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Molecular detection of *Escherichia coli* from ovine aborted fetuses in Mosul city, Iraq

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Abstract

Abortion is one of the major problems in sheep farming. It can result in significant financial losses and presents a major risk to public health. Therefore, the study aimed to isolate *Escherichia coli* as possibly linked to ovine aborted fetuses, confirm the isolates using PCR, identify the virulence genes, and verify the genetic relatedness of local isolates. Between September 2023 and March 2024, a total of fifty swabs were collected from the stomach contents and placenta of twenty-five aborted fetuses. The swabs were cultured on selective media, and the isolates were characterized using biochemical tests and the Vitek2 system. The isolates that targeted the 23SrRNA gene were verified using PCR. Additionally, specialized primers for the *uidA* and *zapA* genes were used. The amplified 23SrRNA gene was sequenced and analyzed. The isolation results showed that *Escherichia coli* was detected in 10% of the specimens. The PCR results for all five isolates were positive for *E. coli*, with a product size of 232 bp. Furthermore, all isolates possessed the *uidA* gene 100%. Nevertheless, the *zapA* gene was present in 40% of the isolates. GenBank accession numbers PQ191249.1 and PQ191250.1 were registered for the 23SrRNA gene. Genetic relatedness shows that the local isolates were closer to *Escherichia coli* strain W170 and *Escherichia coli* O157:H7 strain K1516 from China and USA, respectively. According to these results, a small percentage of pregnant ewes with *Escherichia coli* infection might have an abortion. Consequently, appropriate precautions must be taken to reduce the infection risk.

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Introduction

Sheep are a significant source of both meat and milk for human use. The yearly need for animal-based protein is increasing. Therefore, maintaining excellent reproduction in herds is essential for overcoming the deficit since animal reproductive performance is correlated with the population's nutritional demands for meat, milk, and wool for industry (1). Worldwide, farmers suffer large financial losses as a result of domestic ruminant abortions (2). It is known as the fetus being forced out from the uterus before it is viable and fully developed (3). Ovine abortion can be caused by non-infectious factors such as nutritional deficiencies, genetic disorders, metabolic disorders, physical trauma, damage, and toxins, as well as infectious agents. Zoonotic pathogens like *Toxoplasma gondii*, *Campylobacter* spp., *Chlamydia abortus*, *Coxiella burnetii*, and *Brucella melitensis* are considered to be among the most common infectious causes of abortion in sheep, even though their frequency and occurrence vary depending on the geographic region (4-6). Within the genus *Escherichia* and family *Enterobacteriaceae*, *E. coli* is a rod-shaped, facultative anaerobic, gram-negative coliform bacterium (7). It is also a commensal bacterium species found in the gastrointestinal tract of many different kinds of mammals. However, not all *Escherichia coli* strains are beneficial; some may infect animals and cause illnesses (8,9). There are two types of pathogenic strains of *Escherichia coli*: those that induce

extraintestinal diseases and those that cause intestinal diseases (10,11). At the Veterinary Laboratories Agency-Weybridge, *Escherichia coli* was recovered in pure culture from the placentae and fetal stomach contents of a recently aborted fetus during the lambing season. Additionally, the combined clinical and pathological results indicated that maternal bacteremia and placentitis linked to a verotoxigenic strain of *Escherichia coli* were the cause of the abortion outbreak (12). The findings of Beutin *et al.* (13) indicate that pregnant ewes infected with verotoxigenic *Escherichia coli* may lose over 20% of their lambs. Sensible measures should be taken to reduce the danger of human infection since verotoxigenic *Escherichia coli*, like other causes of ovine abortion, can cause serious illness in humans. Identification of the etiological agent causing an abortion outbreak has important implications for disease control, prevention of future outbreaks, and management of dangers to public health (14).

Therefore, the current study aimed to isolate *Escherichia coli* as possibly linked to ovine aborted fetuses, confirm the isolates using PCR, identify the virulence genes, and verify the genetic relatedness of local isolates via 23SrRNA gene amplification, sequencing, and phylogenetic analysis.

Materials and methods

Ethical consent

The sample collecting methods were authorized in August 2023 under approval issue number UM.VET.2023.121 in 17/8/2023.

Samples collection

Between September 2023 and March 2024, fifty swabs were obtained from the stomach contents (25 swabs) and placenta (25 swabs) of twenty-five fetuses that ewes aborted during the latter stage of pregnancy. The swabs were placed in sterile tubes containing nutrient broth for the conventional isolation processes.

