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Molecular study on acrAB, oqxAB, and marA genes of *Escherichia coli* isolated from patients in the AL-Diwaniyah City, Iraq

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ABSTRACT

The most frequent Gram-negative bacterium that causes urinary tract infections is *Escherichia coli*, recognized as an opportunistic pathogen. *E. coli* strains account for more than 85% of UTI cases. This study's goal is to identify the efflux pump genes (acrAB, oqxAB, and marA) that were isolated from a variety of UTI patients under the supervision of a specialist physician at two healthcare facilities, AL-Diwaniyah General Teaching Hospital and Maternity and Pediatrics Teaching Hospital in the AL-Diwaniyah city, Iraq. The study investigated the prevalence of *E. coli*, which causes UTIs, which included 200 urine samples from individuals with urinary tract infections (UTIs) ranging in age from 5 to 62 years, which were tested for bacterial infection. Cultures of urine samples were isolated from 200 suspected patients with urinary tract infection (UTI) on blood and MacConkey agar and confirmed by the Vitek-2 system. Our results show that only 50 (39%) have been positively diagnosed with *E. coli* out of 200 patients, and an antibiotic sensitivity test was performed using the same system. AcrAB, oqxAB, and marA are efflux pump genes expressed using real-time PCR. The study revealed that 50 (100%) of the *E. coli* isolates had a high rate of oqxAB gene expression (15.22%), while the ratios of acrAB and marA genes were 5.88% and 4.03%, respectively. In conclusion, the majority of *E. coli* isolates studied were obtained from individuals suffering from multiple drug-resistant (MDR) urinary tract infections.

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Introduction

As an opportunistic pathogen, *Escherichia coli* is a type of bacteria that typically lives in the digestive system (Riley et al. 2020; Krawczyk et al. 2021; Foster & Pallen 2022; Ribeiro et al. 2023), and it causes around 90% of urinary tract infections in young women (Ribeiro & Atiyea 2021; Assafi et al. 2022). The rod-shaped, facultatively anaerobic or aerobic *E. coli* bacteria are Gram-negative, non-spore-forming microorganisms (Andhikawati & Permana 2022; Malabadi et al. 2024). These bacteria measure from 1.0 and 3.0 µm in length and 0.5 µm in diameter. There is a single layer of peptidoglycan inside the periplasm. (Rohmah et al. 2018). Based on its genetic composition. Four phylogenetic groupings include *E. coli*: A, B1, B2, and D (Kempf et al. 2016; Yu et al. 2021). Several virulence factors, including adhesions, capsules, siderophores, toxins, and others, affect *E. coli's* ability to cause urinary tract infections. (Parvez & Rahman 2018; Sora et al. 2021). This prokaryotic model organism is well-studied in the domains of microbiology and biotechnology. It is frequently used as a sign of contaminated water since it can persist for long periods in faeces, soil, and water. bacterial pathogenicity" describes a bacteria's genetic capacity to cause illness. Resistance and virulence characteristics determine this (Diard & Hardt 2017). *E. coli* possessed some mechanisms of MDR, such as beta-lactamase production, porin loss, and efflux pumps.

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The efflux pumps are protein carriers located in the cell membrane. They play an important role in transporting and excreting various substances outside the cell to eliminate their harmful effects, so it is an important means of bacterial resistance to antibiotics (Altınöz & Altuner 2019). These pumps give bacteria the ability to be intrinsically resistant within the cell, giving the cell its inherent or intrinsic resistance.

Division Family (RND) - Nodulation - Resistance This is one of the most well-known families of efflux systems, and *E. coli* is the most common type of Gram-negative bacteria that contains it (Auda et al. 2020). The most prevalent efflux system in *E. coli* bacteria is the *care* system. The *crab* genes are an operon component, making it especially active while the bacteria grow in nutrient-poor media or during the stationary phase (Mutanda et al. 2022).

The genes oqxA and oqxB, found in the same operon, encode oqxAB, an efflux pump belonging to the RND family. Li et al. (2019), Ferrand et al. (2020) and Ding et al. (2023) show how different genes and stimuli regulate marA-mediated MDR at different levels, making it intricate and well-tuned and linking it to global cell regulation and metabolism. Control of this kind might target antibiotic-resistant *E. coli* and related pathogens while aiding in the adaptability and spread of MDR strains. Antimicrobial resistance (AMR) poses a threat to undoing the fundamental advantages of antibiotics, affecting not only the human population and jeopardizing decades of progress in healthcare outcomes but also the food production sector. Food safety is essential to enhancing and bolstering global health and ensuring sustainable development as the world progresses towards the Sustainable Development Goals.

