Original Article

# Evaluating the ability of Biofilm formation in Escherichia coli isolated from clinical samples in Zahedan

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Received: 2017/22/04 Revised: 2017/2/07 Accepted: 2017/4/07

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Pars Journal of Medical Sciences, Vol.15, No.1, Spring 2017

Pars J Med Sci 2017;15(1):36-42

## Abstract:

**Introduction:**
Escherichia coli (E.coli) is the main causative pathogen in urinary tract infections (UTIs). Antibiotic resistance based on Extended Spectrum -Lactamase (ESBL) producing Escherichia coli strains is reported to be the cause of community and hospital acquired infections therefore this this is an important problem in public health. The aim of this study was to identify the blaTEM, blaSHV and CTX genes in clinical isolates of E. coli recovered from patients with UTIs in Zahedan, Iran.

**Methods and Materials:**
Escherichia coli isolates (N=60) were analyzed by biochemistry and phenotypic methods. Each of the initial E. coli screening test isolate was investigated for the presence of blaTEM, blaSHV and blaCTX-M genes via polymerase chain reaction (PCR) using gene-specific primers.

**Results:**
Sixty tested isolates; the prevalence of blaTEM and blaCTX-M genes was determined to be 68.3%, 46.6% but blaSHV gene was not detected. Based on phenotypic tests, biofilm capacity was detected in 10 clinical isolates.

**Conclusions:**
The results of this study show that the use of adequate doses of antibiotics in the treatment of infections may be caused by biofilms are emphasized. Rapid detection of biofilm formation, therefore isolates have the ability to decide care and management is necessary.

**Keywords:** Escherichia Coli, Antibiotic Resistance, PCR, Biofilm

## Introduction

E. coli is the most prevalent gram-negative bacillus in fecal flora. E. coli can colonize and sustain in animals, humans and environment. E. coli is a gram-negative rod-shaped bacterium normally found in warm-blooded animals. E. coli is normally non-pathogenic because it is considered a part of the normal flora of human intestine; nonetheless, some of its serotypes can cause serious food poisoning in human (1).

Urinary tract infections caused by E. coli are the second most common bacterial infection and a major cause of patient admission to hospitals. Among all uropathogenic bacterial agents, these gram-negative bacilli of the normal intestinal flora are the most common agents isolated

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in urinary tract infections in 75-90% of outpatients and inpatients (2).

Recognizing the sensitivity pattern of E. coli to different antibiotics is crucial. The sensitivity of the isolated bacteria to antibiotics differs in different regions. The difference in antibiotic sensitivity patterns among different regions lies in the difference in the dose and type of the antibiotics used (3). Nowadays, 20%-50% of E. coli strains have become resistant to the first line antibiotic therapy even in developed countries. Antibiotics should therefore be used based on the region's antibiotic sensitivity pattern (4).

In gram-negative bacteria, β-lactamases play the most significant role in resistance to β-lactam antibiotics. β-lactamase genes in these bacteria, especially ESBLs, are effective factors in increasing resistance to β-lactam antibiotics such as the extended-spectrum cephalosporin. The organisms carrying these genes increase the risk of pathogenicity and mortality in the patients (5). Detecting pathogenic E. coli isolates requires isolating these organisms from non-pathogenic isolates, i.e. normal intestinal flora, which is accomplished through molecular assays, particularly PCR (6).

Bacteria are found as planktonic cells and biofilms in the nature. Bacterial biofilms are complex bacterial aggregates surrounded by a glycocalyx coating and sticking to mucosal surfaces. Biofilms are a collection of microbial cells that irreversibly depend on surface and do not disappear by a gentle wash. Moreover, identifying planktonic cells as mature biofilms causes phenotypic changes with major consequences such as increased resistance to antimicrobial agents and the failure of the host's immune system to eliminate these cells. Over half of bacterial infections reported include biofilm formation (7). Biofilm formation has been observed in different bacterial species, including E. coli and Pseudomonas aeruginosa. The most significant and distinctive characteristic of biofilms is their growth difference, which causes drug resistance and requires different therapies and distinct identification methods (8).

