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Molecular characterization and phylogenetic analysis of *Escherichia coli* isolated from milk of cattle affected by mastitis

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Abstract

This evolution-based study aimed to reliably identify the epidemiological prevalence of *Escherichia coli* that was recovered from affected milk of cattle by mastitis, study the evolution of this bacterium, and describe some isolates using polymerase chain reaction (PCR) technique and DNA sequencing. Here, we collected 50 cattle milk samples and submitted them to conventional bacterial isolation and identification using enrichment culture method and biochemical tests. Then, we confirmed the results by PCR technique based on 16S ribosomal RNA gene. The results showed that *E. coli* was isolated from cattle at (36%), and this was confirmed by PCR that showed highly specific detection of *E. coli* isolates at (100%). DNA sequencing of partial 16S ribosomal RNA gene showed (99%) homological identity with NCBI-Blast *E. coli* isolates and the phylogenetic analysis showed genetic similarity (0.5 genetic changes). In conclusion, this was the first study in Iraq to report genetic relationship between *E. coli* isolated from milk of mastitis-infected cattle. Therefore, it is essential to define the role of animals as an important source in the distribution of some pathogens that are related to public health.

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Introduction

Mastitis is considered as a devastating health problem in dairy animals. The condition is a global issue that affects milk-producing animals around the world leaving important economic crises in countries that relies on dairy production (1,2). This disease condition is characterized by inflammation of mammary gland tissues that is caused by various inflammation inducers such as bacteria and viruses (3). This illness generates multiple problems such as animal health, economic, and even milk quality problem especially when dairy cattle undergo an apparent mastitis such as subclinical mastitis (4).

The disease condition is predisposed via different reasons such as hygiene, management, and milking process errors. Precautionary criteria such as cleaning and disinfecting teats of dairy cattle before milking are important in limiting the occurrence of new infections of mastitis (5). Moreover, mastitis is presented via losses in milk production at 49% and veterinarian services at 37% of total losses (6). When the disease condition is predisposed, causative agents such as bacteria induce mastitis in different stages of peracute, acute, subacute, and chronic levels. Wide range of bacteria that cause inflammation to the mammary gland tissues is presented by both Gram positive and negative. However, certain groups of bacteria play important roles in these infections such as *Staphylococcus* spp and *Escherichia coli* (7).

In some cases, *E. coli* represents the most influential bacterium that causes clinical mastitis (8). The current study was focused on the molecular identification of *E. coli* in the affected mammary glands with mastitis in cattle. Molecular detection of *E. coli* by PCR technique has led to rapid, very sensitive detection of *E. coli* from food samples and samples from clinical test. Many types of food have PCR inhibitors, which effect on purification of the target DNA, so sampling requiring enough samples prepare to eliminate or inactivation inhibitors before PCR test

(amplification). The very low level of *E. coli* in many cases in food samples, lead to amplification step of it treats the low sensitivity, so that make the time to complete the test need at least 24 hours. So that a very sensitive, specific and very rapid test need to isolate and detect *E. coli* we need to find (9).

This study was intended to further understanding the evolutionary history of this bacterium in the Al-Diwaniyah City, Iraq.

Materials and methods

Sampling

Milk was sampled from cattle apparently suffered from mastitis that had been identified via California-mastitis test (CMT) from different locations in Al-Diwaniyah City. The milk of each sample was poured into 25 ml sterile containers.

The milk was collected after cleaning and washing the quarters of udders by disinfectant solution. After that, the milk samples were transported into laboratory and stored in a 4ºC until use for bacterial isolation.

Bacterial isolation

First, the milk samples were inoculated onto brain heart-based infusion-broth and were left in an incubator for 18hrs. Second, the resulted growth was cultivated in EMB-based agar and blood-based agar and was left in an incubator for 18hrs.

