Phenotypic and Genotypic Characterization of Gram negative bacteria Isolated from Birds of Prey (Raptors)

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Abstract

This study was planned to investigate the phenotypic and genotypic characterization of Gram negative bacteria isolated from birds of prey (Raptors).A total of 281 fecal swabs was collected from Raptors in Giza zoo. All isolates were subjected to bacteriological and biochemical examination, and some of them to serological and PCR analysis. The results of bacterial isolation revealed that *E. coli* was isolated with percentage of 51.1%, *SalmonellaTyphimurium* (1.8%), *Proteus* spp.(30.9%), *Shigella* spp.(5.6%), *Enterobacter* spp.(4.6%), *Citrobacter* spp.(3.2%), and *Pseudomonas* spp. (2.8%). The isolated *E. coli* strains, were found belong to O serotypes in order of frequency O26, O55, O63, O27, O151, untypable,O28a, O148 and O112 with percentage of 20.83%, 20.83%, 16.67% 12.5%, 12.5%, 12.5%, 8.33%, 8.33%, and 4.17% isolates ,respectively. All isolated *salmonella* strains were found belong to *Salmonella Typhimurium* serotype. PCR analysis was carried out for all identified serotypes showed that (10/11) 90.9% of tested *E. coli* strains carried (*eae*A) virulence gene, (2/11) 18.18% of the tested *E. coli* isolates were positive to *tsh* gene, (3/11) 27.27% of the examined *E. coli* strains bearing *iss* gene while, *stx*1 gene was not found in any examined *E. coli* strain (0/11) 0%, and *stx*2 was found in (4/11)36.36% of the examined *E. coli* strains.

Introduction:

Raptor is a generic term for all birds of prey. Raptors are carnivorous birds with strong bills, large talons, and exceptional flight capabilities. There are more than 500 species of raptors found throughout the world, and different types of raptors can be found in every type of habitat. From frozen tundras and scorching deserts to dense forests and bustling cities, raptors are key apex predators in every environment ***Gargiulo et al. (2018).***

Enteric pathogens mainly are inherent in the intestinal tract of raptors or opportunistic flesh eating birds who feed on the ground, at places where human waste is released, or live on the fecally contaminated waters ***(Kocijan et al., 2009).***

Detection of the virulence genes by genotypic assays is more essential due to the difficulty to distinguish between pathogenic and non-pathogenic *E. coli* strains, because of the strains were commonly secondary invaders in birds which associated with other stress factors, poor hygienic measures, inadequate feeding and hypovitaminosis A ***(Nakazato et al., 2009).***

EPEC which known as Intamin (encoded ***eaeA*** gene) containing *E.coli* , have the capability of causing attaching and effacing (A/E) lesions on intestinal epithelium , by damage of the microvilli subsequently adherence of bacteria to the apical cell membrane producing sever gastroenteritis ***(Donnenberg et al., 1993).***

STEC strains which carrying (***stx_1***and ***stx_2*** **)** encoded genes have the facility to produce shiga- like toxins (Stx1&Stx2) which can cause a many human and animal diseases ***(Karmali, 1989).*** Most of the pathogenic strains *E.coli* isolated from wild birds were negative for *stx* genes, with few exceptions, while more than 30% of wild birds possess *eaeA*geneby PCR analysis ***(Kobayashi, et al., 2009).***

tsh gene, is an additional adhesion-related factor. The *tsh* gene, encoding a temperature-sensitive hemagglutinin, was firstly isolated and described by Provence and Curtiss ***(Provence and Curtiss, 1994).***And may act as an adhesin, principally in the initial stages of bacterial colonization.

The increased serum survival (iss) gene, first characterized in the ColV plasmid, has a role in serum complement resistance ***(Nolan et al., 2003).*** The gene encodes the Iss protein, which has a pointer sequence specific of outer membrane proteins (OMP) and encodes a 9 to 10 KDa lipoprotein of the bacterial outer membrane ***(Nolan et al., 2003)***.

