Iraqi Journal of Veterinary Sciences

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Molecular detection of *Escherichia coli* isolated from camel milk in Nineveh governorate

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Article information

Article history:
Received 20 June, 2023
Accepted 28 August, 2023
Published online 05 February, 2024

Keywords:
Mastitis
Camel
E. coli
PCR technique

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Abstract

Camel's milk is an extraordinary food with high nutritional value and has therapeutic uses due to its powerful antioxidants. Camels resist many diseases, but bacterial pathogens may cause serious diseases such as mastitis (clinical or subclinical mastitis). *Escherichia coli* is considered the most essential cause of camel mastitis. This study used conventional isolation and PCR methods to isolate *E. coli from* camel milk and detect virulence factors, such as (*Stx2* and *Stx1*). Fifty milk samples were obtained from camels with a single hump in the sparsely populated Badia Al Jazeera area of Al-Anbar and Nineveh provinces over a period ranging from February to May 2023. This study used the classical methods (media and biochemical methods) to isolate and identify *E. coli* and used the polymerase chain reaction (PCR) assay to detect the *uidA*, *Stx1*, and *Stx2* genes. The result explained the ability to isolate *E. coli* in 66% of camels who suffer from subclinical mastitis. Further analysis of the virulence gene reveals that different *E. coli* isolates can bear *Stx1* and/ or *Stx2* with 63% and 27%, respectively. The study concluded that the ability to isolate *E. coli* harboring many different virulence genes with a higher percentage of *Stx1* than *Stx2* is a public health concern.

DOI: 10.33899/ijvs.2023.141222.3106,

Introduction

Camelus dromedaries or Arabian camels are important livestock animals living in semiarid and arid environments (1-5); camel's number reached 12.5 million in 2003, according to WHO (1). In Iraq, Camels are one of the essential demotic animals in a Bedouin desert area and play a significant economic as a source of milk, wool, meat, and skin production (5-8). Camel's milk is an extraordinary food with high nutritional value and has therapeutic uses (5). camel milk is also considered a potent antioxidant (4); camels resist many diseases (9). However, many camels may be affected with mammary gland infection (mastitis), which may occur as clinical mastitis or subclinical mastitis, associated with pain, swelling of mammary glands with composition, and milk color changes (10-14). Many bacterial species can cause mastitis in camel, including many types of *Enterobacteriaceae* (5); camel mastitis is predominantly caused by *Escherichia coli* (*E. coli*), a Gram-negative rod-shaped bacterium. Notably, *E. coli* is commonly present in the intestinal tracts of both animals and humans (15). *Escherichia coli* can be classified into different pathotypes based on their virulence, pathogenicity, and site of infection. These pathotypes include commensal, enteropathogenic, and extraintestinal strains (16-20). Many *E. coli* is armed with virulence factors that enable them to survive in their host and environment (21,22). *E. coli* virulence factors may include capsule, adhesin properties, and production of toxins; many *E. coli* is considered intestinal pathogenic and divided into eight groups according to their infectivity and virulence (23-25). The *Shiga toxin-producing E. coli* (STEC), which contain Shiga toxins (*Stx1 and Stx2*), have foodborne importance as a primary causative agent of hemorrhagic colitis, hemolytic uremic syndrome, and bloody diarrhea; both these toxins encoding in locus for enterocyte effacement (LEE) pathogenicity island which that contain

another gene as *eae A* which less pathogenic than *Stx1* and *Stx*2 which responsible for severe infection in animals (26,27). The *Stx1* and *Stx2* genes produce many toxin subtypes; the *Stx*1 toxin includes three subtypes, while *Stx*2 has nine subtypes (28). Both the *Stx1* and *Stx2* toxins may cause food poisoning in humans after consuming raw milk contaminated with STEC, resulting in various signs and symptoms depending on the virulence of the *Stx* toxin subtypes (29,30).

In many cases, camel milk can be consumed unpasteurized due to its therapeutic worth (31) and rich nutritional value (5). This led to becoming an essential source of zoonotic disease; for this reason, this study aimed to isolate *E. coli* and detect Shiga toxins genes (*Stx1* and *Stx2*) in isolates.

Materials and methods

Sampling

In the present study, conducted between February and May 2023, 50 milk samples were obtained from female one-humped camels exhibiting subclinical - mastitis using the California Mastitis Test. The collection was conducted in the Badia Al Jazeera area of Al-Anbar and Nineveh province. Each sample, comprising 20 ml of milk, was obtained using sterile containers. To maintain sample integrity, they were transported in a cold box with CO_2 ice to the central Laboratory under cooling conditions.

