus* and other spp. are important Pathogens in human and veterinary medicine, beside their importance in regard to food hygiene because of their ability to form staphylococcal enterotoxins (SEs). The present study applied (ISO 6888-1:1999, AM: 2003) which specifies enumeration and detection of Coagulase Positive Staphylococci "among which enterotoxigenic strains are encountered" in products intended for human consumption or feeding of animals. Rosa *et al.* (2001) Suggested that Baird Parker is sufficient to screen the presence of Staphylococcus *aureus* without the need for further identification, resulting in saving time and money. The study investigated eggs for the presence of Coagulase Positive Staphylococci both on the shell and in the contents, 80 isolates were detected with an overall prevalence rate of 40%. Isolation rates were 29(14.5%), 15 (7.5%), and 36 (18%) from on the shell, contents, and both shell and contents, respectively. Higher prevalence rates were recorded by (Stepień *et al.,* 2009) when they reported the isolation of Coagulase Positive Staphylococci from eggs with a rate of 45.7%, of which 2.5%, 38.7%, and 58.8% were detected from white, yolk, and on the shell, respectively.

In the present study, out of the 200 examined eggs 15 (7.5%) harbored both E.*coli* and Coagulase Positive Staphylococci. This result was in agreement with that stated by (Obi and Igbokwe, 2009) who were able to reveal that freshly laid and stored domestic fowl eggs were contaminated by consortia of microorganisms, which migrated and invaded the inner parts of the eggs due to primarily heavy contamination and then prevailing poor storage conditions.

Serotypes of E. *coli* are classified according to the Kauffmann scheme, currently there are approximately 180 O, 60 H and 80 K antigens; The numbers change as new ones are identified and previous ones that are duplicated or attributable to another bacterial species are removed, additional serotypes with O antigens that have not been recognized also are found in most surveys. Even though molecular methods for identifying specific virulence genes are available, serotyping remains a useful tool for epidemiologic studies. Serotyping provides a means of relating previous work with new work. Variations in the distribution of serotypes according to geographic region occur. Many other serotypes have been found less frequently, while some APEC do not belong to known serotypes or are untypeable (Swayne, 2013). In the present study,13 serotypes belonged to 6 different O groups were identified, of which O27 predominated with an isolation rate of (22%), followed by O152 (11%).While, O125, O6, O159 and O169 were isolated with a rate of (8%), each. Finally, each of O91, O166, O145, O25, O153, O115, and O29 was isolated with a rate of (3%). Serotyping with the available kits failed to identify 5 isolates (14%). The result of serotyping agreed with that of (Rosario *et al*., 2004) who failed to identify the serogroups of 15% of isolates.

In the present study, antibiotic susceptibility patterns of E.*coli* and Coagulase Positive Staphylococci isolates were studied. The study revealed the prevalence of MDR isolates among both microorganisms. The study recorded that 34 (94.1%),

and 72 (90%) of E.*coli*, and Coagulase Positive Staphylococci isolates , respectively were considered MDR ; As it was observed that the tested isolates were resistant to 5 and more; And 3 and more antimicrobial drugs, respectively. Also those drugs belonged to different antimicrobial categories. The result was in accordance with (CLSI, 2011) where it was reported that MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories.

The study recorded that 94.1**%** of E.*coli* isolates were resistant to 5 and more antimicrobial drug from varying drug category. This result agreed with that of (Muhammad *et al.,* 2009) who reported the detection of MDR E.*coli* with a rate of 100%. The highest resistance rates were recorded against Trimethoprim-sulphamethazole, doxycyclin, tetracycline, and amoxicillin; 94.1%, 93.2%, 92.9%, and 92.3 %, respectively. While the highest sensitivity rates were detected to colistin, cefotaxim, gentamycin, and ciprofloxacin; 94.4.%, 76.5% 59.8%, and 55.6%, respectively.

The result for trimethoprim- sulphamethazole susceptibility was similar to that of (Li *et al.,* 2007) as they recorded resistance rate of 100%. While lower resistance rate was observed by (Hasan *et al.,* 2011) who reported resistance rate of 26.7%.