Material and Methods:

Sample:

A sterilized waxed paper were placed on the floor of the cages to reduce possible contamination ***(Bangert et al., 1988 b)***, directly after the birds defecated, the exterior of each freshly voided dropping was swabbed aseptically by using of two sterile cotton swabs. First swab was immersed in test tube contained buffered peptone water and the second swab was immersed in test tube contained Rappaport vasilliadis.

Bacteriological examination:

1- Isolation and biochemical identification of *E. coli:*

1.1. Cultivation in liquid media:

The fecal swabs were collected aseptically and immersed in a tube contain buffered peptone water broth. The inoculated media were incubated at 37º C for 24 hours.

Another swab was immersed in Rappaport vasilliadis broth as enrichment broth for selective isolation of salmonella. The

inoculated media were incubated at 37º C for 18 hours.

1.2. Isolatioin on solid media:

A loopfull from the cultured incubated buffered peptone water was streaked onto the subsequent media; MacConkey's agar, Nutrient agar, and Eosin methyline blue media. The other loopfull from Rappaport vasilliadis was taken and streaked onto MacConkey's agar, Hektone enteric agar and Xylose dextrose agar. The inoculated plates were incubated at 37 º C for 24 hours.

The suspected colonies with typical growth of each bacterial type were sub-cultured three times to get pure culture, then pure isolates were sub cultured on semi-solid nutrient agar slant for preservation of isolates and additional identification, according to ***(Wilson and Miles, 1975) .***

2-Serological identification:

2.1. Serotyping of *E. coli* isolates:

The preliminarily identified isolates biochemically as *E. coli* was subjected to serological identification according to ***Quinn et al. (2002)*** for determination of (O) antigen using slide agglutination test.

2.2. Serotyping of *Salmonella* isolates:

The isolates that were identified biochemically as *Salmonella* spp. were serotyped according to Kauffmann-White Scheme ***(Kauffmann, 1974)*** as characterized by ***(Edwards and Ewing, 1972)*** to detect the "O" and "H" antigens

3- Detection of virulence genes in *E. coli* isolates using PCR:

3.1extraction of DNA according to **QIAamp DNA mini kit instructions**.

3.2preparation of PCR master mix according to Emerald Amp GT PCR master mix (Takara).

3.3Cycling conditions of the primers during PCR.

3.4DNA molecular weight marker.

3.5Agarose gel electrophoresis ***(Sambrook et al, 1989).***

Table (1): *Target genes, oligonucleotide sequence and cycling conditions of different primers used in this study*

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
stx1, stx2	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
eaeA	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
Tsh	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
Iss	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.

Results:

1-Incidence of bacterial pathogens isolated from raptors:

Table (2) *Incidence of bacteria isolated from raptors:*

Bacteria	No. of bacterial Isolates	Percentage %
E. coli	143	51.1
Salmonella Typhimurium	5	1.8
Proteus spp	87	30.9
Shigella spp.	16	5.6
*Enterobacter*spp	13	4.6
*Citrobacter*spp	9	3.2
Pseudomonas spp	8	2.8
Total	281	100

2- Prevalence of *Enterobacteriaceae* from each species of raptors:

Table (3) *Prevalence of Enterobacteriaceae from each species of raptors:*

Bacterial isolate(No)	Egyptian vulture		Long legged buzzard		Common kestrel		Golden eagle		Saker falcon		Barn owl		Great horned owl	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%
E.coli (143)	48	33.6	8	5.6	17	11.9	25	17.5	11	7.7	3	2.1	31	21.7
Salmonella (5)	-	-	-	-	-	-	-	-	-	-	2	40	3	60
Proteus (87)	23	26.4	2	2.3	9	10.3	12	13.8	5	5.7	10	11.5	26	29.9
Pseudomonas (8)	-	-	-	-	-	-	-	-	3	37.5	5	62.5	-	-
Shigella (16)	8	50	-	-	-	-	3	18.8	-	-	-	-	5	31.2
Citrobacter (9)	1	11.1	2	22.2	-	-	1	11.1	-	-	1	11.1	4	44.4
Enterobacter (13)	1	7.7	2	15.4	6	46.1	-	-	4	30.8	-	-	-	-

