Research Article

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Molecular serotyping of Escherichia coli in broiler farms in Sulaymaniyah province/Iraq

Bahar Saeed Mohammed[1], Nahla Mohammed Saeed[2], Sadat Abdulla Aziz[3,4]

1-Department of Laboratory, Microbiology, Veterinary Directorate, Sulaimani, Kurdistan/Iraq.
2-Department of Microbiology, College of Veterinary Medicine, University of Sulaimani, Al-Sulaimaniyah City, Iraq.
3-Department of Anesthesia, College of health Sciences, Cihan University-Sulaimaniya, Kurdistan/Iraq.
4 Department of Basic Sciences, College of Veterinary Medicine, University of Sulaimani, As-Sulaimaniyah City, Iraq.

Corresponding Author Email Address: sadat.aziz@univsul.edu.iq
ORCID ID: https://orcid.org/0000-0001-9455-2639
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Abstract

Escherichia coli (*E. coli*) is a gram-negative bacterium that has economic and public health importance. *E. coli* strains have been classified into pathogenic and non-pathogenic strains. The pathogenic strains of *E. coli* can cause colibacillosis, which is a common bacterial disease in the poultry industry and the poultry farms in the region. The objective of the study was to investigate the prevalence of *E. coli,* its serotypes (O1, O2, O18 and O78) and their antimicrobial susceptibility in the colibacillosis cases in Sulaymaniyah province using culture, antimicrobial susceptibility and molecular approaches. A total of 86 broiler farms were investigated from November 2021 to June 2022. From each farm, samples (liver and heart) were taken from 3-5 broilers colibacillosis cases. The results showed that the colonies that had metallic-green sheen morphologies were positive for E. coli (62/86; 72.1%), in which only 23/62 (37.1%) of the isolates were positive for O2 (7/62; 11.3%), O18 (14/62; 22.6%), and O78 (2/62; 3.2%). While O1 was undetectable in the investigated colibacillosis cases. O18 was predominantly (7/86; 8.1%) detected among 20-30 days-old chickens and followed by O2 (4/86; 4.7%) in 10-20 days-old chickens. The results showed that the majorities of the detected *E. coli* in colibacillosis cases were isolated from the imported chicks from Iran (30; 34.9%) and Netherlands (28; 32.6%). In conclusion, the results showed that the majorites of the colibacillosis cases in the region were caused by *E. coli.* The *E. coli* and its serotypes (O2, O18 and O78) had high prevalence in the region.

Keywords: *E. coli*, Sulaymaniyah, broiler farms.

Introduction

Escherichia coli (*E. coli*) is a Gram-negative bacterium that has economic and public health importance. *E. coli* strains have been classified into pathogenic and non-pathogenic strains depending on their pathogenicity and clinical symptoms. Pathogenic strains of *E. coli* are divided into several subspecies, including avian pathogenic *E. coli* (APEC), which is common in broiler chicken (1) and it is one of the major causes of morbidity and mortality in birds (2), particularly they induce colibacillosis (3, 4).

E.coli is characterized by having some serotypes some serotypes, especially O1, O2, O18 and O78, are commonly detected in broiler chickens and they seem to be associated with bird colisepticemia and colibacillosis, severe respiratory tract infection, particularly in young birds.[5, 6]

O1, O2, O18 and O78 are recognized as the common avian pathogenic *E. coli* serotypes that cause high economic loses in poultry industry [7]. However, there was not any scientific data about those serotypes in the region, especially in Sulaymaniyah province/Iraq. Therefore, the focus of the study was to isolate and identify *E. coli* and its serotypes in the region.

Material and Methods

Sample Collection

The sample was collected from November 2021 to June 2022 in Sulaymaniyah province. A total of 86 broiler farms were investigated. From each farm, 3-5 chickens, which had typical signs of colibacillosis, were selected. Samples (liver and heart) were aseptically collected and transported to the higher education laboratory of the College of Veterinary Medicine at the University of Sulaimani in cold boxes. The collected samples were prepared for standard microbiological examination.