Bacterial isolation and culture media

The swabs were cultivated on MacConkey, Eosin Methylene Blue, and Brain Heart Infusion agar. The suspected colonies of *E. coli* were re-cultured based on their morphological characteristics and biochemical tests. The Vitek2 system (BioMerieux, France) was also used for further confirmation (15).

DNA extraction

DNA extraction was performed on all verified isolates to facilitate molecular conformation of the species and detect the virulence genes *uidA* and *zapA*. In accordance with the company's instructions, the AddPrep kit (Addbio, South Korea) was used to extract DNA. The DNA samples were kept at -20°C until utilized.

Polymerase chain reaction

All obtained isolates were molecularly confirmed using the 23SrRNA gene-specific primers ECO223-F and ECO 455-R. Additionally, PCR screening was performed on all isolates to verify the presence of two sets of virulence genes (*uidA* and *zapA*). All primers were obtained from Macrogen, South Korea, and the amplified products and primer sequences were summarized in table 1.

With the exception of the annealing temperature, the standard PCR technique was followed for all primers. In summary, 25 µl was used, which included 12.5 µl of Hot Start Taq Premix (2X) from Addbio, South Korea, 1 µl of each forward and reverse primer, 2 µl of extracted DNA, and 8.5 µl of PCR water. A thermocycler (BioRad, USA) was utilized for amplification. The conditions for PCR cycles are listed in table 2. The 1.5% agarose gel (Bio-Rad, USA) with 3 µl of GelRed safe Dye (Addbio, South Korea) was used to electrophorize the outcomes of the PCR. Each PCR product was placed into a corresponding well of a prepared agarose gel using eight microliters. A volume of 4 µl of DNA standard marker, 100 bp (Addbio, South Korea), was used to identify the acquired products. A UV transilluminator and digital camera (Bio-Rad, USA) were used to view the results.

Sequencing and phylogenetic analysis

Two amplicons were sequenced following PCR amplification of *the Escherichia coli* 23SrRNA gene (Macrogen, South Korea). The acquired gene sequences were analyzed using BLAST against GenBank-recorded *Escherichia coli* 23SrRNA gene sequences from different nations. The MUSCLE program was used to perform multiple alignments using MEGA 11 software. The 23SrRNA gene's phylogenetic analysis was conducted using the MEGA 11 program's Maximum Likelihood approach. The Maximum Likelihood tree's group resilience was assessed using one hundred bootstrap resampling (19).

Table 1: Primers, sequences, and product size utilized in the identification of *Escherichia coli* and virulence genes (*uidA*, *zapA*)

Primer name	Sequence of the primers (5^- to 3^-)		Size (bp)	Reference
ECO	223-F	ATCAACCGAGATTCCCCCAGT	232	(16)
	455-R	TCACTATCGGTCAGTCAGGAG		
uidA	F	AAAACGGCAAGAAAAAG CAG	623	(17)
	R	ACGCGTGGTTACAGTCTT GCG		
zapA	F	ACCGCAGGAAAACATATAGCCC	540	(18)
	R	GCGACTATCTTCCGCATAATCA		

Table 2: The cycling conditions utilized in PCR amplification

Phase	°C	Time	Cycles (n)
Initial Denaturation	95ºC	10 min	1
Denaturation	95ºC	45 sec	
Annealing	*ºC	45 sec	35
Extension	72ºC	1 min	
Final extension	72ºC	5 min	1
Hold	4ºC	∞	

* Annealing temperature at 55ºC for *E. coli* 23SrRNA gene, 58ºC for *uidA* and 59ºC for *zapA* genes.

Results

Bacterial culture and biochemical tests

The *Escherichia coli* was detected in 10% (5/50) of the specimens. There were 12% (3/25) in the contents of the fetal stomach and 8% (2/25) in the placenta. Both media (MacConkey and Eosin Methylene Blue agar) displayed bacterial colonies with a dark pink morphology. Furthermore, Vitek2 confirmed every *Escherichia coli* isolate following positive outcomes from the specific biochemical tests.

PCR for confirmation of the isolates and identification of virulence genes

Following gel electrophoresis, the PCR results for all five isolates were positive for *Escherichia coli*, targeting the 23SrRNA gene with a product size of 232 bp (Figure 1). Furthermore, molecular screening for virulence genes in *Escherichia coli* showed that all isolates possessed the *uidA* gene 100% (Figure 2). PCR for the presence of the *zapA* virulence gene revealed that 2/5 (40%) of the isolates had the gene (Figure 3).