Ethical approval

All samples were collected following owner approval, and the study was carried out based on the ethical guidelines provided by the Institutional Animal Care and Use Committee at Mosul University's College of Veterinary Medicine, with an authorized ID of UM.Vet.2023.016.

Isolation and identification of *E. coli*

The milk samples were examined to separate and identify pathogenic strains of *E. coli*. To do this, all samples were placed in nutrient broth (LAB, United Kingdom) and incubated for 24 h at a temperature of 37°C. To follow the classical method, a single loopful of the nutrient broth was spread onto Eosin Methylene Blue Agar (EMB) and MacConkey agar (LAB, United Kingdom). These plates were then incubated for 24 h at 37°C. Additionally, Brilliance *E. coli*/coliform Agar (Oxoid, United Kingdom) was employed to differentiate between generic *E. coli* and coliform bacteria. To confirm the presence of suspected *E. coli* isolates, various biochemical tests were conducted, including Gram staining, Indole testing, Methyl Red testing, Citrate Utilization testing, Voges-Proskauer testing, as well as Catalase, Oxidase, and Triple Sugar Iron agar (32). The *E. coli* isolates were preserved by being kept at -80ºC and maintained in a Nutrition broth that included 15% glycerol.

DNA Isolation

The subsequent protocols were executed in order to separate and examine dubious *E. coli* isolates. Prior to being incubated for 24 hours at 37°C, all the samples were cultured on Brilliance E. coli/coliform medium. *E. coli* and coliform bacteria can grow and differentiate more easily on this particular agar media. The DNeasy Blood and tissue kit from Geneaid (Korea) was used in accordance with the instructions to isolate the DNA of *E. coli*. With the help of this kit, you can reliably extract DNA from a variety of sources, including bacteria. The Bio-drop gadget was then used to quantify the isolated DNA, enabling precise calculation of the concentration of DNA. Ultimately, in order to preserve its stability and quality for further investigations, the extracted DNA from E. coli was kept at -20°C. By doing this, it is guaranteed that the DNA will stay intact and be prepared for additional research and experimentation.

U*id A, stx1,* and *stx2* Genes Amplification

As shown in table 1, the *uidA, Stx1,* and *Stx2* sequences of the isolated *E. coli* bacteria were amplified using the PCR technique. Twenty-five microliters were used in total for the PCR reaction. One microliter each of primers one and two, 6.5 microliters of DNeasy-free water (Promega Corporation, USA), 4 microliters of the *E. coli* DNA template, and 12.5 microliters of 2× GoTaq Green Mix Master (Promega Corporation, USA) made up the reaction mixture. After that, gel electrophoresis was used to visualize the target sequence amplicons. A 1.5% agarose gel (Peqlab, Erlangen, Germany) was prepared, and the DNA samples were loaded into wells along with a DNA marker (100 bp ladder). Electrophoresis was then performed, allowing for the separation and visualization of the amplified DNA fragments, which were compared to the DNA ladder for size estimation. The mixture was added to an Eppendorf tube, and the total volume was adjusted to 25 µl. The PCR amplification uses appropriate thermal cycling conditions. The specific thermal cycling conditions would depend on the PCR protocol, including the denaturation, annealing, and extension temperatures and durations. These conditions are typically optimized for each primer set, and the DNA template is amplified.

Results

The conventional microbiology diagnosis for camel's milk reveals isolation of 33/50 *E. coli* isolate, all *E. coli* isolate was emphasized using specific *uid A* gene that gives 632bp amplicon specific for genus *E. coli* (Figure 1), with a total isolation rate reaching 60% from all subclinical mastitis. Further analysis of *E. coli* isolates to detect *Stx1* and *Stx2* genes show that positive PCR amplicons (347 bp and 592 bp), respectively, some isolates carrying either *Stx*1 and /or *Stx*2 with positive occurrence reach 63% (21/33) and 27% (9/33) for *Stx1* and *Stx2* respectively (Figures 2 and 3).