Isolation and Identification of *E. coli*

Samples were aseptically inoculated into nutrient broth (Lioflechm, Italy), incubated at 37°C for 24 hrs. Then after, each inoculum was sub-cultured on MacConkey agar at 37°C/18-24 hrs (Lioflechm, Italy). Well-defined colonies, which had typical pink-color, were subcultured on Eosine-methylene blue (EMB) agar (Liofilchem-Italy). Typical colonies were selected and inoculated into nutrient broth (Neogen-UK) and incubated at 37°C [8, 9].

DNA extraction

The boiling technique was used to extract DNA from the isolates. From each sample about one ml of the grown bacteria in nutrient broth was centrifuged (13,000 rpm for 10 min) to pellet the bacteria, then washed in 50-100 µl of ultra-pure water in an Eppendorf tube. After being centrifuged at 13,000 rpm for 10 min, the pellet was resuspended in 100 µl of ultra-pure water by a pulsatile vortexing. The sample was icubated at 100°C for 15 min, then after, the sample was kept on ice. After centrifugation, the supernatant, which contained the DNA, was transferred into a new Eppendorf tube and stored at -20 °C to be used as a template for PCR [10].

Molecular Detection of *E.coli*

E. coli general primer (phoA gene) was used to detect *E. coli* then four serotypes specific primers (Table-1) were used to detect O1, O2, O18 and O78 serotypes by targetting rfbo1, rfbo2, rfbo18, and rfbo78 genes using PCR (Table 1). Briefly, 20µL of PCR reaction mix

was prepared by mixing 10µL of Taq master mix, 1µL for each forward and reverse primers and 2µL of DNA template, the volume was compeleted using nuclease-free water. The mixture was subjected to PCR amplification program started with an initial denaturation step at 94°C/10 minutes, proceeded through 36 cycles of denaturation at 95°C/35 seconds, annealing at 57-60°C/30 seconds, and extension at 72°C/30 seconds. A final extension step at 72°C/5 minutes, and held at 4°C. Then the amplicons were run on 1.5% agarose gel and visualised using gel documentation system (Figure-1)

Statistical Analysis

Cross-tabulation (SPSS) was used to analyze all data. Chi-square was used to find an association between the variables. P-Value less than 0.05 was considered statistically significant.

Results

The results showed that 67/86(77.9%) of the isolates had bright-pink colonies morphology on MacConkey agar, in which 62/86(72.1%) revealed metallic-green sheen morphology, and there was a highly significant (*P*=0.001) association between morphology of the colonies on MacConkey agar and EMB agar. The isolates that had no metallic-green sheen morphology were negative (24/86; 27.9%) for *E. coli*. While all the isolates (62/86; 72.1%), which were positive for *E. coli*, had metallic-green sheen colony morphology. All detected (23/86; 26.7%) serotypes (O2, O18 and O78) were characterised by having metallic-green sheen colony morphology. The results also showed that there was a highly significant (P=0.001) association between morphology of the grown *E. coli* colonies and serotyping (Table 2).

The results revealed that 62/86(72.1%) of the isolates were positive for *E. coli*, in which 23/86(26.7%) or 23/62(37.1%) of the isolates were posative for O2(8.1%), O18(16.3%) and O78(2.3%). O18 (14/23; 60.9%) serotype was found to have higher incidence compared to O2 (7/23; 30.4%) and O78 (2/23; 8.7%). While 24/86(27.9%) of the isolates were negative for all *E. coli* serotypes. The results also showed that there was a highly significant associations (*P*=0.001) between colibacillosis and *E. coli* (Table 3*)*.

Highest rate (32/86; 37.2%) of colibacillosis was found among 20-30 days-old chickens if compared to 10-20 days-old 30(34.9%) and over 30 days-old 24(27.9%) chickens. O18 was predominantly (7/86; 8.1%) detected among 20-30 days-old chickens, which was followed by O2 (4/86; 4.7%) in 10-20 days-old chickens. Highest rate of infection by the isolates was reported among 10-20 days-old chickens (10; 11.7%) and almost all serotypes were detected at this age range. Meanwhile the lowest rate (4; 4.7%) of infection was reported among over 30 days-old chickens. The results showed that there were not any significant (*P*=0.336) associations between colibacillosis and age of the chickens (Table 4).