Extraction of DNA and PCR

To detect *E. coli* isolates via the use of PCR targeting a specific sequence region in the *16S rRNA* gene, the following steps were followed. First, bacteria were exposed to DNA extraction using Presto Mini-DNA Bacteria Kit (Geneaid Biotech Ltd. USA)**.**

The manufacturer's instructions were followed that included making 11mg/ml proteinase k buffer. Second, the extracted DNA was evaluated using NanoDrop to check for its quality and quantity. Primers were designed using NCBI Websites and Primer 3 Plus software. The resulted primers were F: GGAACTGAGACACGGTCCAG and R: CGTCAGTCTTCGTCCAGGG. They targeted a specific region, 438bp, in the studied gene. These primers were purchased from Bioneer Company, Korea.

The master mix was generated using Accu-Power® PCR-PreMix-Kit and following the kit instruction. Amounts of 2.5 µl DNA, 1.25 µl from each primer, and 15µl water for molecular use. The mix was placed in the kit specific tube that contains polymerase, dNTPs, and buffer. The mixes were then vortexed for 3 minutes. Finally, the tubes were transferred to a thermocycler (T100 Thermal cycler BioRad. USA) in which the conditions were 1 cycle of first DNA-based denaturation at 95ºC 5min, 30 cycles of (DNA-based denaturation at 95ºC 30s, DNA annealing at 58ºC 30s, and DNA-based extension at 72ºC 1min), and 1 cycle of finishing extension at 72ºC 5min (According to the primer manufacturer's instructions).

The resulted PCR-based products were examined using 1% gel electrophoresis with the use of ethidium bromide and investigated under UV imager.

16S rRNA gene partial sequencing

The PCR-based product was recovered from the used gel via the use of (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic, Canada).

The resulted samples were sent to sequencing (Macrogen, Korea). The phylogenetic tree was built utilizing the UPGMA-based method, MEGA 6.0.

Results

Cultivation and PCR

E. coli isolation results were 18 (36%) positive out of 50 samples. PCR confirmation of the resulted isolates was successfully generated using the primers that targeted a 438bp region in the 16S rRNA gene in the *E. coli* (Figure 1).

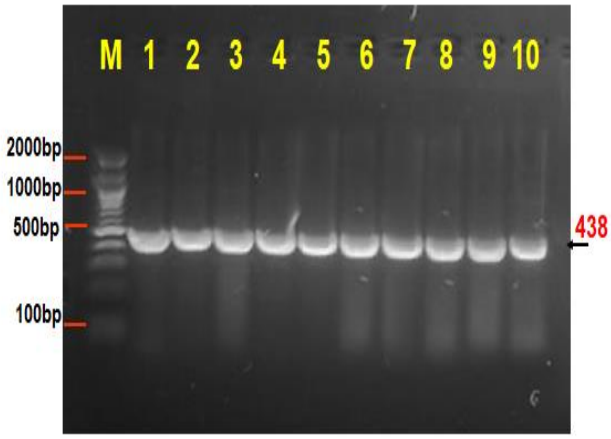

Figure 1: PCR-based gel electrophoresis. The*16S rRNA*-based amplification in *E. coli* isolates from mastitis infected milk. Lane (M) represents the DNA marker (2000-100bp), Lane (1-10) show 10 positive samples at 438bp-size of amplification.

DNA Sequencing analysis results

The results regarding the DNA sequencing further confirmed the identity of the causative agent, *E. coli*, which induced mastitis in the affected dairy cattle.

The current study isolates, when phylogenetic analysis was done, showed homological sequence identity with global isolates at 99% matching rate and which shown as phylogenetic tree (Figure 2).

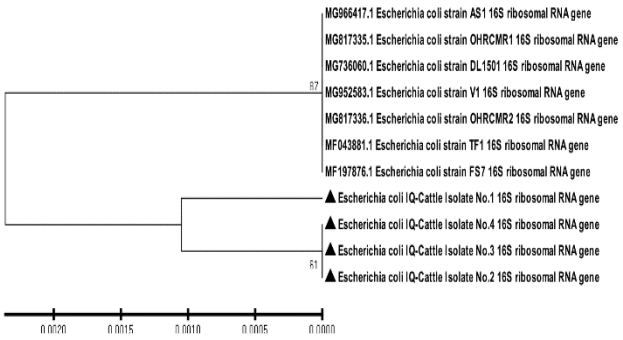

Figure 2: Phylogenetic tree targeting *16S rRNA*. The isolates have 99% identity with global *E. coli* isolates.