Concerning resistance rate against doxycyclin, the result of this study was almost similar to that detected by (Jiang *et al.,* 2009) who reported resistance rate of 95.6%.

The results for resistance profiles against tetracycline were in agreement with that of (Jiang *et al.,* 2009) who reported resistance rate of 93.4%. Lower resistance rate was recorded by (Muhammad *et al.,* 2009) who recorded 52%.

The study of resistance rate against amoxicillin was in agreement with that of (Sheikh *et al.,* 2012) who reported a rate of 92.86%, lower resistance rate was recorded by (Motayo *et al.,* 2013) who recorded a resistance rate of 16.8%.

The result of colistin sensitivity agreed with the result of (Maalej *et al.,* 2011) who observed sensitivity rate to colistin of 100%.

The result of susceptibility concerning cefotaxim was in accordance with that of (Pérez *et al.,* 2014) who recorded resistance rate of 84.5%. In the contrary Oteo *et al. (*2006) recorded resistance rate of 100%.

The result of gentamycin was in agreement with that of (Huang *et al.,* 2009) who detected sensitivity rate of 55.96%.

Higher susceptibility rate to ciprofloxacin was detected by (Hasan *et al.,* 2011) who reported a sensitivity rate of 87.1%. On the other hand, lower resistance rate was recorded by (Li *et al.,* 2007) who recorded 19%.

The study revealed that 72 (90%) of Coagulase Positive Staphylococci isolates were considered MDR. This result was in agreement with that of (Suleiman *et al.,* 2013) who reported that 100**%** of the detected Coagulase Positive Staphylococci isolates were MDR.

The study revealed that the highest sensitivity rate of Coagulase Positive Staphylococci was observed against ampicillin 87%. This result disagreed with that of (Lee, 2003) who recorded resistance rate of 73.3%.

The antibiotic susceptibility profile of Coagulase Positive Staphylococci isolates revealed 73.3% sensitivity rate to ciprofloxacin. This result was in accordance with that of (Suleiman *et al.,* 2013) who detected sensitivity rate of 100%.

The detected sensitivity rate to gentamycin was 70%. This result was in agreement with that of (Suleiman *et al.,* 2013) who observed sensitivity rate of 100%.

The study detected that the highest resistance rate of Coagulase Positive Staphylococci was against oxytetracyclin 90%, this result agreed with that of (El-Jakee *et al.,* 2008) who observed resistance rate against oxytetracyclin of 80%.

The study also observed 86.7% resistance rate of Coagulase Positive Staphylococci against trimethoprime-sulphamethazone. This result disagreed with that of (Lee, 2003) who succeeded to detect 100% sensitivity rate for trimethoprime-sulphamethazone. On the other hand, the result was in accordance with that of (Nam *et al.,* 2011) who detected resistance rate of 100%.

The observed resistance rate of Coagulase Positive Staphylococci against oxacillin was 86.7%.This result was similar to that of (Lee, 2003) who detected 100% resistance rate against oxacillin. While, this result disagreed with that of (Nam *et al.,* 2011) who detected resistance rate against oxacillin of (6.2%).

Control programs do not address laying hens whose eggs are produced for personal consumption or local sale, control measures should not forget home-produced eggs, as there is a risk of infection from their consumption, (Hardy *et al.,* 2012).

The *eae*A gene was tested to speculate the virulence of the isolated E. *coli* strains. There were 100% positive results for this gene. This result assured the virulence of these isolates because the *eaeA* gene encodes for intimin protein which is considered as a bacterial adhesion molecules that leads to the emersion of the A/E lesions (Kilici *et al.,* 2007). The high incidence rate of *eae*A gene detection was recorded by many authors as (El-Jakee *et al.,* 2012)

who detected *eae*A gene in 95.9% of the tested E. *coli* O157:H7 isolates.