The results showed that the majorities of the detected *E. coli* in colibacillosis cases were isolated from the imported chicks from Iran (30; 34.9%) and Netherlands (28; 32.6%). O18 was although predominantly isolated from Netherlands (7; 8.1%) and Iran (4; 4.7%) oriented chicks. The chi square analysis revealed no significant (P=0.515) association between *E. coli* and the sources of the chickens (Table 5).

Table 5: Colibacillosis and *E. coli* serotypes rate of detection according to the source of the chickens.

Colibacillosis VS Sources of the chickens																									
Colibacillosis			Sources																				Total	P-Value	
			Hawler		Netherlands		Iran		Portugal		Belgium		Turkey		Jordan										
E. coli	Negative	Count	5(20.8%)	2(2.3%)	8(33.3%)	9(10.4%)	8(33.3%)	7(8.2%)	2(8.3%)	1(1.2%)	1(4.2%)	3(3.5%)	0(0.0%)	1(1.2%)	0(0.0%)	0(0.0%)							24(100%)	0.515	
		Total%	5.8%		9.3%		9.3%		2.3%		1.2%		0.0%		0.0%								27.9%		
	O2	Count	2(28.6%)		2(28.6%)		2(28.6%)		0(0.0%)		1(14.3%)		0(0.0%)		0(0.0%)								7(100%)		
		Total%	2.3%		2.3%		2.3%		0.0%		1.2%		0.0%		0.0%								8.1%		
	O18	Count	0(0.0%)		7(50.0%)		4(28.6%)		0(0.0%)		2(14.3%)		1(7.1%)		0(0.0%)								14(100%)		
		Total%	0.0%		8.1%		4.7%		0.0%		2.3%		1.2%		0.0%								16.3%		
	O78	Count	0(0.0%)		0(0.0%)		1(50.0%)		1(50%)		0(0.0%)		0(0.0%)		0(0.0%)								2(100%)		
		Total%	0.0%		0.0%		1.2%		1.2%		0.0%		0.0%		0.0%								2.3%		
	Other Serotypes	Count	2(5.1%)		11(28.2%)		15(38.5%)		3(7.7%)		6(15.4%)		1(2.6%)		1(2.6%)								39(100%)		
		Total%	2.3%		12.8%		17.4%		3.5%		7.0%		1.2%		1.2%								45.3%		
Total			9(10.5%)		28(32.6%)		30(34.9%)		6(7.0%)		10(11.6%)		2(2.3%)		1(1.2%)								86(100%)		

