

Detection of *aggR* and *agg4A* Genes and Antibiotic Resistance Profiles in Biofilm-Forming *Escherichia coli* Clinical Isolates from Miandoab Hospitals

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Abstract

Introduction: *Escherichia coli* (*E.coli*) is a non-pathogenic facultative bacterium that is the predominant pathogen in the human intestine, however, some of its strains are known to be the cause of many human infections. The major adhesins of enteroaggregative strains has *aggR* gene encodes AAF-I. This strain has too *agg4A* encodes AAF-IV, each of which has a specific role. Various factors, such as the high volume of antibiotic use, poor hygiene conditions contribute to the development and spread of antibiotic resistance.

Methods: Urine samples were taken from 100 patients referred to Miandoab Hospital in Iran and cultured in Eosin methylene blue(EMB) selective culture medium. *E. coli* differential test was performed for positive samples. Antibiogram test was used to investigate antibiotic resistance. PCR was performed for investigation the presence of *aggR* and *agg4A* genes.

Results: In this study, 80% of the samples were resistant to the antibiotic ampicillin and 20% were sensitive to the antibiotic ampicillin. In this study, 20% of the samples formed weak biofilm, 20% of the samples formed medium biofilm, 50% of the samples formed strong biofilm, and 10% of the samples formed very strong biofilm. One sample (5%) had the *aggR* gene and six samples (30%) had the *agg4A* gene.

Conclusion: According to the results of this study, *aggR* and *agg4A*, as genes effective in biofilm biosynthesis, have different effects on biofilm formation of clinical isolates of *E.coli* by PCR method, and these effects are exerted through the influence on the expression of genes involved in biofilm formation.

Keywords: *Escherichia coli*, biofilm, Antibiogram, *aggR*, *agg4A*.

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Introduction

Escherichia coli (*E.coli*) is a non-pathogenic facultative bacterium that is the predominant pathogen in the human intestine, however, some of its strains are known to be the cause of many human infections such as sepsis, meningitis, biliary tract infections, urinary tract infections, and pneumonia.¹ This bacterium lives mainly in the lower intestine of warm-blooded organisms, including humans, but it also has the ability to colonize and persist in animal hosts and the environment, and is often discharged into the environment through feces or sewage effluent.² This bacterium does not cause disease as long as it is in its

original habitat, but some strains of this bacterium can separate from its commensal strains and cause more serious and pathogenicity in host tissues and organs.³

E.coli colonize the gastrointestinal tract of human infants within hours of birth and typically coexist with their hosts for decades with mutual benefit.⁴ The genes encoding the pathogenicity factors of the different pathotypes of these bacteria and their ability to exchange genetic information are considered to be the most important factors in the pathogenicity of *E.coli*.⁵

Enteric pathogenic *E.coli* are divided into two major groups: diarrheagenic *E.coli* (DEC) and extraintestinal *E.coli* (ExPEC). DEC bacteria are among the most common pathogens known in the world and are an important cause of traveler's diarrhea, dysentery, and urinary tract infections.⁶ ExPEC is an important human and animal pathogen that causes extraintestinal diseases including urinary tract infections and sepsis.⁷ Enteropathogenic *E.coli* bacteria are well classified into 6 strains: enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). All these strains differ in symptoms and pathogenicity. EAEC are considered the most important pathotypes of these bacteria.⁸

EAEC is a heterogeneous emerging pathogen associated with acute and persistent diarrhea in children, traveler's diarrhea, and outbreaks in industrialized countries. Characteristic adhesion, biofilm formation, production of enterotoxins and cytotoxins, and mucosal inflammation are four main characteristics of this pathogen.⁹ The attachment of this bacterium is mediated by proteins and complex structures encoded by AafA molecules and a short surface protein called.¹⁰ The major adhesins of enteroaggregative strains, which are classified into 5 groups and encoded by different genes, are called adhesive fimbriae (AAF). For example, aggR encodes AAF-I, aafA encodes AAF-II, agg3A encodes AAF-III, and agg4A encodes AAF-IV, each of which has a specific role.¹¹

E.coli is a bacterium that is commonly found in the intestines of humans and some animals and is mostly harmless. However, some of its isolates can escape from the symbiotic state and become pathogenic through transferable genetic factors such as plasmids, transposons, bacteriophages and genes that are effective in pathogenesis.¹² Various factors, including the high volume of antibiotic use, poor hygiene conditions, and the use of antibiotics in animal nutrition, contribute to the development and spread of antibiotic resistance. Although various antibiotics are used in the treatment of *E.coli* infections and the biofilms caused by them, however, antibiotic resistance is spreading due to inappropriate use and most microbial and biofilm species that were previously sensitive to drugs have gradually become resistant to them.¹³ In recent decades, antibiotic resistance has increased significantly among *E.coli* species. Therefore, choosing appropriate antibiotics for treatment

and implementing appropriate infection control strategies in hospitals is essential.¹⁴ In this study we investigated the presence of aggR and agg4A coding genes in biofilm *E.coli* clinical isolates and their relationship with the antibiotic resistance pattern of East Azerbaijan-Miandoab hospitals.