According to Ferrer et al. (2017), AMR is one of the top ten dangers to global health, according to the World Health Organization (WHO). Several common illnesses from antibiotic resistance include respiratory and urinary tract infections. This condition is considered tough to cure because of the increased antibiotic resistance of the bacteria. Investigating the regulation of these genes may lead to the development of strategies to combat antibiotic resistance. The regulation of efflux pump genes is affected by many things, such as genetics, the environment, and antibiotics (Auda et al. 2020).

The current work intends to quantify the resistance gene expression rate in patients with UTIs about the *E. coli* efflux pump genes, acrAB, oqxAB, and marA. Since every bacterial isolate included every gene, real-time PCR—a modern technique—was used. The concentration of the sample, the circumstances of sample collection, and the degree of patient inflammation all impacted on the gene expression rate, which differed amongst isolates.

Materials and Methods

Sampling

The present study enrolled 200 samples from patients with urinary tract infections (UTIs) in the age range of 5-62 years; various genders were investigated for bacterial infection using bacteriological culture of urine samples under the supervision of a specialist physician at two healthcare facilities: AL-Dewaniyah General Teaching Hospital and Maternity and Paediatrics Teaching Hospital in the city of AL-Diwaniyah.

The data collection occurred from October 20, 2023, to January 15, 2024. All urine samples were inoculated on specific media (blood and MacConkey agar) using a sterile loop. The Blood and MacConkey were manufactured by an Indian company (Himedia), which is differential media. After that, the agar plates undergo a 24-hour aerobic incubation at 37°C. Moreover, after that, the bacteria were identified by laboratory tests, and then the automated approach Vitek®2 AST-N222 (BioMeneux, Turkey) was used to make the diagnosis, and a facilitation document was issued by the College of Medical Biotechnology.

Antibiotic susceptibility test

The Vitek system (BioMeneux, Turkey) is a widely used automated system for performing antibiotic susceptibility testing (AST) on bacteria. Here's a general overview of the protocol but refers to your specific instrument's manual for detailed instructions**:** Vitek instrument, appropriate Vitek test card for the bacteria being tested (e.g. GN card for gram-negative bacteria), isolated bacterial colony, sterile saline solution, and McFarland standards for inoculum standardization (Al Bulushi et al. 2021; Gajic et al. 2022).

Quantitative Reverse Transcription Real-Time PCR (RT-qPCR)

We used the quantitative real-time PCR method to measure the efflux pump gene expression of drug-resistant *E. coli* isolates and a housekeeping gene as a standard. The strategy adhered to the steps described in the research was according to Gomes et al. (2018) and Nove et al. (2020).

Total RNA extraction

The following steps, which were exactly as the manufacturer instructed, were used to get total RNA from MDR *E. coli* isolates using total RNA extraction kits. After adding 2 µg/ml of inducible ethidium bromide to bacterial isolates in Luria Bertani broth and letting them sit at 37ºC for a while to make bacterial cells (OD600: 0.8–1.0), the supernatant was obtained by centrifuging the bacterial cells at 10,000 rpm for one minute. 1 ml of

Trizol reagent was added to the bacterial pellets, and they were violently vortexed for ten seconds at room temperature. After adding 200 µl of chloroform to each tube, they were shaken vigorously for a minute. We incubated the mixture on ice for five minutes. We centrifuged the mixture for fifteen minutes at 13,000 rpm at 4°C. 500 µl of isopropanol was added to a new 1.5 ml microcentrifuge tube containing the supernatant. After integrating the mixture, rotate the tube four or five times and let it sit at 4°C for ten minutes. The tube should be centrifuged for 10 minutes at 4°C and 13,000 rpm. After discarding the supernatant once more, we added 1 ml of 80% ethanol and vortexed it. Next, we centrifuged the mixture at 13,000 rpm for 15 minutes at 4°C. We allowed the RNA pellet to air dry and disposed of the supernatant. In each sample, 100 µl of free nuclease water was mixed with the RNA pellet. The extracted RNA was then kept at -80°C.