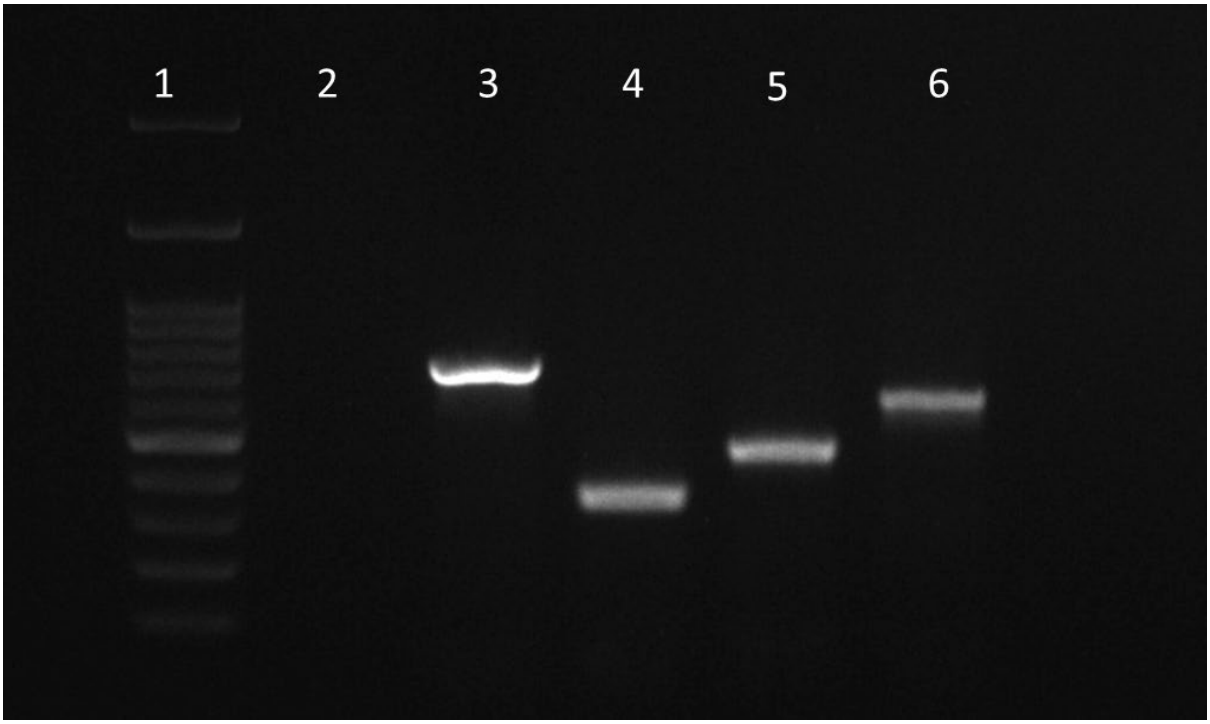

Figure 1: Gel electrophoresis image of *E.coli* and *E. coli* serotypes. Lane-1; DNA ladder-100, lane-2; *rfbO1* (255 bp), lane-3; *phoA E.* coli general (720 bp), lane-4; *rfbO2* (335 bp), lane-5; *rfbO18* (459 bp), lane-6; *rfbO78* (623 bp).

Table 1: Primers used to detect E. coli and O1, O2, O18, and O78 serotypes .

Names	Primers	Genes	Sizes	References
E. coli Universal primers	CGATTCTGGAAATGGCAAAAG CGTGATCAGCGGTGACTATGAC	phoA	720	Kaspersen, *et al* [26]
Serotype O1	CGATGTTGAGCGCAAGGTTG CATTAGGTGTCTCTGGCACG	rfbO1	255	Hu, Tu [27]
Serotype O2	CGATGTTGAGCGCAAGGTTG GATAAGGAATGCACATCGCC	rfbO2	335	Wang, Meng [28]
Serotype O18	CGATGTTGAGCGCAAGGTTG AGAAGCATTGAGCTGTGGAC	rfbO18	459	
Serotype O78	CGATGTTGAGCGCAAGGTTG TAGGTATTCCTGTTGCGGAG	rfbO78	623	

Table 2: Colibacillosis isolation and identification using MacConkey agar, EMB agar and PCR.

Colibacillosis			EMB								P-value
			Metalic-green sheen			Nonmetalic-green Sheen			Total		
MacConkey agar	Bright-pink colony	Count	62(92.5%)	23(26.7%)		5(7.5%)	0(0.0%)		67(100%)	23(26.7%)	0.001
		Total%	72.1%			5.8%			77.9%		
	Yellow Colony	Count	0(0.0%)			19(100%)			19(100%)		
		Total%	0.0%			22.1%			22.1%		
	Total		62(72.1%)			24(27.9%)			86(100%)		
E. coli PCR	Negative	Count	0(0.0%)			24(100.0%)			24(100%)		0.001
		Total%	0.0%			27.9%			27.9%		
	O2	Count	7(100.0%)			0(0.0%)			7(100%)		
		Total%	8.1%			0.0%			8.1%		
	O18	Count	14(100%)			0(0.0%)			14(100%)		
		Total%	16.3%			0.0%			16.3%		
	O78	Count	2(100%)			0(0.0%)			2(100%)		
		Total%	2.3%			0.0%			2.3%		
	Other Serotypes	Count	39(100%)			0(0.0%)			39(100%)		
		Total%	45.3%			0.0%			45.3%		
	Total%		62(72.1%)			24(27.9%)			86(100%)		

Table 3: *E. coli* and *E. coli* serotypes distribution according to the colibacillosis cases.