Materials and Methods

Patient selection and urine culture

After obtaining informed consent urine samples from 100 patients who were hospitalized or outpatients in hospitals in Miandoab– Iran due to urinary tract infection were examined. Urine samples prepared from patients were cultured on a selective culture medium for gram-negative bacteria, EMB agar, and placed in an incubator for 24h at 37°C. After 24h, the bacterial culture medium was examined for the growth of bacterial colonies. Colonies that grew on EMB agar were considered as gram-negative bacteria of the sample. Gram staining was performed to confirm the selection of Gram-negative bacteria. Therefore, 20 positive samples of gram-negative bacteria of the sample were isolated for examination and stored in a medium containing 20% glycerol and a temperature of -20°C.

Differential tests

Differential tests including oxidase test, catalase test, SIM medium, motility and indole production test, urease medium, MR-VP medium, culture on Simon citrate medium, TSI medium change pattern, and lysine test were performed to differentiate *E.coli* from other gram-negative bacteria.

Antibiogram test

To determine antibiotic susceptibility, 3-4 colonies of bacteria were removed and transferred to a tube containing sterile physiological serum. The tubes were placed in a 42°C incubator for 30min to prepare a suspension equivalent to half McFarland turbidity. Using a sterile swab, the bacteria were streaked on Mueller-Hinton agar medium in all directions. Five antibiotic discs include Imipenem(IMP), Sulfamethoxazole(SMX), Nalidixic acid(NA), Ciprofloxacin(CIP) and Ampicillin(AMP) were placed on the surface of the plate next to the flame and under the laboratory microbial hood. The plates were transferred inverted to a 37°C incubator and the results were examined after 18h. Finally, the results were determined as sensitive, resistant, and intermediate based on the measurement of the diameter of

the growth inhibition zone using a millimeter ruler according to the Clinical & Laboratory Standards Institute (CLSI) protocol.

Screening for biofilm-forming bacteria

The microplate titer method was used to screen biofilm-forming bacteria. The isolated bacteria were inoculated in TSA 1% glucose culture medium in special microplate wells and incubated for 24h at 37°C. The resulting suspension was diluted 1:100 to reach a turbidity of 0.5 McFarland. TSB solution was poured into the first two wells of the microplate as a positive control. Then, 200µl of the diluted suspension was transferred to the microplate wells and the microplate was incubated without movement for 24h at 37°C. We filled two wells for each sample to be sure. After the incubation time, the wells were carefully emptied under aseptic conditions and washed three times with PBS to remove planktonic cells. In order to stabilize the biofilm structures, 200µl of 96% ethanol were added to the wells and drained after 15 min. Then, the microplate wells were filled with crystal violet and methylene blue dyes and drained after 15 min. For decolorization, the microplate wells were washed 6 times with phosphate-buffered saline (PBS) and to separate the biofilm, the wells were filled with 200µl of 33% acetic acid and read using an ELISA reader at a wavelength of 570nm. The calculation was performed using the formula of Edward et al.¹⁵

DNA extraction

In this study, the Vira gene extraction kit was used daily as follows 0.5 McFarland was poured into the microtube. 200µl of lysis solution was added to the cell containing the sample. 20µl of proteinase k was added to the sample and incubated for 10min at 70°C. 100µl of isopropanol was added to the contents and mixed. The mixed contents were centrifuged at 1200rpm. The supernatant was discarded. Then 500µl of washing solution 1 was added to the contents and centrifuged again at 1200 rpm for one min and then the supernatant was discarded. Then 500µl of wash solution 2 was added to the contents and centrifuged again at 1200 rpm for one min and then the supernatant was discarded. Collection tube was placed into the contents of the mixture and collected the mixture. 100µl of wash buffer was added to the collected mixture. The centrifugation was repeated again at 1200 rpm for 1min. Template DNA was collected. The quality and quantity of extracted DNA were assessed using Nanodrop.