The Real-Time PCR primers

The NCBI-Gene bank sequence and Primer3 Plus were used in this study to make real-time PCR primers that were used to find and measure efflux pump genes in *E. coli* isolates (Table1). The Iraqi Scientific Researcher Company designed all primers using Primer 3 Plus.

Table 1. qPCR detection gene primers for *E. coli* with their nucleotide sequence.

Gene	Sequence (5'-3')	Size	NCBI-Reference code
gap A gene	F: AGGCGAAATGAAAGGCGTTC R: AAGTGCAAACTTCGCCGTTG	77 bp	EU014639.1
acrAB gene	F: AAAATTCCCTGGCGATCCAC R: TGCACAACAGCCATTTCTCC	144bp	EU904994.1
oqxAB gene	F: AAAGTGACCGCCCCTATTGAC R: ACTCGTCGACGTCAAAGTAGAC	142bp	OK668389.1
mar A gene	F: GATGACAACCTGCATCAACCG R: AAAACAGCCGTTGCAGATGC	85bp	MW999402.1:294-635

Statistical analysis

Version 26 of the Statistical Package for the Social Sciences (SPSS) statistically analyzed the data and then presented the findings. We expressed the qualitative variables as numbers and percentages. All normally distributed variables were presented as mean and standard deviation (SD). To estimate the relationship between any two categorical variables, we use the chi-square test. To estimate the difference in the Mean between any two normally distributed variables, we used the independent sample t-test. The level of significance was found when the P-value was 0.05 or less. A 95% Confidence Interval (CI) and an odds ratio (OR) were also found for factors that were significant and those that were not (Rehak et al. 2008).

Ethical disclosures

In this research, the College of Medical Biotechnology at Al-Qadisiyah University in Iraq issued a facilitation document (document number 1576, 2023/09/17), and we followed the regulations of the Clinical Research Ethics Committee. The authors declare that they did not use artificial intelligence in the preparation of this manuscript for any purpose. The authors also affirm that all data presented in this study were collected and analyzed in accordance with ethical guidelines, ensuring transparency and integrity throughout the research process. Furthermore, they acknowledge the contributions of their research team and the support received from the university.

Results

Two hundred urine samples were collected from people of different ages and genders. The current study found that 126 isolates (63%) of the 200 samples were infected with bacteria, with *E. coli* bacteria being the most common type of infection (39.7%). The rest of the percentages were due to other types of bacteria. The current study showed that all *E. coli* isolates were resistant to antibiotics and contained efflux pump genes. The purified real-time PCR products for DNA extraction of E. coli isolates by using the *gapA* gene.

Sensitivity test by Vitek-2 system

The VITEK-2 system conducted a sensitivity test on the *E. coli* isolates to detect multidrug resistance (MDR). The present results show 25 isolates (50.0%) of *E. coli* isolates were resistant to several drugs and considered MDR isolates; the current study's findings are in line with a study done in Iran by researchers (Moini et al. 2015), where the MDR rate for *E. coli* was 50%. It is an understandable ratio.

Table 2 shows the expression of three genes in the recovered *E. coli* isolates. The mean of acrAB gene expression was 5.88±1.40, while the mean of oqxAB gene expression was 15.22±3.83, and the mean of marA gene expression was 4.03±0.82 respectively.

Table 2. The comparison between gene expression in *E. coli* isolates.

Characteristic	*acr*AB	*oqx*AB	*mar*A	*P value*
Gene expression				
Mean± SD	**5.88 ± 1.40^{A}**	**15.22 ± 3.83^{B}**	**4.03 ± 0.82^{A}**	**0.003 †S**
Range	1.18 – 13.64	1.38 – 41.93	1.45 – 9.45	
Different letters denote the significant differences at p< 0.05				

n: number of cases; SD: standard deviation; †: one way ANOVA; ¥: Chi-square test; S: significant at P > 0.05

Real-time PCR is the modern technique used for the gene expression of the gene efflux pumps, and the figures show the real-time PCR stages (Webb et al. 2018; Umar et al. 2019).