PCR			Colibacillosis						
		Other Serotypes	O1	O2	O18	O78	Total	P-Value
E.coli	Positive	Count	39(62.9%)	0(0%)	7(11.3%)	14(22.6%)	2(3.2%)	62(100%)	0.001
					23(37.1%)				
		Total%	45.3%	(0%)	8.1%	16.3%	2.3%	72.1%	
	Negative	Count	24(100%)	0(0%)	0(0.0%)	0(0.0%)	0(0.0%)	24(100%)	
		Total%	27.9%	(0%)	0.0%	0.0%	0.0%	27.9%	
	Total		63(73.3%)	0%	7(8.1%)	14(16.3%)	2(2.3%)	86(100%)	
						26.7%			
	Serotypes			0(0%)	7(30.4%)	14(60.9%)	2(8.7%)	23(100%)	

Table 4: Colibacillosis and *E. coli* serotypes rate of detection according to the age ranges in broiler chickens.

Colibacillosis VS age range of the chickens													
Colibacillosis		Age-ranges								Total			P-Value
		10-20 days old			20-30 days old			Over 30 days old					
Negative	Count	6(25%)			8(33.3%)			10(41.7%)			24(100%)		
	Total %	7.0%			9.3%			11.6%			27.9%		
O2	Count	4(57.1%)			2(28.6%)			1(14.3%)			7(100%)		
	Total %	4.7%			2.3%			1.2%			8.1%		
O18	Count	4(28.6%)			7(50.0%)			3(21.4%)			14(100%)		
	Total %	4.7%	10(11.7%)		8.1%	9(10.4%)		3.5%	4(4.7%)		16.3%		
O78	Count	2(100.0%)			0(0.0%)			0(0.0%)			2(100%)		0.336
	Total %	2.3%			0.0%			0.0%			2.3%		
Other serotypes	Count	14(35.9%)			15(38.5%)			10(25.6%)			39(100.0%)		
	Total %	16.3%			17.4%			11.6%			45.3%		
Total		30(34.9%)			32(37.2%)			24(27.9%)			86(100.0%)		

Discussion

Avian pathogenic *E.coli* was isolated from colibacilosis cases in broiler farms in Sulaymaniyah province. The results of our study revealed that 72.1% of colibacillosis cases in the broiler farms in the region were caused by *E. coli*. The same pattern of infection by *E. coli* in colibacilossis cases was also reported by other researchers (11, 13). 88.6% of APEC isolates were classified into *E. coli* serotypes in Korea. The proportion of *E. coli* serotypes isolates from chicken cloaca was 71.05% in Vietnam (7). In Poland, 23% of colibacillosis cases in broiler chickens were positive to *E.coli* (14). The incidence of *E. coli* in broiler chickens in the winter was 15.7% in healthy, 37.1% in sick and 55% in freshly dead chickens. However, it was 15.8%, 17.5% and 18.7% in healthy, diseased and freshly dead chicken in the Summer (15). High prevalence of *E. coli* infections in broiler chickens might be related to accumulation of *E.coli* aerosols in the atmosphere of chicken barns, which increase the chance of infection through inhalation (12, 16. 17).

The prevalence of the serotypes among *E. coli* positive samples were 37.1% (O2; 11.3%, O18; 22.6%, O78; 3.2%). A study in poland revealed that the prevalence of *E. coli* serotypes was 4%(O1), 1%(O2), 8%(O18) and 8%(O78) in broiler chickens (14). It was 23.79%(O78), 14.89%(O2), 12.63%(O1) in Jordan (18), 20.3%(O78) and 8.9% (O2) in Korea (19), 28.7%(O2) and 14.7%(O78) in Germany (20), and it was 10.56%(O18), 9.44%(O2), 7.79% (O1), and 6.56% (O78) in Southern Vietnam. The serotype O18 was mostly isolated from the wild animals, especially in geckos (7). While O78 was reported as a dominant serotype in Poland (14). A study in Iran showed that O1 (21.25%) and O78 (37.5%) were the most prevalent serotypes if compared to O2 (17.5%), O18(10%) and other serotypes (13.75%) (21).

