



Isolation and Identification of *Escherichia coli* from Buffalo's Milk using PCR Technique in Nineveh Governorate

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Abstract

SUBCLINICAL mastitis (SCM) poses a considerable burden and challenge to modern dairy management. The occurrence of *Escherichia coli* (*E. coli*) in numerous categories of food such as meat, egg, and milk also increases a public health concern, particularly through the consumption of unpasteurized dairy products, impacting human health. The primary objective of current study was to detect *E. coli* from states of sub-clinical mastitis in buffalo. The researchers employed classical methods for isolation and identification. Additionally, they utilized PCR (Polymerase Chain Reaction) techniques to detect specific virulence factors encoding genes, namely *Stx1*, and *Stx2*, which are associated with the pathogenicity of *E. coli*. Between March and July 2023, a total of fifty milk specimens were taken from buffalo. These specimens were obtained from diverse regions, including Kenitra, Haoy Al-Kanysa, Busyf, Al-Shamseat, Badoush, Kuba, and Tilkeif, all situated in Nineveh province. The findings of the current study indicated the proportion of *E. coli* detected in the milk was 42% (21 out of 50 specimens). Additionally, during the molecular monitoring for an exact *E. coli* gene, it was observed totally isolates (100%) have the *uidA* gene. One isolate of *E. coli* possessed the *Stx1* gene 1/21 (4.8%). The *Stx2* gene was not discovered in *E. coli*. The results of this research could contribute to addressing a key factor in subclinical mastitis in buffalo, offering potential benefits for the private sector in effectively managing the disease. *E. coli* isolates carry virulence factors that are responsible for causing food poisoning in humans.

Keywords: PCR method, subclinical mastitis, *E. coli*, Buffalo's milk.

Introduction

Buffalo's milk is important nutrition value because that it contains unique composition and numerous health benefits such as large amount of fat, protein, and minerals such as phosphorus, calcium, and iron [1]. Buffaloes play a significant role in global milk production, accounting for a large majority of it. Iraq is considered to be a highly significant country both in terms of milk production and its consumption. Buffalo's milk is more consumption in Iraq to a significant extent, perhaps even more so than cow's milk. Buffaloes in Iraq are categorized based on their habitat as either "marsh" or "city" buffaloes. The marsh buffaloes are known

as Al Ahwar marsh buffaloes and are specifically adapted to the marshland environment. On the other hand, the urban areas in Iraq are home to larger and more productive river-type buffaloes, which are known for their higher milk yield. There is also considerable diversity in the coloration of buffaloes that are found in urban areas [2].

Mastitis is widely acknowledged as one of the commonly ailments affecting dairy cattle, resulting in significant damages within the industry [3]. The primary reasons for the incurred losses are primarily attributed to a decline in both the quality and quantity of milk produced which are accompanied by an upsurge in expenses related to

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veterinary care and labor, other than an elevated ratio of rejecting [4]. Sub-clinical mastitis (SCM) is distinguished by alterations of the several properties of milk, along with pathological transformations occurring within the mammary gland [5]. The subclinical form of mastitis is reported to be significantly more common, with the prevalence rates estimated to be 15 to 40 times higher when they are compared to the clinical manifestation of mastitis [6]. While the specific causative agents of sub-clinical mastitis (SCM) can vary across different countries and studies [7]. Pathogenic *E. coli* is regarded as a common microorganisms accountable for environmental mastitis. It tends to infect the mammary gland during the initial phases of lactation, particularly if they were not promptly treated. If they were left untreated, this infection can have severe consequences and even be life-threatening [8]. Typically, this infection initiates as a sub-clinical infection and improvements to cause different forms of clinical mastitis for the period of the firstly months of lactation. Approximately 51% of clinical mastitis cases caused by *E. coli* were observed to originate during the dry stage [9]. In Iraq, previous research has identified *E. coli* as the primary factors responsible for causing mastitis in cattle [10]. Additionally, *E. coli* detected in milk of cattle and dairy farms [11]. Numerous strains of *E. coli* possess a virulence factors that enhance their ability to survive within their host organisms as well as in the surrounding environment. These virulence factors contribute to their pathogenicity and aid in their adaptation and persistence [12]. *E. coli* virulence factors encompass a variety of characteristics such as capsule formation, adhesion properties, and toxin production. *E. coli* are mostly associated with intestinal pathogenicity and are divided into eight collections depended on their infectivity and virulence traits. Based on the virulence factors, *E. coli* were divided to the different groups [13]. The raw milk was contaminated via STEC has ability to cause a various disease which related to the public health such as hemolytic uremic syndrome and hemorrhagic colitis especially when drinking or eating unpasteurized or non-sterilized milk and its products. [14, 15].