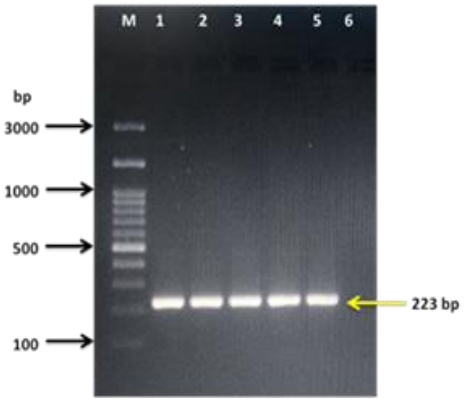

Figure 1: Agarose gel electrophoresis of PCR products. Lane M, DNA marker (100 bp); lanes 1-5 positive samples of *Escherichia coli* giving 232 bp product size; lane 6 negative control.

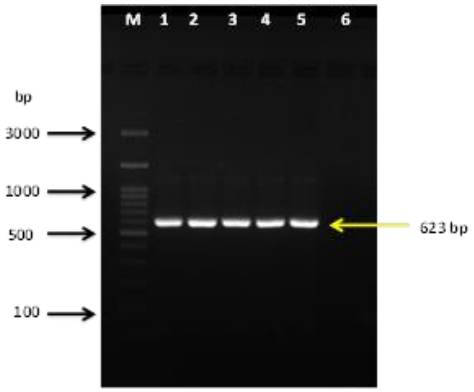

Figure 2: Agarose gel electrophoresis of PCR products. Lane M, DNA marker (100 bp); lanes 1-5 positive samples of *uidA* gene giving 623 bp product size; lane 6 negative control.

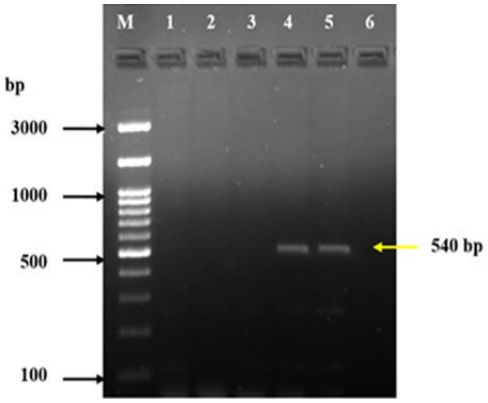

Figure 3: Agarose gel electrophoresis of PCR products. Lane M, DNA marker (100 bp); lanes 1-3 negative isolates; lanes 4 and 5 positive isolates of zapA gene giving 540 bp product size; lane 6 negative control.

Sequencing and phylogenetic analysis

The local two *Escherichia coli* strains (AOB-EC1-M24) with accession numbers PQ191249.1 and PQ191250.1, identified by sequence analysis of the 23SrRNA gene, exhibited 100% similarity with the previously published strains from China, USA, United Kingdom, Switzerland, Germany, South Korea, Japan, Turkey, Belgium, Finland, Australia, New Zealand and Spain (Table 3).

Additionally, fifteen 23SrRNA gene sequences from various *Escherichia coli strains* were combined into a Maximum Likelihood phylogenetic tree. The tree's confidence was ensured by using a 100 times bootstrap value. The phylogenetic tree showed the existence of two clades, with 100 similarities among the 23SrRNA gene sequence members, namely clade 1 and clade 2. Clade 1 consists of the strains CP114893.1, CP122648.1, CP140008.1, LR890508.1 and CP026027.1 from Germany, Belgium, Finland, Australia and Spain, respectively. Clade 2

consists of the strains PQ191249.1, PQ191250.1-CP163029.1, CP049612.1, CP099209.1, CP100525.1, CP114893.1, CP098217.1, AP021895.1, CP062924.1 and LR778148.1 from Mosul, China, USA, United Kingdom, Switzerland, South Korea, Japan, Turkey and New Zealand, respectively (Figure 4). Based on the generated phylogenetic tree, it has been determined that the 23SrRNA gene is preserved in all *Escherichia coli* strains, and local strains were more closely to *Escherichia coli* strain W170 (CP163029.1) and *Escherichia coli* O157:H7 strain K1516 (CP049612.1) from China and USA, respectively (Figure 4).

Table 3: Sequence identity between local *Escherichia coli* strain AOB-EC1-M24 (PQ191249.1, PQ191250.1) and other strains have recorded in the GeneBank.