The *iss* gene had the 2nd incidence degree as 76.92% of the E. *coli* isolates showed positive amplification of the specific 266 bp of this gene. *Iss* (increased serum survival gene) is considered as a promising virulence gene that is usually associated with the APEC strains. Its rule was studied by (Lynne *et al.*, 2007) when they observed a significant drop in E. *coli* resistance to serum with the *iss*- mutant. A bor-mutant also showed a drop in serum resistance but the drop in serum resistance was more violent in *iss*-mutant which indicates that *iss* contributes more to serum resistance than bor in the E. *coli* strains. This effect was assured when the level of serum resistance was restored after the *iss* was reintroduced into the iss- mutant.

The *tsh* (temperature-sensitive hemagglutinin) gene was detected in 4 isolates. This gene encodes for an autotransporter protein secreted by avian-pathogenic E. *coli*. This gene is rarely detected in commensal E. *coli* (Delicato *et al.,* 2002) and is frequently found in highly pathogenic avian E. *coli.* The presence of this gene increases the danger of the harboring strains as it has a potential role as an adhesin (Provence & Curtiss, 1994) and it also has the ability to degrade haemoglobin (Otto *et al.,* 1998).

None of the thirteen tested E. *coli* isolates showed positive PCR results for the *stx*1 gene. However, 5 isolates (38.46%) was recorded as positive for the *stx*2 gene. A close result was reported by (Zahraei *et al.,* 2007) who suggested that *Stx*2 may be widespread among APEC as they detected *stx*1 only in one isolate (8.33%) out of the 12 tested isolates, but stx2 was detected in 9 (75%) isolates. AL-Ashmawy, (2013) has much correlated results as she detected *stx*2 in 37/39 of the E. *coli* isolates from table eggs. The correlation was high also because none of the positive *stx*2 isolates showed positive results for stx1 gene. The heat stable toxin *(Stx)* causes disruption of chloride channels in the cell and secretion of fluid and electrolytes into the intestinal lumen causing diarrhea (Gaastra and Svennerholm, 1996). However the current results differed markedly from that obtained by many authors as (Wani *et al.,* 2004; Zahraei *et al.,* 2007 and AL-Ashmawy, 2013) who reported that all *stx*2 positive isolates were negative for *eae* genes, our results were supported by that obtained by (Dutta *et al.,* 2011) who recorded *eae* and *stx*1 genes in 2 toxin producing Escherichia *coli* (STEC) isolates. The results of the current study also was supported by the study done in Egypt by (Galal *et al.,* 2013) who detected both of the stx1 and stx2 genes with *eae*A gene in 2/19 (10.52%) of the samples, and detected either stx1 or stx2 with *eae*A gene in 3/19 (15.78%) of the samples.

The *pap*C gene which encodes for the P fimbriae was detected in 3 isolates. Rocha *et al.* (2008) reported that *pap*C operon is located in bacterial colonization in respiratory epithelium which directly affects the intensity of infection.

The gamma haemolysine (*hlg*) gene was positively amplified in 45.45% of the tested Coagulase Positive Staphylococci isolates. This gene is considered as one of the most important virulence genes of Staphylococci as it lead to generation of pores in the erythrocytes after initial binding of the two synergistically acting proteins *hlg*B and *hlg*A (Dickinson and Bisno, 1993).

The risk of Coagulase Positive Staphylococci isolates was accounted through the 81.81% positive percentage of the *spa* gene. This gene encodes for the protein A which antagonize the function of the immune system through hindering of the antibody mediated immune clearance of the organism through binding to the Fc receptor of IgG. Also, protein A interferes with the phagocytosis of opsonized bacteria via binding IgG (Murray *et al.,* 2002).

The PCR result of the *coa* gene was enough to confirm the virulence of the Staphylococci isolates as it is considered as a marker for its virulence. The coagulase aids in the formation of a fibrin layer around a focal staphylococcal abscess, which leads to the localization of the infection and protecting the organism from phagocytosis (Sawai *et al.,* 1997).

The results of the enterotoxin genes were so interesting, as 45.45% of the isolates were positive for *Seb* and *Sec*, while 27.27% of the isolates showed positive results for the *See* gene. The presence of these entrotoxins is so threatening as they resist the hydrolysis by gastric and jejunal enzymes and also they are heat stable at 100°C for 30 minutes which can explain why the staphylococcal food poisoning is the leading cause of food-borne microbial intoxication worldwide (Holmberg and Blake, 1984).