The main goals of this study was to detect *E. coli* in the sub-clinical mastitis in buffalos that was regarded as a major causative agent in the sub-clinical mastitis in animals, to use the traditional

methods to isolation and identification of pathogenic bacteria, and to discover of specific – specie *uidA* gene and Shiga toxin genes (*Stx1* and *Stx2*).

Material and Methods

Sampling

In the current study, the milk specimens from Buffalo that had subclinical mastitis using the California Mastitis Test that were obtained from March to July 2023. The milk specimens were collected from various regions, namely Kenitra, haoy Al-Kanysa, Busyf, Al-Shamseat, Badoush, Kuba, and Tlkeif) located in Nineveh province. Each specimen that was consisted of 25 ml of milk and was obtained using a sterile tube. The milk specimens were immediately transferred to the central laboratory in a cooled state using a cold box with CO₂ ice.

For Isolating and Identifying *E. coli*

The analysis involved isolating and identifying pathogenic bacteria in the milk specimens. This was accomplished by immersing all specimens in nutrient broth obtained from LAB (UK) and then incubating them (24 hours at 37°C). Following the conventional method, a small amount of the nutrient broth was spread onto EMB and Mac-Conkey agar from LAB (UK). These agar plates were then incubated for 24 hours at 37°C. Additionally, Brilliance *E. coli*/coliform Agar from Oxoid (UK) was utilized to separate amongst generic *E. coli* and coliform bacteria. Various biochemical tests, including Gram staining, Indole testing, Methyl Red testing, Citrate Utilization testing, Voges-Proskauer testing, as well as Catalase, Oxidase, and TSI agar [16], were conducted to approve the existence of suspicious *E. coli*. To preserve *E. coli*, they were kept in Nutrition broth containing 15% glycerol and were maintained at a temperature of -80°C to utilize to next exam.

DNA extraction

To extract and analyze the doubtful *E. coli* isolates, the following procedures were carried out. For amplification of the suspected *E. coli* isolates, all isolates were streaked on Brilliance *E. coli*/coliform media for 24 hours at 37°C to identify *E. coli*. Using the directions supplied, the DNeasy Blood and Tissue Kit from Qiagen (Germany) was used to extract DNA from *E. coli*. Next, for

estimation of the DNA concentration of extracted DNA, we used the English Bio-drop instrument, which offers precise measurement. The isolated DNA from *E. coli* was then kept in storage at -20°C to maintain its purity and stability during additional examinations.

Amplification of the Genes

The *uidA*, *Stx1*, and *Stx2* gene sequences were amplified using the PCR technique (Table 1). The entire 30 µl reaction of PCR was carried out. The reaction mixture included 15 µl of 2× GoTaq (Green Mix Master) from Promega Corporation (USA), primer F (1 µl), primer R (1 µl), double distillate water (9 µl) from Promega Corporation (USA), the *E. coli* DNA template (4 µl). Subsequently, the amplicons of the target sequences were visualized through gel electrophoresis.

To perform gel electrophoresis, a 1.5% agarose gel from Peqlab (Germany) was prepared, and the DNA samples, along with a DNA marker (100 bp ladder), were loaded into wells. Electrophoresis was conducted to separate and visualize the

amplified DNA fragments, which were compared to the DNA ladder for size estimation. The entire mixture was added to an Eppendorf tube, and the total volume was adjusted to 30 µl. The PCR amplification was implemented utilizing appropriate thermal cycling conditions. The specific circumstances, including denaturation, annealing, and extension temperatures and durations, varied depending on the PCR protocol used. These conditions are typically optimized for each specific primer set and DNA template being amplified.

Results

The conventional microbiology diagnosis for buffalo's milk reveals isolation of 21/50 *E. coli* isolate, all *E. coli* isolate was emphasized by using the PCE assay to detect the *uidA* gene which have the molecular weight 632 bp (Figure 1), with a total isolation rate reaching 42% from all subclinical mastitis (Table 2). In addition, the results of our work appeared that the only one isolate of *E. coli* had been possessed the *Stx1* gene 1/21 (4.8%) which have the molecular weight 347 bp (Fig. 2), and None of isolates have the *Stx2* gene (Fig. 3).