The present research was conducted in 2015 to determine antibiotic resistance and biofilm formation in E. coli species isolated from clinical samples in Zahedan.

## Materials and Methods

### Patients and Sampling:

Sixty isolates were collected from the fecal samples of patients with diarrhea, who were suspected of having E. coli infections, in the medical laboratories of Zahedan. The samples were transferred to the Microbiology Laboratory in specially covered sterile containers at 4 ℃. The fecal samples were homogenized with a phosphate-buffered saline in the laboratory. The solution was then centrifuged at 3000 rpm for five minutes to precipitate solids in the stool. The supernatant was then transferred to a sterile container at 4 ℃.

### Microbial Phenotypic Identification:

The samples were cultured on MacConkey agar, Xylose Lysine Desoxycholate (XLD) agar, Salmonella Shigella Agar (SSA) and Thiosulfate citrate bile salts-sucrose (TCBS) agar and incubated for 24 hours at 37 ℃ for isolating E. coli, which appeared as red colonies on MacConkey agar. The colonies suspected to be E. coli were re-cultured on the agarose medium, and negative samples were then placed on biochemical media such as TSI, MR-VP Simmon's citrate and SIM for the final confirmation.

**PCR:**

Sixty samples were prepared for DNA extraction and PCR. The kits made by the National Center of Genetics and Biological Reserves of Iran were used for DNA extraction. Specific primers used in the literature were used to track β-lactamases, including CTX, TEM and SHV (9-11). Table 1 presents the process of the PCR. The thermal cycle used in the PCR comprised one cycle at 95 ℃ for 15 seconds, 35 cycles at 94 ℃ for 30 seconds, 60 ℃ for 40 seconds, 72 ℃ for one minute and 72 ℃ for ten minutes. The results obtained were then examined using 1% agarose gel electrophoresis.

Table 1: The volumes needed for PCR

| Substance | Volume | Concentration |
|---|---|---|
| PCR buffer | 2.5 µL | 10X |
| $MgCl_2$ | 2 µL | 25 mM |
| dNTPs | 1 µL | 2 mM |
| Taq polymerase | 1 µL | 5 U/µL |
| Primer F*3 | 1.5 µL | 20 p.mol |
| Primer R*3 | 1.5 µL | 20 p.mol |
| DNA | 2 µL | |
| Distilled water | 8 µL | |
| Total | 25 µL | |

**Assessment of Biofilm Formation**

To investigate the biofilm formation ability of E. coli isolates, a biofilm formation test was performed in the laboratory setting according to the following procedure. The isolates were incubated at 37 ℃ after being cultured on TSB for 24 hours. A microbial suspension adjusted to the turbidity of a 0.5 McFarland was obtained from each of the isolates, and all wells in a 96-well plate were filled with 200 µL of the medium. A total of 200 µL of the antibiotic stock of each of the two antibiotics at the maximum concentration, i.e. penicillin 32 mL/g and flucloxacillin 64 mL/g, was then added to each well and a serial dilution was prepared. The plate surfaces were covered and incubation was performed at 30 ℃ for 24 hours. After removing their content, the wells were rinsed thrice using PBS. After 15 minutes, alcohol was discharged and the plate was dried in the air. One hundred µL of 2% crystal violet was added to all the wells and 20 minutes later, the plates were washed with water to remove the additional dye. A total of 150 µL of 33% acetic acid was then added to the plates to remove the additional dye, and the optical density (OD) of the wells was read at a wavelength of 570 nm using an ELISA reader. Moreover, suspension-free antibiotics and the positive control ATCC-25923 were respectively used as negative control and positive control. The ability to form OD was also recorded over a 4-hour period. At the end, the biofilm reduction caused by antibiotics could be calculated using the OD of the treated, control and sham control wells as per the following formula.

Reduction percentage=[(C-B)-(T-B)]×100; where C is the mean OD of control wells; B the mean OD of sham control wells and T the mean OD of treated wells. (C-B)-(T-B)=C-B-T+B=C-T?!!