Primer designing

In this study, the primer sequences of Moraes et al. were used.¹⁶ To ensure the accuracy of the primer sequences, the primers were blasted in the NCBI database. The sequences of the primers used are shown in Table 1.

Table 1. Gene name and primer sequences.

Gene Name	Seq (5-3)	TM(°C)	Length
agg4A-F	TGAGTIGTGGGGCTAYCTGGA 6304	61.78	21
agg4A-R	CACCATAAGCCGCCAATAAGC	60.25	22
aggR-F	GCAATCAGATTAARACAGCGATACA T	59.30	24
aggR-R	CATTCTTGATTGCATAAAGGATCTGG AT	59.70	25

Polymerase chain reaction (PCR)

PCR was used to amplify genes. In this design, Amplicon-Denmark Master Mix was used, which contains NA polymerase, buffer, dNTP, and MgCl₂. 13µl of Master Mix (2x), 2µl of forward and reverse primer mix, and 1µg of extracted DNA were poured into a microtube and diluted to a final volume of 25µl with distilled water. Amplification was performed according to a temperature protocol including one cycle for 10min at 95°C and thirty-five cycles of 60 seconds at 95°C, 45 seconds at 60°C, and 60 seconds at 72°C. 2% agarose gel electrophoresis was used to examine the amplified fragments.

Statistical analysis

The obtained data were analyzed with SPSS version 27 statistical software, and GraphPad Prism software was used to draw the relevant graphs and analyze the data. T test and one-way ANOVA were performed on the data, and a significant value of P<0.05 was considered.

Results

Of the 100 urine samples taken from volunteer patients participating in this project, 20 samples contained *E. coli* bacteria. Gram staining revealed the bacillary form of *E. coli* to be pinkish-red under the light microscope. Figure 1 shows a microscopic image of *E. coli* at 50x magnification. The results of differential tests for *E. coli* in all samples were as follows: citrate negative, lysine positive, indole positive, urease negative, MR positive, VP negative, oxidase negative, and catalase positive.

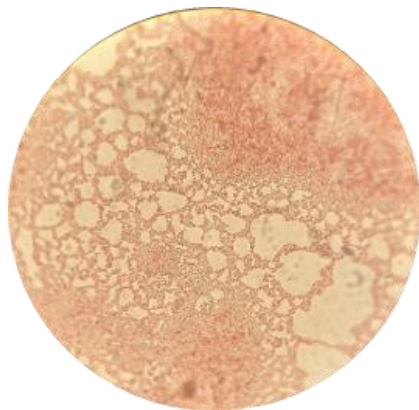


Figure 1. *E.coli* (50x magnification).

Antibiogram test results

Antibiogram test results for 5 antibiotics (IMP, SMX, NA, CIP and AMP) used in 20 *E.coli* positive bacteria were evaluated according to the CLSI table. The results of the antibiogram test showed that the highest antibiotic resistance was observed to the antibiotic AMP, with 80% of the samples being resistant to AMP, and the lowest antibiotic resistance was observed to the antibiotic IMP, with 20%. Figure 2A shows an image of the antibiogram of a sample. shows and Figure 2B the frequency of antibiotic resistance of *E. coli* positive samples.

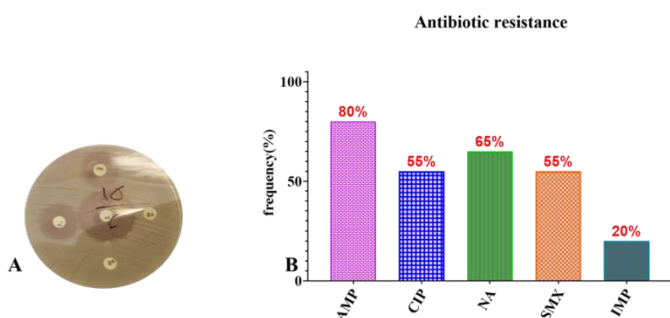


Figure 2. Frequency of antibiotic resistance of *E.coli* positive samples.

In this study, 80% of the samples were resistant to the antibiotic AMP and 20% were sensitive to the antibiotic AMP. 55% of the samples were resistant to the antibiotic CIP, 30% were sensitive to the antibiotic CIP, and 15% were semi-sensitive to the antibiotic CIP. 65% of the samples were resistant to the antibiotic NA and 35% were sensitive to the antibiotic NA. 55% of the samples were resistant to the antibiotic SMX and 45% were sensitive to the antibiotic SMX. Only 15% of the samples were resistant to IMP antibiotic, 60% were sensitive to IMP antibiotic, and 25% were semi-sensitive to IMP antibiotic. Figure 3 shows the frequency of resistance or sensitivity to each antibiotic separately.