TABLE 1. The sequence Primers and PCR program used for detecting of the genes

Gene	Primer	Sequence (5' - 3')	Molecular weight [bp]	Program	Reference
<i>uidA</i>	uidA-F	5- CCAAAGCCAGACAGAGT -3	623	I	[17]
	uidA-R	5- GCACAGCACATCAAAGAG -3			
<i>Stx1</i>	Stx1-F	5- AGTTAATGTGGTGGCGAAGG -3	347	II	[17]
	Stx1-R	5- CACCAGACAATGTAACCGC -3			
<i>Stx2</i>	Stx2-F	5- CCATGACAACGGACAGCAGTT -3	779	III	[18]
	Stx2-R	5- CCTGTCAACTGAGCAGCACTTTG -3			

PCR program: I = 35 cycles (94 °C – 60 s, 57 °C – 60 s, 72 °C – 60 s), II = 35 cycles (94 °C – 60 s, 55 °C – 60 s, 72 °C – 60 s), III = 35 cycles (94 °C – 60 s, 62 °C – 60 s, 72 °C – 60 s)

TABLE 2. The percentage of the genes detected in *E. coli*

Gene	Positive <i>E. coli</i> (No.)	Positive <i>E. coli</i> (%)
<i>uidA</i>	21/50	42%
<i>Stx1</i>	1/21	4.8%
<i>Stx2</i>	0/21	0%

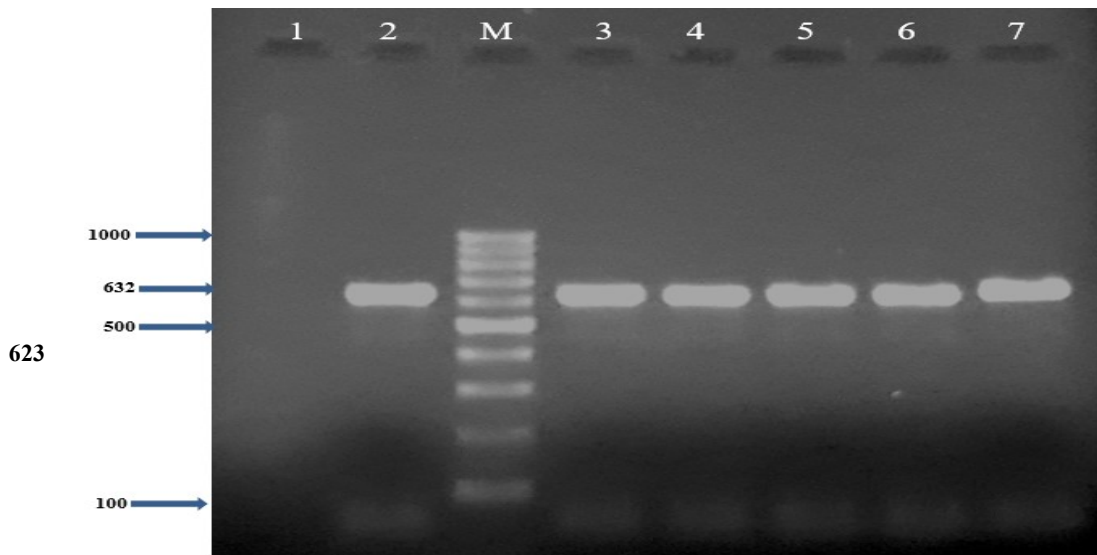


Fig. 1. The molecular weight of the typical amplicon of the *uidA* gene in *E. coli* was 623