3-Results of serological identification of isolated *E. coli*:

Table (4) *E. coli serovars isolated from raptors:*

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Serotype	Number	Percentage%
O26	2	8.33
O27	1	4.17
O28a	2	8.33
O55	4	16.67
O63	4	16.67
O112	1	4.17
O148	2	8.33
O151	3	12.5
O158	3	12.5
Untypable	2	8.33
Total	24	100

Genotypic characterization of *E. coli* strains using conventional polymerase chain reaction (PCR) for detection of the virulence genes:

A. Detection of attaching and effacing gene (*eaeA* gene) of *E. coli*:
The *eae*A virulence gene was carried by 90.9% (10/11) of the examined *E.coli* strains isolated from raptors.

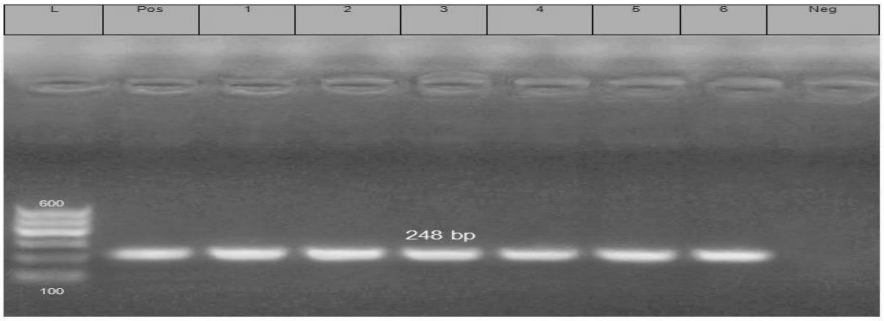

Figure (1): Agarose gel electrophoresis for amplified products of *E.coli eae*A gene at lane L: 100bp DNA ladder, lanes Pos., Neg: positive and negative controls, respectively. lanes 1-6: positive *E.coli* isolates at 248bp

B. Detection of. **Temperature sensitive hemagglutinin gene (*tsh*) of *E. coli*:**
The *tsh* virulence gene was carried by 18.18% (2/11) of the examined *E.coli* strains isolated from raptors.

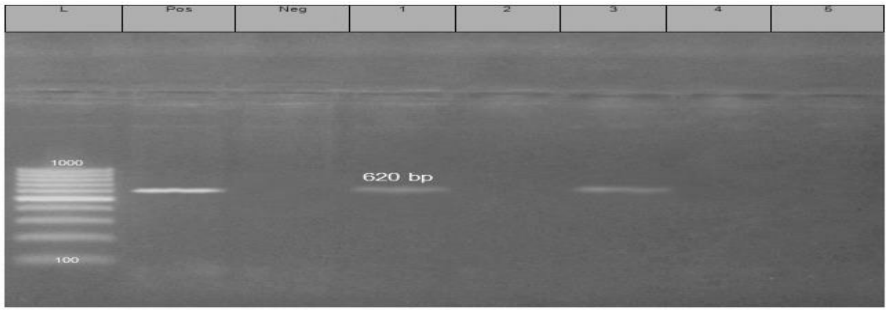

Figure (2): Agarose gel electrophoresis for amplified products of *E.coli* tsh gene at
lane L: 100bp DNA ladder, lanes Pos., Neg: positive and negative controls, respectively. lanes 1,3: positive *E.coli* isolates at 620bp

C. Detection of iron serum survival gene (*iss*) of *E. coli*:
The *iss* virulence gene was carried by 27.27% (3/11) of the examined *E.coli* strains isolated from raptors.