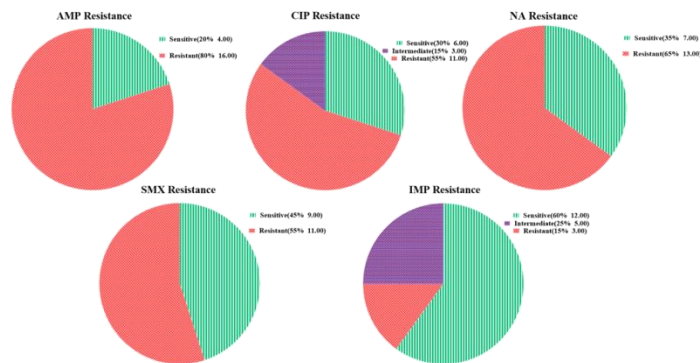


Figure 3. The frequency of resistance or sensitivity to each antibiotic separately.

By performing a disk diffusion test on samples that were sensitive to antibiotics, it was determined that the highest average growth inhibition zone was related to the antibiotic CP with an average of 33.833 mm and the lowest average growth inhibition zone was related to the antibiotic AMP with an average of 19.25mm. Figure 4 shows the comparison of the Disk diffusion test.

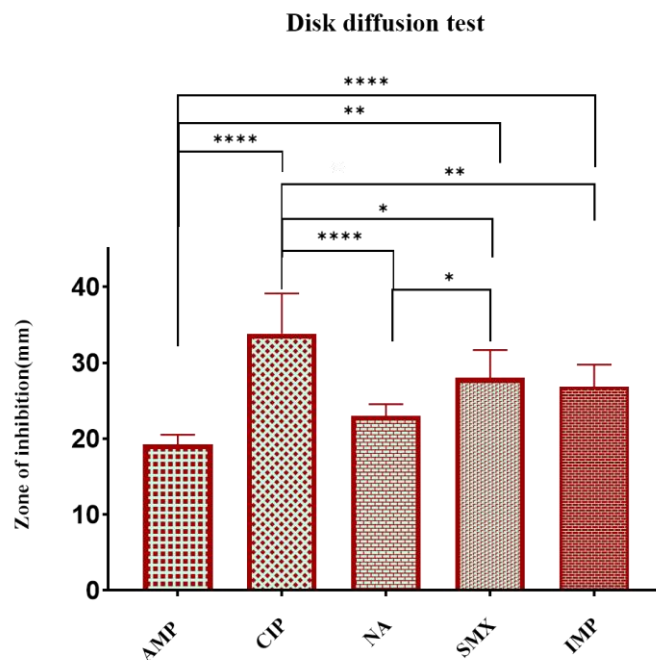


Figure 4. The comparison of the Disk diffusion test. (* indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$, ns indicates non-significance).

Results of biofilm formation of samples

In this study, 20% of the samples formed weak biofilm, 20% of the samples formed medium biofilm, 50% of the samples formed strong biofilm, and 10% of the samples formed very strong biofilm. Figure 5 shows Frequency of biofilm formation of *E.coli* positive samples.

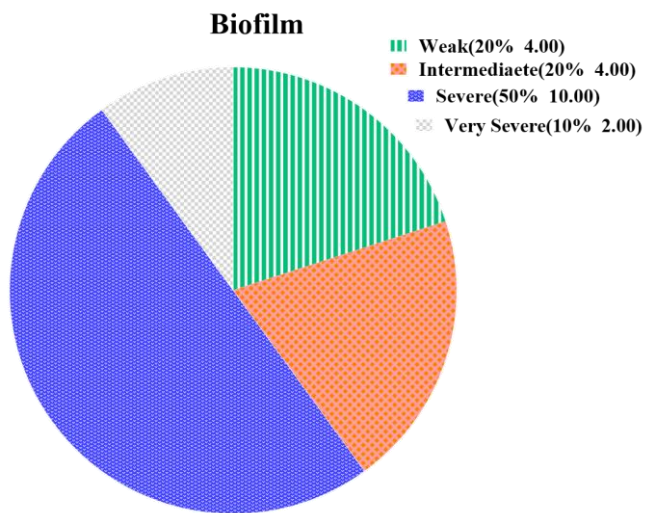


Figure 5. Frequency of biofilm formation of *E.coli* positive samples.