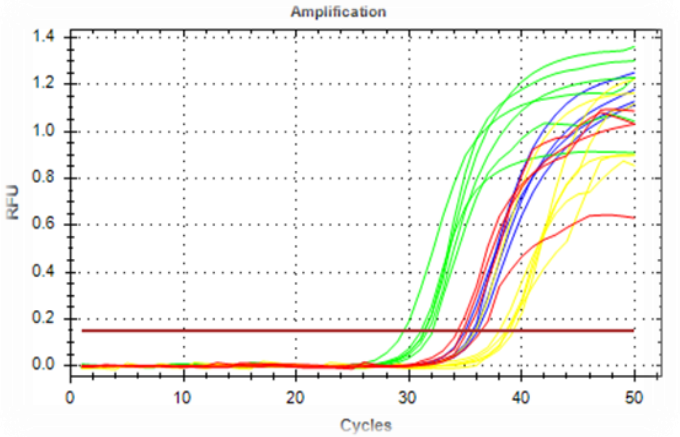

Fig. 1. The Real-Time PCR amplification plots of efflux pump genes MDR *E. coli* isolates. The blue plots (*acr*AB), the green plots (*oqx*AB), the yellow plots (*mar*A), and the red plots (*gap*A).

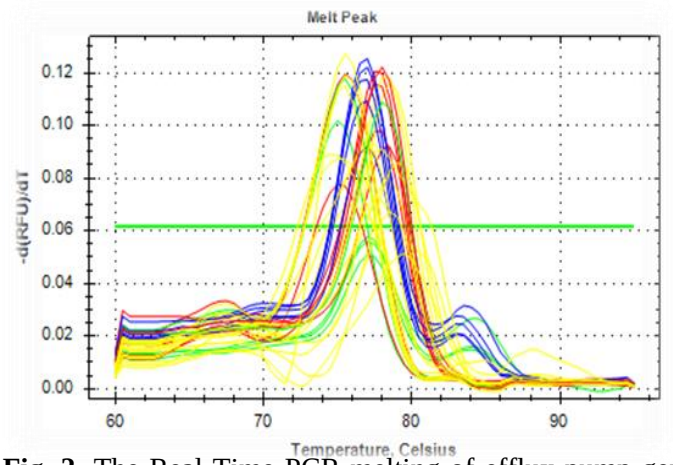

Fig. 2. The Real-Time PCR melting of efflux pump genes MDR *E. coli* isolates. The blue plots (*acr*AB), the green plots (*oqx*AB), the yellow plots (*mar*A), and the red plots (*gap* A).

This figure shows the comparison between the rates of genes.

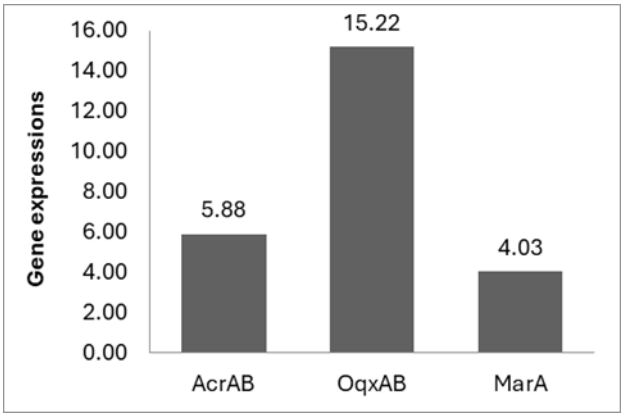

Fig. 3. The comparison between gene expression in *E. coli* isolates.

Figure (3) illustrates that the oqxAB gene exhibited the highest percentage of gene expression among the bacterial isolates, succeeded by acrAB and marA.

Figures 4-6 also depict the logistic slope, which illustrates how the proportion of gene expression affects each gene.

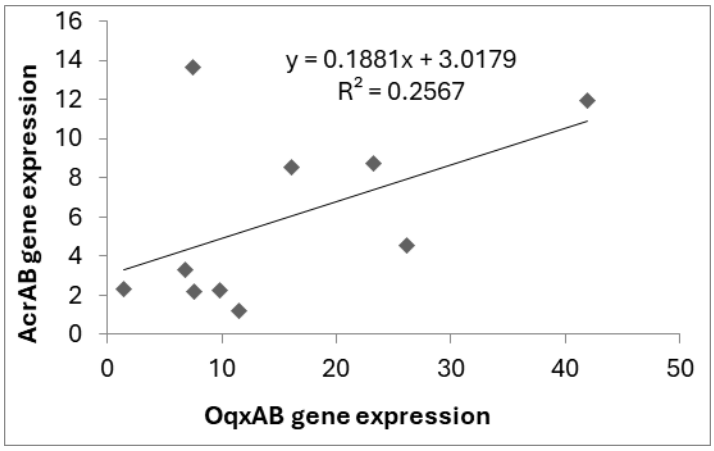

Fig. 4. The Logistic scatter blot acrAB gene and oqxAB *gene expression* among *E. coli* isolates.