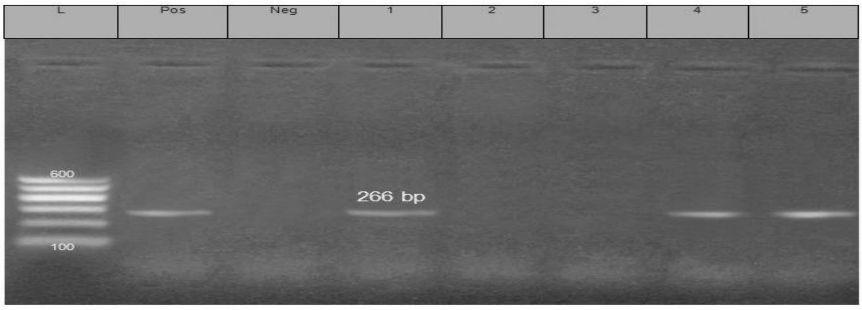

Figure (3): Agarose gel electrophoresis for amplified products of *E.coli iss* gene at lane L: 100bp DNA ladder, lanes Pos., Neg: positive and negative controls, respectively. lanes 1,4,5: positive *E.coli* isolates at 266bp

D. Detection of shiga toxin 1(*stx1*) of *E. coli*:

The *stx1*virulence gene was not detected in the examined *E.coli* strains isolated from raptors by zero % (0/11).

E. Detection of shiga toxin 2(*stx2*) of *E. coli*:

The *stx2* virulence gene was carried by 36.36% (4/11) of the examined *E.coli* strains.

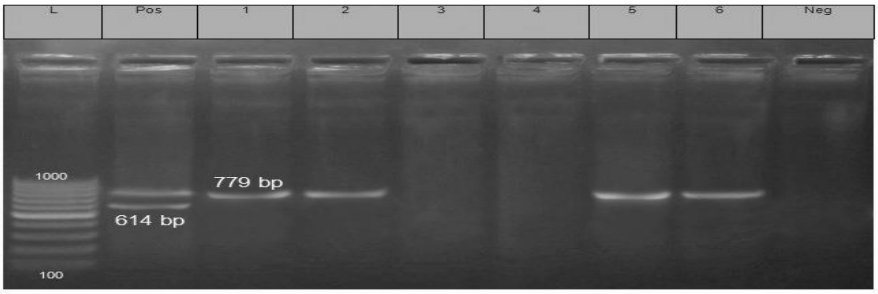

Figure (4): Agarose gel electrophoresis for amplified products of *E.coli stx1* and *stx2* gene at lane L: 100bp DNA ladder, lanes Pos., Neg: positive and negative controls, respectively.
lanes 1-6: Negative *E.coli* isolates for stx1 gene at 614bp
Lanes 1,2,5,6: positive *E. coli* isolates for stx2 gene at 779bp

Table (5): *Incidence of virulence genes (eaeA, tsh ,iss ,stx1 and stx2) detected by cPCR among isolated E.coli strains from raptors:*

Virulence gene	*E.coli* isolates	Percentage (%)
eae A	10/11	90.9
tsh	2/11	18.18
iss	3/11	27.27
stx1	0/11	0
stx2	4/11	36.36

Discussion:

As the number of raptors decreases in many parts of the world ***(Molina-Lópezet al., 2011)***, knowing which pathogens may be causing disease could also provide important information on the conservation and welfare of these species.

Among *Enterobacteriaceae*, *E. coli* and *Salmonella spp*. are the most potential pathogens affecting humans, animals and birds concerning zoonoses and food poisoning. Previously conducted studies revealed that *E. coli* and *Salmonellae* havebeen isolated from free- living birds ***(Refsum et al., 2002 and Fukuyama et al., 2003)***.

In the present study, we have been trying to throw the light on phenotypic and genotypic characterization of Family Enterobacteriacea isolated from birds of prey.A total of (281) fecal swab is collected from birds of prey in Giza zoo. The resultsof Incidence of bacterial pathogensisolated from Raptorsin Table (2) revealed that *E.coli* was isolated by (51.1%), Salmonella typhimurium(1.8%), Proteus spp(30.9%), Shigellaspp(5.6%), Enterobacterspp(4.6%), Citrobacterspp(3.2%), and Pseudomonas spp (2.8%).