Molecular isolation and identification results

The results of electrophoresis of PCR products showed that out of 20 positive *E.coli* samples, one sample (5%) had the *aggR* gene and six samples (30%) had the *agg4A* gene. None of the samples contained the *aggR* and *agg4A* genes at the same time. Figure 6 shows frequency of *aggR* and *agg4A* of *E.coli* positive samples.

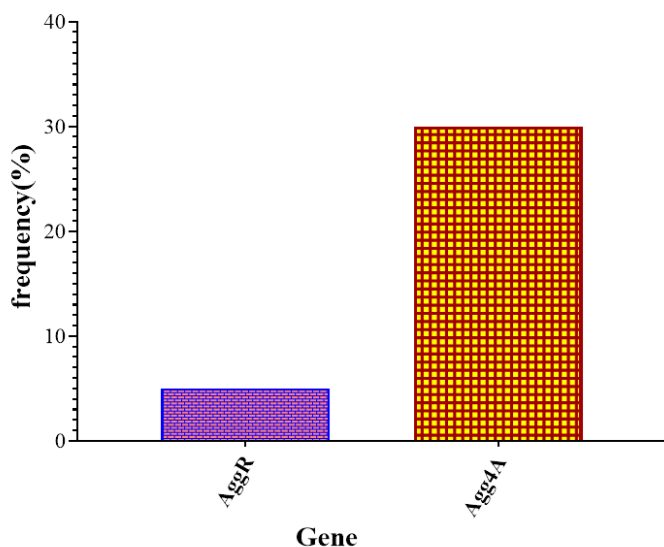


Figure 6. Frequency of *aggR* and *agg4A* of *E.coli* positive samples.

Discussion

The aim of this study was to investigate the presence of genes encoding *aggR* and *agg4A* in biofilm formation in clinical isolates of *E.coli* and their relationship with antibiotic resistance patterns in patients referred to hospitals. The resistance of the samples to 5 types of antibiotics was examined, and the size of the zones of non-growth was compared with the CLSI standard table.

By performing this disk diffusion test on samples that were sensitive to antibiotics, it was determined that the highest average growth inhibition zone was related to the antibiotic CIP with an average of 33.833 mm and the lowest average growth inhibition zone was related to the antibiotic AMP with an average of 19.25 mm. The microplate titer method was used to screen for biofilm-forming bacteria. Comparing the study with the mean absorbance reading formula for biofilm production in an ELISA reader, the evidence obtained indicated that 10% of the samples formed very strong biofilms, 50% strong, 20% medium, and 20% weak biofilms. The presence of *aggR* and *agg4A* genes was investigated by PCR method, and the results of identifying *aggR* and *agg4A* genes in biofilm formation in clinical *E.coli* isolates were observed as follows: the frequency of the *aggR* gene in positive *E.coli* samples that had formed biofilm was 5%, and the frequency of the *agg4A* gene in positive *E.coli* samples that had formed biofilm was 30%.

In a study conducted in 2015 by Fattahi et al., 100 *E.coli* isolates collected from patients with urinary tract infections were examined for biofilm formation on the surface of 96-well microplates. The presence of *fimA*, *papC*, and *hly* genes was also determined by PCR. 92% of the isolates formed biofilms, and the frequencies of *papC*, *fimA*, and *hly* genes were estimated to be 43%, 94%, and 26%, respectively. Also, the biofilm formation rates among strains expressing these genes were 100%, 93%, and 100%, respectively.¹⁷ In our study, by examining biofilm formation in the *aggR* and *agg4A* genes, it was observed that the frequency of *aggR* genes in biofilm formation in positive *E.coli* samples was 5% and in *agg4A* it was 30%. By comparing the studies conducted in 2015 by Fattahi et al. with our study, it was observed that the percentage of biofilm formation among strains expressing the genes of Fattahi et al. was above 50% and even 100%, while the percentage of biofilm formation among strains expressing the *aggR* and *agg4A* genes was below 50%, and this difference in the percentage of biofilm formation among strains expressing the genes is likely due to the difference in the type of genes. In 2016, Tajbakhsh et al. isolated 130 *E.coli* strains from individuals with symptoms of urinary tract infection. They then examined the cases for antibiotic resistance patterns, serogrouping, and detection of pathogenic genes. 61.53% of the strains formed biofilms. 87.5% of the strains were also resistant to AMP, and the frequencies of *fimH*, *pap*, *sfa*, and *afa* genes in strains that had the ability to form biofilms were calculated to be 93.33%, 86.66%, 86.66%, and 66.66%, respectively.¹⁸ In our study, the highest resistance of strains was to the antibiotic AMP, which was observed to be 80%. In the study by Tajbakhsh