The *blaTEM* gene was tested for the 11 selected E. *coli* isolates to assess its resistance to amoxicillin. Interestingly, the positive PCR percent (92.3%) was highly related to the phenotypic positive percent (92.3%) which confirmed the high degree of the resistance of these isolates to amoxicillin.

However, the PCR showed high positivity for the *Tet*A(A) gene (100%) than the positive resistance percent obtained for the tetracycline by the antibiotic susceptibility test (92.3%), which may be related to the sensitivity of PCR itself. This also may be related to the antibiotic susceptibility test which may be influenced by several factors, some of which include the medium used for bacterial culture, type of drug tested, and the type of organism.

Conversely, the antibiotic susceptibility test showed higher positive percent for the trimethoprim-sulphamethazole (84.61%) than that showed for *sul*1 gene by PCR (61.53%). This was elucidated by

(Gündoğdu *et al.,* 2011) who recorded 10 strains of 96 carrying *sul*2 *and intl*1 were not positive for the presence of the *sul*1 gene. This was explained by (Grape *et al.,* 2005) who referred to the *sul*1 gene as a semi-conserved segment.

Out of the 11 tested Staphylococci isolates, 4 were positive for *mec*A gene. This can increase the ferocity of this isolates. This was also reported by (Pyzik *et al.,* 2014) who detected *mec*A gene in two S. *aureus*-like strains isolated from table eggs. To determine the ability of MRSA strains to infect human, (Lee, 2003) performed RAPD PCR and the results showed that their genome was very closely related to some human strains considering these isolates may be a possible source of food borne human infections.

However, the PCR positive percent for *mec*A gene (36.36%) was somewhat far from that obtained by antibiotic susceptibility test which showed 81.81% positive percent for the resistance to oxacillin. This was clarified by (Mathews *et al.,* 2010) who reported two types of strains that show phenotypic resistance to oxacillin however they don't harbor the *mecA* gene. The 1st type of those two strains is called borderline oxacillin resistant S.*aureus* (BORSA) which hyper produces betalactamase and while they appear oxacillin resistant, do not possess the usual genetic mechanism for such resistance. They reported also that another type of strains known as modified S. *aureus* (MODSA) which possess a modification of existing penicillin binding proteins rather than the acquisition of a new PBP as is the mechanism for classical MRSA.

The *tet*K gene was tested for the evaluation of the antibiotic resistance to tetracycline. The PCR result showed a 72.72% positive result which was lower than the resistance percent encountered by the antibiotic susceptibility test (90.9%). Schmitz *et al.* (2001) illustrated the mechanisms of tetracycline resistance for Staphylococcus species in two models. The 1st one is the active efflux resulting from the acquisition of the *tet*K and *tet*L genes located on a plasmid; and the 2nd one is the ribosomal protection mediated by *tet*M or *tet*O determinants located on either a transposon or the chromosome. And as recorded by (Duran *et al.,* 2012), the *tet*M gene had higher positive percent (78%) than that recorded for *tet*K (43%) in the study performed to evaluate the association between the antibiotic susceptibility patterns and the antibiotic resistance genes in staphylococcal isolates.

The *bla*Z gene was positively amplified in 54.54% of the Staphylococci isolates. This result was dissimilar to the recorded penicillin resistance by the antibiotic susceptibility test (81.81%). The discrepancy between those results may also be related to the different mechanisms (other than *bla*Z) for the resistance of staphylococci to penicillins. Duran *et al.* (2012) mentioned those 2 mechanisms where the most important mechanism one is the production of beta-lactamase which inactivates penicillin by hydrolysis of its beta-lactam ring, another mechanism is associated with penicillin-binding protein 2a (PBP2a), encoded by *mec*A.