No.	*Escherichia coli* strain	GenBank accession number	Country	Sequence identity
1.	*Escherichia coli* strain W170	CP163029.1	China	100%
2.	*Escherichia coli* O157:H7 strain K1516	CP049612.1	USA	100%
3.	*Escherichia coli* strain RHB16-SO-C08	CP099209.1	UK	100%
4.	*Escherichia coli* strain LH13-b	CP100525.1	Switzerland	100%
5.	*Escherichia coli* strain CM13	CP114893.1	Germany	100%
6.	*Escherichia coli* strain Z0117EC0032	CP098217.1	South Korea	100%
7.	*Escherichia coli* 2018-11-3CC DNA	AP021895.1	Japan	100%
8.	*Escherichia coli* strain 179	CP062924.1	Turkey	100%
9.	*Escherichia coli* strain ETEC4077	CP122648.1	Belgium	100%
10.	*Escherichia coli* strain ATCC 11303	CP140008.1	Finland	100%
11.	*Escherichia coli* isolate MSB1-9I-sc-2280417	LR890508.1	Australia	100%
12.	*Escherichia coli* isolate SC475	LR778148.1	New Zealand	100%
13.	*Escherichia coli* strain LIM	CP026027.1	Spain	100%

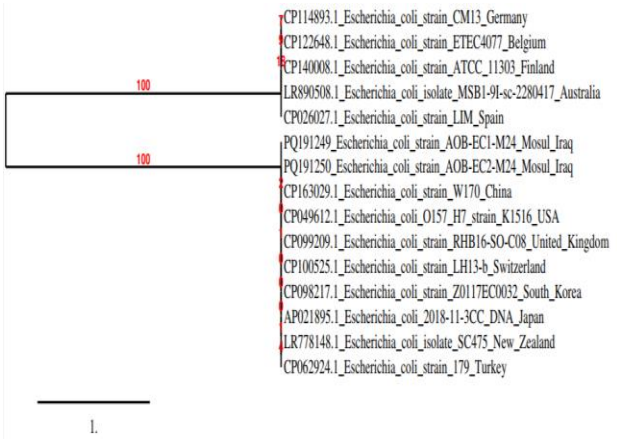

Figure 4: Phylogenic tree of local *Escherichia coli* strain AOB-EC1-M24 (PQ191249.1, PQ191250.1) and other strains have been recorded in the GeneBank. The 23SrRNA gene is preserved in all *Escherichia coli* strains, and these sequences' phylogenetic analysis mostly identified two clades with 100 similarities among the 23SrRNA gene sequence members.

Discussion

The most serious problem is herd abortion since it has a significant adverse economic impact on animal fertility, meat production, and milk supply (20). The present investigation detected *Escherichia coli* in 10% (5/50) of the specimens. This finding is consistent with the study by Esmaeili *et al.* (21), who found that the prevalence of *Escherichia coli* was low in aborted flocks, and this bacterium is the main cause of sporadic abortions (22). Also, this study was in line with the study's findings, which showed that 13 isolates, or 7% of the total, came from fetuses that had been aborted and showed signs of tissue degradation (23). According to earlier studies, endotoxins from gram-negative bacteria like *E. coli* can induce abortions through prostaglandin release (24,25). Conversely, *Escherichia coli* and other bacteria associated with sporadic abortions are probably opportunists (26). This outcome is also consistent with research by Van Engelen *et al.* (27), who found that *Escherichia coli* was the cause of 5% of sheep abortions among 98 ovine fetuses. However, the presence of *Escherichia coli* in aborted fetuses may be due to a lack of knowledge, unsanitary conditions in most rural regions, and environmental factors (28). The final confirmation of *Escherichia coli* was determined by the polymerase chain reaction technique, which has a high sensitivity for detecting bacteria based on DNA (29). Furthermore, molecular screening for virulence genes in *Escherichia coli* showed that all isolates possessed the *uidA* gene (100%). At the same time, PCR for the presence of the *zapA* virulence gene revealed that 2/5 (40%) of the isolates had the gene. *Escherichia coli* is recognized and categorized based on these genes that produce virulence factors. In order to detect and diagnose the genes involved in pathogenicity, the polymerase chain reaction is utilized, which sets it unique from other assays (30,31). These findings are consistent with the conclusions established by researchers regarding the

significance of virulence genes, which are essential for diagnosing bacteria because they produce the enzymes that the bacteria use in biochemical reactions.