et al. in 2016, strains were also resistant to the antibiotic AMP with a percentage similar to the percentage in our study, which was 87.5%. This indicates that from 2016 until now, the resistance of *E. coli* bacteria in urinary tract infections to the antibiotic AMP has remained high, showing resistance with almost the same percentage. In 2017, Lara and colleagues screened 258 *E. coli* isolates from extraintestinal infections for EAEC strains and UPEC/EAEC hybrid genotypes. 21 pathogenic genes were detected, which contained molecular markers of EAEC and UPEC strains. A biofilm assay was also performed to assess biofilm formation. The UPEC *chuA* and *fyuA* genes were detected in more than 70% of the strains. The EAEC *aggR* and *aatA* genes were detected in only 3.4% of the strains. The *pap* gene was detected in all hybrid strains, and UPEC/EAEC hybrid strains formed biofilms to a small extent.¹⁹ In our study, the frequency of the *aggR* gene in biofilm formation in positive *E. coli* urinary tract infection samples was 5%. However, in a 2017 study by Lara et al., who studied 258 *E. coli* isolates related to extraintestinal infection, the *aggR* genes and another gene called *aatA*, which were related to EAEC, were detected in only 34% of the strains.

In 2017, a study was conducted by Khaleque et al. in which 56 UPEC strains were examined for their biofilm-forming ability and the presence of pathogenicity genes related to urinary tract infection, including *papC*, *fim1*, *afa*, *sfa*, and pathogenicity genes related to diarrhea, using multiplex-PCR. The results showed that 21 strains had the ability to form biofilms, 42% of which were positive for the *pap* gene, and 11% were positive for *aafA*. DEC genes were found in only 7 isolates. One isolate had the *aatA* gene.²⁰ In our study, all 20 *E. coli* strains from urinary tract infection samples formed biofilms, with percentages of very strong, strong, moderate, and weak biofilms, while in a study conducted by Khaleque et al. in 2017, only 21 of 56 *E. coli* strains from urinary tract infection samples had the ability to form biofilms.

Nunes et al. (2017) evaluated 92 stool samples collected from children with diarrhea and healthy children. They detected a cluster of EAEC strains with extraintestinal genetic markers (ExPEC). The results showed that all 92 EAEC strains were positive for some of the EAEC pathogenic genes (*aggR*, *aggA*, *aafA*, *aap*, *astA*, *pet*). 35.9% of the strains were positive for EAEC genes. 17 EAEC strains were found with UPEC markers.²¹ In our study, the *aggR* and *agg4A* genes were investigated for pathogenicity in the form of urinary tract infection by *E. coli* bacteria, and the presence of the genes in these samples was 5% in the *aggR* gene and 30% in the *agg4A* gene in the positive bacterial samples with biofilm formation. By comparing the study by Nunes et al. in

2017, it can be seen that these two genes, *aggR* and *agg4A*, which are among the pathogenic genes of EAEC, are detected in both stool samples and urinary tract infection samples.

Conclusion

According to the results of this study, *aggR* and *agg4A*, as genes effective in biofilm biosynthesis, have different effects on biofilm formation of clinical isolates of *E. coli* by PCR method, and these effects are exerted through the influence on the expression of genes involved in biofilm formation. This result challenges the previous idea that the production of pathogenic factors is reduced in the presence of *aggR* and *agg4A* genes.

Highlights

What Is Already Known?

Antibiotic resistance to *E. coli* varies in different parts of the world, and studies in different parts of the world have shown different results.

What Does This Study Add?

This study reports antibiotic resistance to *E. coli* in Miyadoab, East Azerbaijan Province, Iran.

Authors' Contributions

M.B was involved in planning and supervised the work and proof outline H.S and M.B drafted the manuscript and designed the figures. Z.H was responsible for the substantive review of the article and the project. All authors discussed the results and commented on the manuscript.

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Conflicts of Interest Disclosures

The authors declare no competing interests.

Consent For Publication

The authors declare their consent for publication.

Ethics approval

This project was approved by the university's ethics committee with ID IR.IAU.TABRIZ.REC.1403.298

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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The extent of AI use

AI was not used.

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