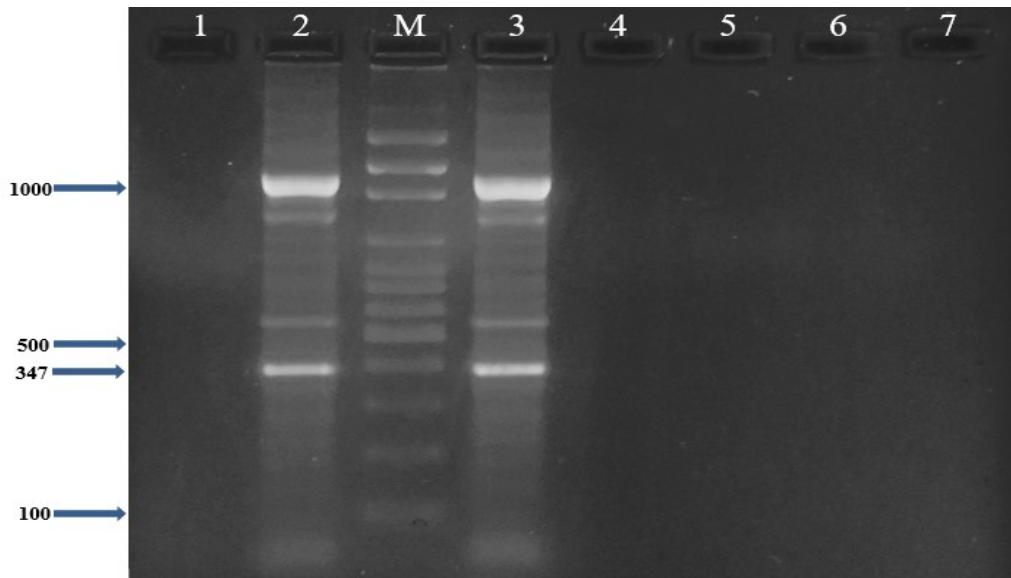


Fig. 2. The molecular weight of the typical amplicon of the *Stx1* gene in *E. coli* was 347

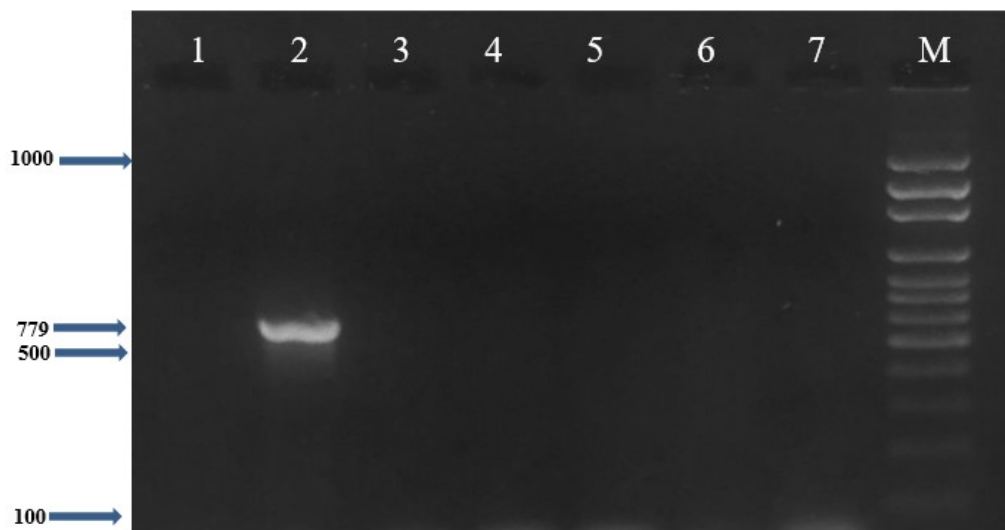


Fig. 3. The molecular weight of the typical amplicon of the *Stx2* gene in *E. coli* was 779

Discussion

Subclinical mastitis proves to be a troublesome issue on dairy farms, accounting for nearly seventy percent of economic losses from mastitis disease, and its occurrence is strikingly higher, ranging from 15 to 40 times more than clinical mastitis [19]. Many of previous study that appeared *E. coli* is the causative agent of the most occurrence diseases (sub-clinical mastitis) in animals [20]. Our results revealed that the occurrence rate of *E. coli* from sub-clinical mastitis in buffalo was 42%. This result is agreement with the study reported 42% [21]. While, our results were nearest from the previously studies found the occurrence rate of *E. coli* in buffalo was 43.3% [22] and 44.4% [23]. In addition, the present study appeared that our results were higher from another studies declared rate of *E. coli* in buffalo was 9.8% [24], 20% [25]. Furthermore, the result of present study was lower than the other research that showed the occurrence rate *E. coli* in subclinical mastitis in buffalo was in Pakistan 59.6% [26], in Iraq 66.6% [27]. The present study appeared that 4.8% (1/21) of *E. coli* possessed the *Stx1* gene. Many of studies revealed that among all *E. coli* isolates from buffalo's milk, those that lacked the *Stx1* gene were identified [28]. While, our result was lower from the previous studies which showed *E. coli* isolates possessed the *Stx1* was 28.6% in Egypt [29], in Iraq was 93.1% [30], and in India was 95.8% [31]. Additionally, the *E. coli* isolates did not possess the *Stx2* gene, our results was agreement with another studies which appeared the *Stx2* not found in all *E. coli* isolates [28, 32]. The results of recent study was lower than the previously studies which found the *Stx2* in *E. coli* isolates was 28.6% in Egypt [33], and 38.5% in Brazil [34].