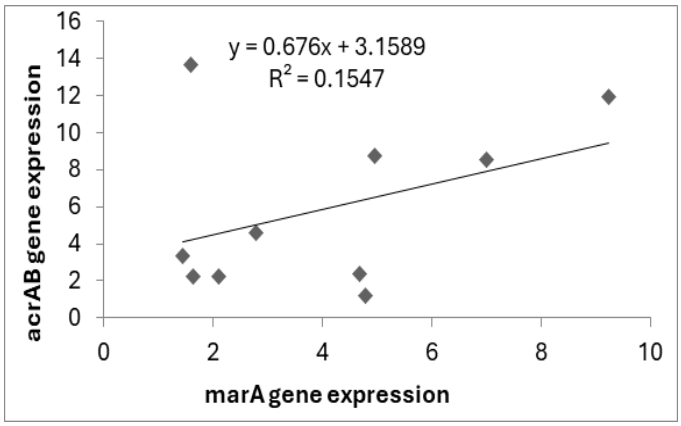

Fig. 5. The Logistic scatter blot acrAB gene and marA gene expression among *E. coli* isolates**.**

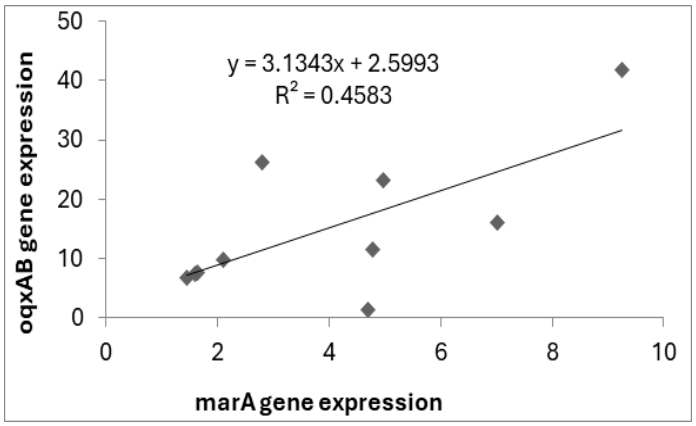

Fig. 6. The Logistic scatter blot oqxAB gene and marA gene expression among *E. coli* isolates.

Discussion

The present study enrolled 200 samples from patients with urinary tract infection (UTI) with an age range of 5-62 years were investigated for bacterial infection using bacteriological culture of urine samples. The study involved the culture of urine samples from 200 suspected patients with urinary tract infection (UTI) using blood and MacConkey agar, which were then incubated overnight at 37°C to identify the bacterial agent. The present study shows that 126 (63%) of urine samples have a positive diagnosis of bacterial infection out of 200 patients with UTI. The VITEK-2 system was used to identify ID-Gram Negative (ID-GN cards) according to the manufacturer's instructions. The present results showed that 50 isolates (39.7%) of UTI patients have *E. coli* bacterial infection because *E. coli* bacteria are the main cause of UTIs.

It has been determined how the acrAB gene is expressed in *E. coli* isolates and the mean of acrAB gene expression was 5.88±1.40 in *E. coli* isolates; the recovered bacterial isolates all possessed the acrAB gene (100%). There was a study in Egypt that found that the *acrAB* gene was expressed at a rate of 78.6% in *E. coli* bacteria (Abdelhamid & Abozahra 2017). This is consistent with the study conducted by the other researchers (Gawad et al. 2018), which indicated that 80% of *E. coli* isolates possessed the *acrAB* gene. *AcrAB*, a type of bacterial efflux pump, is in charge of both *E. coli* bacteria's natural and acquired resistance to a wide range of antibiotics. This is why the percentages are different.