Table 1: The sequence Primers and PCR program used for detecting the *uidA*, *stx1*, and *stx*2 gene

Gene	Primer	Sequence (5- 3)	Amplicon Size [bp]	Program	Reference
uid A	*uidA-1* *uidA-2*	5-CCAAAAGCCAGACAGAGT-3 5-GCACAGCACATCAAAGAG -3	623	I	(33)
stx1	*stx1-1* *stx1-2*	5-AGTTAATGTGGTGGCGAAGG-3 5-CACCAGACAATGTAACCGC-3	347	II	(33)
stx2	*stx2* *stx2*	5- TTCGGTATCCTATTCCCGG-3 5- CGTCATCGTATACACAGGAG-3	592	II	(34)

PCR program**:** I=35 times (94°C – 30s, 57°C – 30s, 72°C – 30s), II=35 times (94°C – 30s, 55°C – 30s, 72°C – 30s).

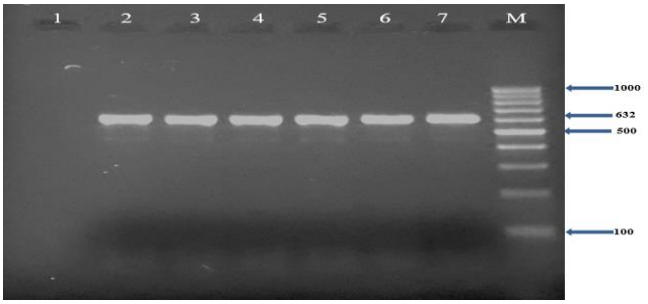

Figure 1: Length of Gene Sequences in Base Pairs: uidA (623 bp), M Lane: DNA ladder, 1 Lane: Negative Control, 2-6 Lanes: Positive *E. coli* isolates, 1 Lane: Positive Control (Shiga Toxin-Producing *E. coli*).

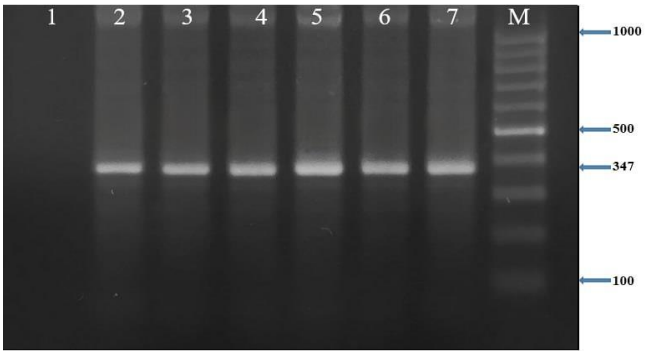

Figure 2: Length of Gene Sequences in Base Pairs: *Stx1* (347 bp), M Lane: DNA ladder, 1 Lane: Negative Control, 2-6 Lanes: Positive *E. coli* isolates, 1 Lane: Positive Control (Shiga Toxin-Producing *E. coli*).

Discussion

Like other dairy animals, camel mastitis is considered a severe disease that affects the quantity and quality of milk and increases the cost of treatment, leading to economic loss. Although the diagnosis of clinical mastitis could be made quickly with some tests, relatively little research has been focused on camel mastitis, particularly those caused by bacteria. Subclinical mastitis required more significant attention to confirm the health and safety of camels, particularly in desert areas (35).

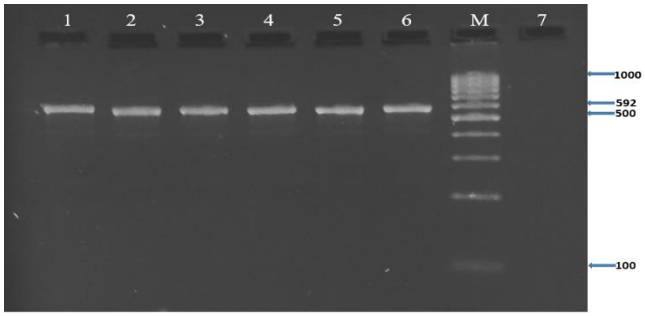

Figure 3: Length of Gene Sequences in Base Pairs: *Stx2* (592 bp), M Lane: DNA ladder, 1 Lane: Negative Control, 2-6 Lanes: Positive *E. coli* isolates, 1 Lane: Positive Control (Shiga Toxin-Producing *E. coli*).