The allover incidence of *E. coli* isolation from raptors was 51.1% (143/281). The attained results were in harmony with the results stated by ***Ludovico et al. (2015)*** who detect *E.coli* in the pellets of birds of prey by ratio 65.8%. Also, this result was similar to that achieved by ***Anna et al(2017)*** which isolated *Escherichia coli* from free living raptors by ratio (35%), ***Hajer et al.,(2012)*** thatisolated *E.coli* from common buzzard by ratio 85%**. *Bangert et al., (1988a)*** who isolated *Escherichia coli* from the feces of 42 of the 47 raptors by ratio of 89%. On the other hand ***Dubravka et al.,(2013)*** isolated *Escherichia coli* from four samples (26.6%) isolated from Erusian griffon vultures.

In the current study *Salmonella Typhimurium*were isolated only from five (1.8%) fecal samples originating from raptors (great horned and barn owl) in Giza zoo. this result is coincided with that of ***Reche et al. (2003)*** who isolated salmonella from captive raptors by percentage of 7.36%, while in free living ones by percentage of 4.19%. Also ***Ludovico et al. (2015)*** who isolated 2/73 (2.7%)Salmonella spp. from raptor pellets that inspected and serotyped as Salmonella entericaserovarTyphimurium. In contrast, this result less than that mentioned by ***Dubravka et al. (2013)*** in which*Salmonella* were isolated from five (33.3%) fecal samples originating from Eurasian griffon vultures. And ***Mikaelian et al. (1997)*** detected Salmonella Typhimurium in great horned owl, They were displaying lethargy, diarrhea, eye swelling, poor body condition and sudden death in some cases.

Determination of Shigella spp. from wildlife well detected and ultimately

shows that animals (e.g. birds, rodents) are consider as vectors for Shigella spp. and have the probability to cause zoonotic infection ***Wong, N.K. (2010)*** .

Shigella spp. was isolated from raptors 16/281 by ratio 5.6%. nearly similar result was recorded by ***Wong, N.K. (2010)*** who detected shigella in Four samples from a total of fifty swab samples (8%) collected from the wildlife(rats, squirrels, birds, and bats).

Enterobacterspp was isolated from raptors 13/281 by ratio 4.6%. nearly similar results stated by ***Maria et al. (2017)*** who detected Enterobactercloacea by ratio 17.2% in migratory Passeriformes. Higher incidence was documented by ***Ludovico et al. (2015)*** who isolated Enterobacter spp. by ratio 52.1% from pellets of raptors.And ***Krysta H. R. (2006)*** who isolated Enterobacterspp39/243by ratio (16%) from wild birds.

Citrorobacter spp. was isolated from raptors 9/281 by ratio 3.2%. This result is lesser than that detected by ***Ludovico et al. (2015)*** who isolatedCitrobacter spp.by ratio 38.3%from raptor pellets. And ***Krysta H. R. (2006)*** who detected Citrobacter in wild birds by ratio(27/243; 11%).

Pseudomonas spp. was isolated from raptors 8/281 by ratio 2.8%. This result was in harmony with ***Gierse (2001)*** who detected pseudomonas infection with ratio (4.31%)***, Anna et al (2017)*** who isolated pseudomonas aeroginosa by ratio 7% from raptors. while pseudomonas spp was isolated by higher percentage from wild birds by ratio 22% by ***Brittingham et al. (1988)***.

In contrast ***Ludovico et al. (2015)*** documented that Pseudomonas spp was never isolated from raptors pellets. Also ***Zwart (2000)*** who is stated that falcons were not affected by *Pseudomonas* infections.

Table (3) showed thePrevalence of *Enterobacteriaceae* from each species of raptors as follow: The total incidence of*E.coli* (51.1%) divided as 33.6% from Egyptian vulture, 21.7% from Great horned owl ,17.5 % from Golden eagle, 11.9% from Common kestrel, 7.7% from Saker falcon, 5.6% from Long legged buzzard and 2.1% from Barn owl. While, the total incidence of *Salmonella typhimurium*(1.8%) divided as 60% , from Great horned owl and 40% from Barn owl respectively.