The age of the broiler chickens also appears to have an effect on the type of the isolated serotypes, as Our results showed that almost all serotypes, especially O78, were isolated from 10-20 days-old chickens, and 50% of O18 serotypes were isolated from 20-30 days-old chickens. Previous studies determined that age might have an effect on getting an infection with a specific serotype (22).. Even an outbreak and mortality by colibacillosis appears to be affected by the age of the birds [23], the mortality rate by colibacillosis was higher (93%) among 11-15 days old chicks if compared to 6-10 days-old (83.33%) and 1-5 days-old chicks (21.42%) (24). A study in Bangladesh revealed that the prevalence of colibacillosis in broiler chickens was higher (1%) in 25-30 days-old chickens if compared to 31-35 days-old chickens (0.5%) (25).

In conclusion, the results showed that the majorities of the colibacillosis cases in the region were caused by *E. coli*. The *E. coli* serotypes, including O2, O18 and O78, had high prevalence in the region. The isolates, especially O78, were mostly isolated from 10-20 days-old chickens.

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التنميط المصلي الجزيئي لبكتيريا *Escherichia coli* في مزارع الدجاج اللاحم في محافظة السليمانية/العراق

بهار سعيد محمد[1], نهلة محمد سعيد[2] سادات عبد الله عزيز[3,4]

[1]- قسم مختبر الأحياء الدقيقة البيطرية بمديرية السليمانية في إقليم كردستان/العراق.
[2] - قسم الأحياء الدقيقة-كلية الطب البيطري، جامعة السليمانية، السليمانية، العراق.
[3] - قسم التخدير-كلية العلوم الصحية جامعة جيهان-السليمانية في إقليم كردستان/العراق.
[4] - قسم العلوم الأساسية-كلية الطب البيطري، جامعة السليمانية، السليمانية المدينة، العراق.

الخلاصة

الإشريكية القولونية (E. coli) هي بكتيريا سالبة الجرام لها أهمية اقتصادية وصحية عامة. تم تصنيف سلالات الإشريكية القولونية إلى سلالات مسببة للأمراض وغير ممرضة. يمكن أن تسبب سلالات الإشريكية القولونية المسببة للأمراض داء العصيات القولونية، وهو مرض بكتيري شائع في صناعة ومزارع الدواجن في المنطقة. كان الهدف من هذه الدراسة هو دراسة مدى انتشار بكتيريا الإشريكية القولونية وأنماطها المصلية (O1 وO2 وO18 وO78) وقابليتها لمضادات الحياتية في حالات داء العصيات القولونية في محافظة السليمانية باستخدام الزراعة وفحص الحساسية لمضادات الميكروبات والأساليب الجزيئية. تم فحص 86 مزرعة دجاج فروج في الفترة من نوفمبر 2021 إلى يونيو 2022. من كل مزرعة، تم أخذ عينات (كبد وقلب) من 3-5 حالات داء العصيات القولونية في دجاج اللاحم. أظهرت النتائج أن المستعمرات التي تحتوي على مورفولوجيات لمعان معدني أخضر كانت إيجابية بالنسبة للإشريكية القولونية (86/62؛ 72.1%)، حيث كانت 62/23 فقط (37.1%) من العزلات إيجابية بالنسبة لـ O2 (62/7؛ 72.1%). 11.3%)، 14/62) O18؛ 22.6%)، و2/62) O78؛ 3.2%). بينما كان O1 غير قابل للاكتشاف في حالات داء العصيات القولونية التي تم التحقيق فيها. تم اكتشاف O18 في الغالب (86/7؛ 8.1%) بين الدجاج بعمر 20-30 يومًا، يليه 4/86) O2؛ 4.7%) في الدجاج بعمر 10-20 يومًا. أظهرت النتائج أن أغلبية الإشريكية القولونية المكتشفة في حالات داء العصيات القولونية تم عزلها من الكتكوت المستورد من إيران (30؛ 34.9%) وهولندا (28؛ 32.6%). وفي الختام أظهرت النتائج أن غالبية حالات داء العصيات القولونية في المنطقة كانت ناجمة عن بكتيريا الإشريكية القولونية. وكانت بكتيريا الإشريكية القولونية وأنماطها المصلية (O2 وO18 وO78) منتشرة بشكل كبير في المنطقة.

الكلمات المفتاحية: الاشريكية القولونية, السليمانية, مزارع الدجاج اللاحم.