Many research showed the ability to isolate *E. coli* in subclinical mastitis as the main causative agent (36). The present result showed that *E. coli* isolates, which PCR confirmed, reached 66% of all milk samples, which was higher than 3% in Oman (37), 7% in Saudi (38), and 36% in Egypt (39). In addition, the results of this study were near to the results of the previous study in Kenya, which found the percentage of *E. coli* isolated from camels' milk was 56.5% (40). The higher isolate percentage in the current study may be related to the fact that subclinical mastitis occurred in higher prevalence than clinical mastitis and required more time to be detected (35), farther than most information for subclinical bacterial mastitis depends on milk bacteriological examination and show that *Enterobacteriaceae* was predominant in mastitis (37,41); this lead to different bacterial type and isolation percentage comes from different research that can cause either clinical or subclinical mastitis.

The result showed that *E. coli* isolated harboring 63% and 27% for *Stx1* and *2*, respectively; many research listed different percentages for either *Stx*1 and *2* from different sources. Onlen *et al*. (42) reported that the percentage reached 15.6 % and 6.3% for *Stx2* and *Stx1*, respectively, in *E. coli* isolated from food; other research shows a higher percentage of *Stx2* and *Stx1* in *E. coli* isolated from dairy cattle which reach to 100% and 93.1% respectively (43) different result showed by other research includes 15% and 13% for *Stx2* and *Stx1* genes, respectively from human isolate (44). In comparison, Bulgarian cattle show 9.08% and

4.54% for *Stx2* and *Stx1* (45); in camel milk, the frequency of *Stx2* and *Stx1* may reach 77.7, 46.2 (39), while, another study found that 23.1% of *E. coli* isolates possessed the *Stx1* gene and no one of these isolates have the *Stx2* gene (40). This study agreed with Njage *et al.* (40) that the difference in virulence gene occurrence is closely related to animal species and sources of *E. coli* isolated. Diab *et* al. (39) mention that *Stx*1 was a frequently detected virulence gene among *E. coli* camel isolate, which comes compatible with the present results.

Conclusion

E. coli causes serious illness in camels and results in subclinical mastitis that may affect the quality and quantity of milk and result in economic and public health problems; our isolated *E. coli* harbor many different virulence genes with a higher percentage of *Stx1* than *Stx2*.

Acknowledgment

The authors greatly thank University of Mosul, College of Veterinary Medicine for providing facilities to achieve this study.

Conflict of interest

There is no conflicting interest.

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التشخيص الجزيئي لجراثيم الإيشريكيا القولونية المعزولة من حليب الجمال في محافظة نينوى

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الخلاصة

يعد حليب الجمال غذاء غير عادي فهو يمتلك قيمة غذائية عالية وله استخدامات علاجية بسبب مضادات الأكسدة القوية التي يمتلكها، على الرغم من ان الإبل لديها مقاومة للعديد من الأمراض ولكن بإمكان أن تصاب بأمراض خطيرة مثل التهاب الضرع (التهاب الضرع السريري أو تحت السريري) نتيجة مهاجمتها من قبل مسببات الامراض الجرثومي. وتعد جراثيم الإيشريكيا القولونية من اهم مسببات لالتهاب الضرع في الجمال. في هذه الدراسة تم استخدام الطرق التقليدية (الأوساط الزرعية والطرق البيوكيميائية) لعزل وتشخيص جراثيم الإيشريكيا القولونية واستخدمت طريقة تفاعل البلمرة المتسلسل (PCR) للكشف عن جينات *uidA* و *Stx1* و *Stx2* . هدفت هذه الدراسة إلى عزل جراثيم الإيشريكيا القولونية من حليب الجمال والكشف عن بعض عوامل الضراوة (*Stx1* و *Stx2*) باستخدام طرق الزرع التقليدية والطرق الجزيئية. شملت الدراسة جمع خمسين عينة من حليب الجمال ذات السنام الواحد المتواجدة في منطقة بادية الجزيرة بمحافظة الأنبار ونينوى في الفترة من شباط الى ايار ٢٠٢٣. اظهرت نتائج الدراسة بان نسبة عزل جراثيم الإيشريكيا القولونية كانت ٦٦٪ من حليب الجمال التي تعاني من التهاب الضرع تحت السريري، اضافة الى ذلك فقد اظهرت نتائج التشخيص الجزيئي لجين الفوعة أن عزلات جراثيم الإيشريكيا القولونية المختلفة كانت تمتلك على جين *Stx1* أو *Stx2* بنسبة ٦٣٪ و ٢٧٪ على التوالي. خلصت الدراسة إلى القدرة على عزل جراثيم الإيشريكيا القولونية التي تحتوي على العديد من جينات الفوعة المختلفة بنسبة عالية لجين*Stx1* مقارنة بـ *Stx2* والتي لها التأثير على الصحة العامة.