The total incidence of proteus (30.9%) divided as 29.9% from Great horned owl, 26.4% from Egyptian vulture, 13.8% from Golden eagle, 11.5% from Barn owl, 10.8% Common kestrel, 5.7% from Saker falcon and 2.3% from long legged buzzard. While, the total incidence of Pseudomonas (2.8%) divided as 62.5% from Barn owl and 37.5% from Saker falcon respectively.

The total incidence of Shigella (5.6%) divided as 50% from

Egyptian vulture, 31.2% from Great horned owl and 18.8% fromGolden eagle. While the total incidence of Citrobacter (3.2%) divided as 44.4% from Great horned owl, 22.2% from Long legged buzzard and 11.1% from Egyptian vulture, Golden eagle and Barn owl respectively.

Finally, the total incidence of Enterobacter (4.6%) divided as 46.1% from Common kestrel, 30.8% from Saker falcon, 15.4% from Long legged buzzard and 7.7% from Egyptian vulture respectively.

Table (4) showed the results of serological typing in the current study in which, 24 *E.coli* isolates recovered from raptors were distributed among 9 different O serotype groups besides untypable ones. The most prevalent serogroups were O55 and O26 (20.83%) and followed by O63 (16.67%) then O151,O27 and untypable (12.5%), followed by O28a, and O148(8.33%) and O112 and O158 (4.17%).

These results somewhat agreed with ***Ludovico et al. (2015)*** whoisolatedO55 (4.2%), O164 (8.3%), O145 (12.5%), O26 (16.7%), and O103 (29.2%)from raptors**.** And ***Maysa et al. (2013)*** isolated O119 (2) from cattle egrets, and from sparrows one strain from each serogroup (O55 , O111 , O26, O128.Additionaly**,** ***Ahmed (2016)*** obtained 10 strains from House crows (1) O119 ,(3) O125, (2) O157 , (3) O158 and (1) O166, 6 strains obtained from Cattle egrets (1) O25, (1) O27 ,(1) O86 ,(1) O166, (1) O168 and (1) O169 and 8 strains from House sparrows (2) O6 , (3) O44 , (1) O126 and (2) O146 and 6 strains identified as untypable (20%), which were 4 strains from Cattle egrets and 2 strains from House sparrows

Thepresent study was directed to detection of some virulence genes *(eaeA, tsh, iss, stx1 and stx2 genes*) in *E. coli* isolated from raptors by using of molecular biological techniques (PCR).

Results of PCR analysis showed in table (5) were as follow :(11/11) 100% of tested *E.coli* strains isolated from raptors carried (eaeA) virulence gene as shown in photo (1). This result agreed with ***Dhanashree and Mallya(2007)*** who recorded 110 positive *eaeA*gene from total samples 140 (77.5%). And this result was higher than that stated by ***Kobayashi et al*****,*****(2009)*** who stated that over 30% of wild birds carried *eaeA*by PCR analysis. And ***Knobl et al, (2011)*** which was (8.3%).

The results of examination of *E.coli* isolates for the presence of temperature sensitive hemagglutinin gene (*tsh*) as shown in photo (2)dicovered that (2/11) 18.18% of the examined *E.coli* strains from raptors bearing the virulence gene (*tsh*). This results are in harmony with ***knobl et al (2011)*** who detected tsh gene in 3/24 E. coli isolates by ratio 12.5% from psittassine birds. However ***Saidenberg et al (2013)*** identified tsh gene in just one isolate (1/22) by

ratio 4.5% of *E. coli* isolates recovered from healthy Alagoas Curassows (*Pauximitu*) in Brazil.

The result of examination of *E.coli* isolates for the presence of the increased serum survival gene (*iss*) as shown in photo (3) revealed that (3/11) 27.27% of the examined *E.coli* strains from raptors bearing the virulence gene (*iss*).this results is similar to that of ***Knobl et al (2011)*** who detected iss gene in 7/24 *E. coli* isolates in psittassine birds by ratio 29.2%. while higher percentage was detected by ***Saidenberg et al (2013)*** who found that the most frequent virulence factor was *iss*(11/22 isolates)by ratio 50% of *E. coli* isolates recovered from healthy Alagoas Curassows (*Pauximitu*) in Brazil

The result of investigation of *E.coli* isolates for the presence of (stx1and stx2) as shown in photo(4) recorded that stx1 was not found in any examined *E. coli* strain (0/11) 0%, while stx2 was found in (4/11)36.36% of the examined *E.coli* strains.