In conclusion, this study confirm that local house hold produced chicken eggs which are consumed as food can harbor resistant bacterial pathogen of zoonotic importance, those pathogens may impose public health hazard. The study recommends rising public awareness to the importance of proper thermal processing and cooking of eggs specially for immune-compromised group as pregnant women, children and old ages. The study also recommends regular monitoring and surveillance of house hold sector together with guidance programs to the public targeting rising awareness for safe house hold rearing procedures of chicken, storage and handling of eggs, in order to prevent dissemination of dangerous pathogens through environment and the transfer of infection to other animals and human.

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تواجد المسببات المرضية البكتيرية المقاومة للمضادات الحيوية في بيض دجاج التربية المنزلية

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استهدفت الدراسة الوقوف على مدى تواجد البكتريا الممرضة متعددة المقاومة للمضادات الحيوية بالبيض المنتج من الدجاج المربى بقطاع التربية المنزلية. وأظهرت نتائج الفحص المعملي لعدد ٢٠٠ بيضة تواجد الميكروب القولوني النموذجي والمكور العنقودي ايجابي التجلط بنسبة ١٨% ،٤٠ % على التوالي، بينما لم يستدل على تواجد ميكروب السالمونيلا في أي من العينات. أظهرت نتائج الفحص السيرولوجي عزل ١٣ نمط مصلي للأيشريشيا كولاي بنسب مختلفة كان أكثرها تواجد O27 بنسبة ٢٢% ،بينما عجز الفحص المعملي عن تصنيف نسبة ١٤% من المعزولات. أظهرت نتائج اختبار الحساسية أن نسبة العترات متعددة المقاومة للمضادات الحيوية كانت ٩٠%، ٩٤.١% بين معزولات المكور العنقودي الذهبي والقولوني النموذجي على التوالي. أعلى نمط مقاومة بين معزولات المكور العنقودي كان تجاه الأوكسيتتراسيكلين ، الأوكساسيلين ، والترايميثوبريم سالفاميثازول بنسبة ٩٠%،٨٦.٧%،٨٦.٧% على التوالي. بينما كانت أعلى نسب الحساسية تجاه الأمبيسيلين ٨٧%. أثبتت الدراسة أن أعلى نسب مقاومة معزولات الميكروب القولوني النموذجي كانت تجاه الترايميثوبريم سلفاميثازول ، الدوكسيسيكلين ، التتراسيكلين واللأموكسيسيكلين بنسب ٩٤.١%، ٩٣.٢%،٩٢.٩% ،و ٩٢.٣% على التوالي. بينما سجلت الدراسة أعلى نسب لحساسية معزولات الميكروب القولوني النموذجي تجاه الكوليستين ،السيفوتاكسيم ،الجنتامايسين والسيبروفلوكساسين بنسب ٩٤.٤%، ٧٦.٥%، ٥٩.٨% ،و ٥٥.٦% على التوالي. وباستخدام اختبار البلمرة المتسلسل للكشف عن جينات الضراوة وجينات المقاومة للمضادات الحيوية بعدد ممثل للمعزولات اظهرت النتائج تواجد جينات الضراوة *seb, sec,see,spa,coa,* and *hlg* بنسب ٤٥.٤٥%، 27.27%,٤٥.٤٥% ,81.81% ,100% و ٤٥.٤٥% على التوالي. وبينما أظهر الأختبار عدم تواجد جيني *sea,sed* بمعزولات المكور العنقودي الذهبي كما اظهرت تواجد جينات المقاومة للمضادات الحيوية *tet*k *blaz,mec*A, بنسب ٥٤.٥٤%،% ٣٦.٣٦ ،٧٢.٧٢% على التوالي. أظهرت نتائج اختبار البلمرة المتسلسل على معزولات الميكروب القولوني النموذجي تواجد جينات الضراوة *tsh,iss,stx*2,*eae*A, و *papC* بنسب ٣٠.٧٦%، ٧٦.٩٢% ،٣٨.٤٦% ،١٠٠% و ٢٣.٠٧% على التوالي ،بينما لم يستدل الأختبار على تواجد جين *sxt*1 في أي من المعزولات. وقد كانت نسب تواجد جينات المقاومة للمضادات الحيوية *blaTEM,sul*1,*tet*A بنسب ٩٢.٣% ،٦١.٥٣%،٩٢.٣% على التوالي.