Moreover, several virulence features that *Escherichia coli* possess, such as *uidA* and *zapA*, enable the bacteria to cause fetal mortality as well as antibiotic resistance (32-34). Two strains have the *zapA* gene, which produces the enzyme protease. It is considered one of the important enzymes because it can break down IgA and IgG antibodies, reducing the immune response in fetuses and causing abortion. For this reason, the enzyme is a significant virulence factor for the *Escherichia coli* that produces it (35,36). Table 3 displays the results of the 23SrRNA gene BLAST of the two strains against several strains accessible in Genbank. The 23SrRNA gene alignment data demonstrates the similarity (100%) between the aligned nucleotide sequences. The two strains under study tended to have a nucleotide sequence similar to those of the same species.

Furthermore, the phylogenetic tree showed the existence of two clades, with 100 similarities among the 23SrRNA gene sequence members. It has also been determined that the 23SrRNA gene is preserved in all *Escherichia coli* strains, and local strains were more similar to *Escherichia coli* strain W170 (CP163029.1) and *Escherichia coli* O157:H7 strain K1516 (CP049612.1) from China and USA, respectively. Based on these results, it is thought that the fifteen strains of the tree have similar genetic and metabolic characteristics and have very close relationships (37). The same findings were also demonstrated by earlier research by Abuelhassan *et al.* (38), and Ayoade *et al.* (39), who noted in their phylogenetic tree that the *Escherichia coli* isolates under investigation were very similar to those found in other regions of the world (40).

Conclusion

Escherichia coli should not be ignored as a cause of abortion; rather, it should be appropriately handled because it has been linked to abortion in pregnant ewes. Furthermore, the isolated *Escherichia coli* possess the virulence genes *uidA* and *zapA*, which enable the bacteria to cause fetal death and abortion. According to phylogenetic analysis, the local isolates of *Escherichia coli* seemed more like American and Chinese strains.

Acknowledgment

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Conflict of interest

The authors declare that they have no conflicts of interest related to the publication of this work.

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الكشف الجزيئي للإيشريكيا القولونية من أجنة الضأن المجهضة في مدينة الموصل، العراق

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الخلاصة

يعد الإجهاض من المشاكل الرئيسية في تربية الضأن، حيث يسبب خسائر اقتصادية كبيرة كما يشكل خطرا كبيرا على الصحة العامة. لذلك، هدفت الدراسة إلى عزل الإيشريكيا القولونية التي قد تكون مرتبطة بأجنة الضأن المجهضة، أكدت العزلات باستخدام تفاعل البلمرة المتسلسل، تحديد جينات الضراوة، والتحقق من الصلة الوراثية للعزلات المحلية. ما بين أيلول ٢٠٢٣ وأذار ٢٠٢٤، تم جمع خمسين مسحة من محتويات المعدة والمشيمة لخمسة وعشرين جنينا مجهضا. نميت المسحات على الأوساط الزرعية الانتخابية وصنفت العزلات باستخدام الاختبارات الكيموحيوية وجهاز الفايتك. تم التحقق من العزلات باستخدام تفاعل البلمرة المتسلسل مستهدفا جين 23SrRNA. فضلا عن ذلك، تم إستخدام بادئات متخصصة لجينات الضراوة (*uidA, zapA*). تم إجراء التسلسل الجيني والتحليل الوراثي بالاعتماد على نواتج تضخيم الجين 23SrRNA. أظهرت نتائج العزل بأن الإيشريكيا القولونية تم استنباتها من ١٠٪ من العينات. كانت نتائج تفاعل البلمرة المتسلسل لجميع العزلات الخمس إيجابية لجرثومة الإيشريكيا القولونية بحجم ٢٣٢ زوجا قاعديا. علاوة على ذلك، امتلكت جميع العزلات جين الضراوة *uidA* ١٠٠٪، بينما أظهرت ٤٠٪ من العزلات امتلاكها للجين *zapA*. سجلت تسلسلات الجين 23SrRNA بأرقام انضمام PQ191249.1 و PQ191250.1 في بنك الجينات. أظهرت العلاقة الوراثية بأن العزلات المحلية كانت أقرب إلى عزلة الإيشريكيا القولونية W170 وعزلة الإيشريكيا القولونية O157: H7 K1516 من الصين والولايات المتحدة الأمريكية على التوالي. وفقا لهذه النتائج، فأن نسبة قليلة من النعاج الحوامل المصابة بجرثومة الإيشريكيا القولونية قد تعرضت للإجهاض. وبالتالي، يجب اتخاذ التدابير المناسبة للحد من خطر الإصابة.