Similar results are obtained by ***(Lilian et al., 2017)*** who revealed that 3/401of *E.coli*samples are positive for *stx*2 gene (0.75%) distributed among the orders of Psittaciformes, Strigiformes and Columbiformes. None of strains were positive for *stx*1 gene. and ***Ahmed (2016)*** whostated that 33.3% of *E.coli* strains isolated from wild birds was carried each shiga toxin producing genes(stx1,stx2),Also ***(Foster et al., 2006)*** who stated that one out of 231 composite fecal samples collected from wild birds was *stx*positive.

However, the results were differ from that mentioned by ***Knobl et al (2011)*** didn't record any isolates had shiga toxin genesfrom 24 positive *E.coli*isolates from *amazonaaestiva*. And ***Mona et al. (2013*)** whio found only one strain from nine strains of *E.coli* (**O6**) carried *stx1*gene and they didn't record any strain carried *stx2*gene.

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الملخص العربى

التوصيف الظاهرى والجينى للبكتريا سالبة الجرام المعزولة من الطيور الجارحة

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هدفت هذه الرسالة الى دراسة التوصيف الظاهرى والجينى للبكتريا سالبة الجرام التى تصيب الطيور الجارحة لذا فقد تم تجميع عدد 281 عينة (مسحات بكتريولوجية من المجمع) من الطيور الجارحة المتواجدة فى حديقة حيوان الجيزة. .وكانت النتائج كالتالى: اظهرت النتائج عزل الميكروب القولونى بواقع 143 من 281 بنسبة 51.1%. وعزل ميكروب السالمونيلا بواقع 5 من 281 بنسبة 1.8%. وعزل ميكروب البروتياس بواقع 87 من 281 بنسبة 30.9%. وعزل ميكروب الشيجيللا بواقع 16 من 281 بنسبة 5.6%. وعزل ميكروب الانتيروباكتر بواقع 13من 281 بنسبة 4.6%. وعزل ميكروب السيتروباكتر بواقع 9من 281 بنسبة 3.2%. واخيرا ميكروب السودوموناس بواقع 8 من 281 بنسبة 2.8%.وبإجراء الإختبارات السيرولوجية لاربعة وعشرون معزولة من الميكروب القولونى التى تم عزلها من الطيور الجارحة أوضحت النتائج ان المعزولات تشمل عترة واحدة تنتمى الى (O158 - O112–O28) و عترتان تنتميان الى (O148–O27 -O26) ثلاثة عترات تنتمى الى (O151) ثلاثة عترات غير مصنفة , كما تم عزل اربع عترات تنتميان الى (O63) وعزل خمس عترات تنتمى الى (O55). كما ان التصنيف السيرولوجى لمعزولات السالمونيللا اظهر ان جميع العترات تنتمى سيرولوجيا الى سالمونيلا تيفيميوريم بواقع 5/5 بنسبة 100%.وبإستخدام تفاعل إنزيم البلمره المتسلسل للتسعة معزولات التى سبق تصنيفها سيرولوجيا للكشف عن وجود الجينات الخاصة بالميكروب القولونى (eaeA), (tsh),(iss),(stx1)و(stx2) وجد أن المعزولات بواقع 11/10(بنسبة 90.9%) إيجابية وتحمل جين الضراوة eaeA, المعزولات بواقع 11/2 بنسبة 18.18% تحمل جين الضراوة (tsh), وجد ان المعزولات بواقع 11/3 بنسبة 27.27% تحمل جين الضراوة (iss),وجد ان جميع المعزولات لاتحمل جين السميةstx1 واخيرا وجد ان المعزولات بواقع 11/4 بنسبة 36.36% تحمل جين السمية stx2.