The average expression of the oqxAB gene in *E. coli* isolates varied from 15.22 to 3.83, signifying a substantial increase in gene expression. A study by Zhong et al. (2014) in China revealed that the oqxAB efflux pump gene is present in all *E. coli* isolates, and noted that mechanisms facilitating efflux, particularly the high expression of the efflux pump, significantly contribute to tigecycline resistance: oqxAB. Rodriguez-Martnez et al. (2013) identified the oqxAB efflux pump gene in 73% of the *E. coli* samples in a separate study. The varying ratios result from several factors, including the geographical location, the extent of bacterial species infection, the quantity of isolates, and the methodology employed for their collection.

The average expression of the marA gene in *E. coli* isolates was 4.03±0.82. The gene expression rate for the efflux pump gene marA varies due to differences in the concentrations of the isolates and the methods of collection. Previous research by Ruiz & Levy (2010) and Albarri et al. (2022) exhibited that all *E. coli* isolates possessed the *marA* gene. The marA gene functions as a transcription factor that employs random transdermal mutations to regulate the transport of various drugs and enhance membrane impermeability.

The mean gene expression levels for acrAB, oqxAB, and marA were 5.88±1.40, 15.22±3.83, and 4.03±0.82, respectively. The oqxAB gene exhibited a significantly higher mean expression compared to the other genes ($P<0.05$). Nonetheless, the expression exhibited a non-significant difference between the sacred and marA genes ($P < 0.05$); the variations in the functions of these genes elucidate the discrepancies in the findings of the current study. In the presence of dangerously elevated levels of antibiotics, the oqxAB gene exhibits significantly higher expression than the acrAB and marA genes. The oqxAB gene may demonstrate elevated and high expression levels due to factors such as genetic organization, biological and functional context, and gene expression rate. When analyzing specific genetic relationships between the sacred and marA genes (De Majumdar et al. 2013; Li et al. 2019).

The disparity in gene expression rates for the aforementioned genes may arise from multiple factors.

Researchers have discovered that polymyxin significantly enhances the expression of the acrAB efflux pump gene through protein analysis. Studies carried by Ramos et al. (2016) and Liu et al. (2020) indicate a strong correlation between the marA and acrAB genes. The expression levels of the two genes are approximately equivalent in *E. coli* isolates. This is attributed to the hypothesis that the marA gene induces the acrAB gene in *E. coli* bacteria. However, a prior study conducted by Ruiz & Levy (2010) showed that the oqxAB gene is expressed more rapidly than other genes in *E. coli* isolates. This gene is crucial for bacterial function and plays a crucial role in antibiotic resistance.

The *E. coli* bacteria possess efflux pump genes acrAB, oqxAB, and marA. The Real-Time PCR illustrates the modulation of gene expression rates. Among the genes depicted in these charts, the oqxAB gene exhibits the highest expression rate. Nonetheless, prior research employing this methodology failed to provide precise data on the gene expression rates for these genes (Chetri et al., 2019; Albarri et al., 2022; Ciusa et al., 2022). Previous research utilized the conventional PCR technique, which is regarded as less precise than the current method (Vinué et al. 2018).

There is a link between gene expression and gene expression in *E. coli* isolates. The acrAB gene expression is directly linked to the oqxAB and marA genes. This finding could mean that the recovered isolates of *E. coli* have higher levels of the oqxAB and marA genes than the acrAB gene.

Conclusion

We concluded that patients with multiple drug-resistant (MDR) UTIs provided the majority of the isolates of *E. coli* bacteria under study. The efflux pump genes, acrAB, oqxAB, and marA, which encode efflux pump proteins, were present in all *E. coli* isolates. The study found that the oqxAB gene had the highest rate of expression (15.22%) compared to the acrAB and marA genes. In the recommendations, studies must be continued periodically on *E. coli* bacteria to determine their high resistance to antibiotics. We need to conduct more precise studies to understand the impact of efflux pump genes on the effectiveness of bacteria and their resistance to antibiotics. This study also recommends the role of health awareness as well as taking care of public hygiene to reduce the effect of virulence factors. Antibiotic resistance genes are linked to *E. coli* bacteria, so people should be careful not to use antibiotics unless their doctor tells them to. If they do, strains of bacteria that are resistant to antibiotics will start to appear.

Conflict of Interest

The authors stressed that there is no conflict of interest, but rather that there is a scientific and health benefit to serving society.

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