Discussion

The condition, Mastitis, is manifested by the inflammation-based effects of udder gland tissues, and it still represents among the most major health problems that affect cattle around the world. The disease condition causes huge losses in various aspects of dairy industries from health of affected animals, losses in the veterinary services, and to the reduced quality and quantity of the produced milk (10-13). This illness is caused by various bacterial and viral agents such as *Staph. aureus*, *Streptococcus agalactiae*, and *E. coli* (14). The current study was focused on the common causative agent, *E. coli*, which influences cattle udder glands causing mastitis. Such attention to this microorganism was made to study the evolution of this bacterium in the affected cattle in Al-Diwaniyah City, Iraq. Consistent with other previously made studies (15-17), our results showed the presence of this bacterium in an important rate, 36%, in the milk of the affected cattle. The PCR showed high ability to identify the causative agent in this study. This agrees with Gianneechini *et al*. (11) who detected, using *16S rRNA* gene-based PCR method, *E. coli* as a leading inducing bacteria for mastitis. The results of the partial sequencing targeting a specific region in the *16S rRNA* gene showed the reliability of the used method to correctly identifying the presence of *E. coli* in the affected cattle in the current study and showed close relationship to certain isolates of this bacterium from the world. The 99% identity presented in this study may indicate tiny nucleotide differences between our isolates and the world bacteria. This also indicates that these bacteria in the current study might have suffered certain deleterious mutations that led to such differences in the nucleotide sequences. This result matches with previously works which identified that these bacteria may arise from the environment contaminated with gut bacteria (18-21). This study confirms the *E. coli*-based infection in the tested milk of the affected cattle in the studied city, and it provides valued information about the trusted use of methodology in detecting the causative agents that infect mammary glands of dairy animals.

Conclusion

This was the first study in Iraq to report genetic relationship between *E. coli* isolated from milk of mastitis-infected cattle, and it is essential to define the role of animals as an important source in the distribution of some pathogens that are related to public health.

Acknowledgments

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Conflict of interests

The authors have not received any funding or benefits from industry, agency of financing, or elsewhere to conduct this study.

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الصفات الجزيئية والتحليل الوراثي للاشيريشية القولونية المعزولة من حليب الأبقار المصابة بالتهاب الضرع

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الخلاصة

هدفت هذه الدراسة القائمة على التطور إلى تحديد الانتشار الوبائي للإشريشية القولونية التي تم جمعها من حليب الماشية المصاب بالتهاب الضرع، ودراسة تطور هذه البكتيريا، ووصف بعض العزلات باستخدام تقنية تفاعل البوليمير المتسلسل وتسلسل الحمض النووي. هنا، قمنا بجمع ٥٠ عينة من حليب الماشية وعزل البكتيريا التقليدية وتحديد الهوية باستخدام طريقة الاستزراع والاختبارات البيوكيميائية. بعد ذلك، أكدنا النتائج تقنية تفاعل البوليمير المتسلسل بناء على جين للحمض النووي الاوكسجيني الريبوسومي ١٦س. أظهرت النتائج أن الإشريشية القولونية تم عزلها من الأبقار بنسبة ٣٦٪، وهذا ما أكده تفاعل البوليمير المتسلسل الذي أظهر الكشف النوعي العالي لعزلات الإشريشية القولونية عند ١٠٠٪. أظهر تسلسل الحمض النووي للجين الجزئي للحمض النووي الاوكسجيني الريبوسومي ١٦س ٩٩% هوية متجانسة مع عزلات الإشريشية القولونية وأظهر تحليل النشوء والتطور تشابهًا وراثيًا ٠,٥ تغيرات جينية. في الختام، كانت هذه أول دراسة في العراق تشير إلى وجود علاقة وراثية بين الإشريشية القولونية المعزولة من حليب الأبقار المصابة بالتهاب الضرع. لذلك، من الضروري تحديد دور الحيوانات كمصدر مهم في توزيع بعض مسببات الأمراض المتعلقة بالصحة العامة.