The elevated occurrence of environmental pathogens, such as *E. coli*, identified in cases of subclinical mastitis, suggests inadequate farming practices and an overall deficiency in farm hygiene and sanitation [35]. Preventing and managing subclinical mastitis can be achieved through either before – milking or after - milking decontamination or by implementing alteration in milking methods, for example the use of a milking tool [36]. The infected cow treated with antibiotics is crucial for controlling of disease, as it has demonstrated success in eliminating current intra-mammary glands infections and preventing the emergence of new ones. Typically, intra-mammary or program of antibiotics are administered during the dry period as part of this therapy [37].

Conclusion

The *E. coli* causes serious illness in buffalo and result to subclinical mastitis that may effect of quality and quantity of milk and resulting to economic and public health problems, our isolated

E. coli harboring many different virulence genes with one isolate have the *Stx1* gene while none *E. coli* have the *Stx2* gene. The elevated incidence of *E. coli* isolated from disease suggests inadequate hygiene and substandard management practices on the farms. Buffalo milk may cause potential health risk to human.

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Conflict of Interest

I declare no conflicts of interest.

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عزل وتشخيص جراثيم الإشريكية القولونية من حليب الجاموس باستخدام تقنية تفاعل البلمرة المتسلسل في محافظة نينوى

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الخلاصة

يشكل التهاب الضرع تحت السريري (SCM) عبئاً وتحدياً كبيراً لإدارة منتجات الألبان الحديثة. يؤثر وجود جراثيم الإشريكية القولونية في الحليب أيضاً قلماً على الصحة العامة، لا سيما من خلال استهلاك منتجات الألبان غير المبسترة، مما يؤثر على صحة الإنسان. الهدف الأساسي من هذه الدراسة هو عزل والتعرف على الإشريكية القولونية من حالات التهاب الضرع تحت السريري في الجاموس. استخدم الباحثون طرقاً كلاسيكية للعزل والتعرف على الجراثيم. بالإضافة إلى ذلك، استخدموا تقنيات تفاعل البلمرة المتسلسل للكشف عن عوامل ضراوة معينة ترميز الجينات، وهي *Stx1*، و *Stx2*، و *rfb*، والتي ترتبط بأمراضية جراثيم الإشريكية القولونية. بين آذار وتموز 2023، تم جمع ما مجموعه خمسون عينة حليب من الجاموس. تم الحصول على هذه العينات من مناطق مختلفة، منها القنيطرة وحوي الكنيسة وبوسيف والشمسيات وبادوش وكوبا وتلكيف، وجميعها تقع في محافظة نينوى. أشارت نتائج الدراسة الحالية إلى أن نسبة عزل جراثيم الإشريكية القولونية المسببة للالتهاب الضرع تحت السريري الموجودة في عينات الحليب بلغت 42٪ (21 من 50 عينة). بالإضافة إلى ذلك، فإن طريقة تفاعل البلمرة المتسلسل أشارت إلى أن جميع جراثيم الإشريكية القولونية تمتلك الجين *uidA* وبنسبة 100٪. أظهرت الدراسة بان عزلة واحدة من جراثيم الإشريكية القولونية تمتلك جين *Stx1* 21/1 (4.8%). بينما لا تمتلك اي عزلة من جراثيم الإشريكية القولونية على جين *Stx2*. نتائج هذا البحث قد تساهم في التعامل بشكل جدي مع أحد المسببات الرئيسية في حدوث التهاب الضرع تحت السريري لدى الجاموس، مما يقدم فوائد محتملة للقطاع الخاص في إدارة الأمراض بشكل فعال. أظهرت نتائج الدراسة بان عزلات جراثيم الإشريكية القولونية تمتلك العوامل الضراوة التي تكون مسؤولة عن حدوث التسمم الغذائي في البشر.

الكلمات المفتاحية: تقنية تفاعل البلمرة المتسلسل، التهاب الضرع تحت السريري، جراثيم الإشريكية القولونية، حليب الجاموس.