It is worth noting that all ethical principles associated with sampling, clinical data and the research itself were observed in the present study.

## Results

Sixty isolates of the fecal samples of patients with diarrhea, who were suspected of E. coli infections, were cultured and confirmed using biochemical tests. Forty-one (68.3%) of these samples carried the CTX β-lactamase gene and 28 (46.6%) carried TEM, while no SHV-positive cases were detected.

Ten samples exposed to penicillin with a minimum inhibitory concentration (MIC) of 0.63 µg/µL were found to be a strong biofilm producer, 6 a moderate biofilm producer and 4 a weak producer (Table 2). Moreover, 3 samples exposed to ciprofloxacin with an MIC of 1.9 µg/µL were found to be a strong biofilm producer, 6 a moderate producer and 11 a weak biofilm producer (Table 3).

Table 2: The effect of penicillin on the OD of isolated E. coli biofilms

| Source | Genus and Species | Frequency | Mean biofilm MIC (µg/mg); ≤0.25: sensitive 0.25: semi-sensitive ≥0.5: resistant | Biofilm formation in the microtiter plate | | | | Mean OD |
|---|---|---|---|---|---|---|---|---|
| | | | | Negative | Weak | Moderate | Strong | |
| Clinical Samples | E. coli | 20 | 0.63 | - | 4 | 6 | 10 | 0.32 |
| ATCC 25923 | E. coli Positive controls with no effects of penicillin | 1 | - | - | - | - | 1 | 0.29 |

Table 3: The effect of ciprofloxacin on the OD of isolated E. coli biofilms

| Source | Genus and Species | Frequency | Mean biofilm MIC (µg/mg); ≤1: sensitive 2: semi-sensitive ≥4: resistant | Biofilm formation in the microtiter plate | | | | Mean OD |
|---|---|---|---|---|---|---|---|---|
| | | | | Negative | Weak | Moderate | Strong | |
| Clinical Samples | E. coli | 20 | 1.9 | - | 11 | 6 | 3 | 0.26 |
| ATCC 25923 | E. coli Positive controls with no effects of penicillin | 1 | - | - | - | - | 1 | 0.29 |

## Discussion

Determining the antibiotic resistance caused by β-lactamase genes and the biofilm formation in E. coli species is crucial. Extra-intestinal E. coli strains contribute to intestinal diseases and a wide variety of extra-intestinal diseases in human and animals. β-lactamase genes in these bacteria, especially ESBLs, are effective factors in increasing resistance to β-lactam antibiotics such as the extended-spectrum cephalosporin. All the factors involved in creating and changing the antibiotic pattern of a region thus contribute to this major health problem, which should be constantly monitored using accurate methods.

The present study found 41 (68.3%) samples to be CTX-positive and 28 (46.6%) to be TEM-positive, while no SHV-positive cases were observed. These results are inconsistent with those obtained in the study conducted by Fang, et al. in Sweden, on the frequency of ESBLs in nosocomial E. coli isolates over a five-year period. These researchers reported a frequency of 63% for TEM and 92% for CTX, which are higher than the figures obtained in the present research. The one-year period in which the present study was conducted can explain these discrepancies in the results (12). Hoori et al. used the RAPD-PCR method in Ilam Province, Iran to investigate the isolates in terms of carrying blaTEM and blaSHV. They found 20 (64.5%) isolates to carry blaTEM and 6 (19.3%) to contain blaSHV, which are higher than the corresponding present results. The lowest antibiotic resistance was also reported to be against ciprofloxacin (13). Furthermore, the study conducted by Feizabadi in Tehran, Iran estimated the frequency of blaSHV and TEM respectively at 22% and 9% in ESBL isolates with an MIC of 16 to Ceftazidime, which are lower than the figures found in the present study (14).

The frequency of CTX was estimated at 87.1%, that of TEM at 68.8% and blaSHV at 70.6% in the study conducted by Nazemi in Tehran, which are different from those found in the present study and by Feizabadi (15). In addition, the study conducted by Valadbeigi in Ilam suggested a frequency of 52.5% for TEM, which is close to the present finding and different from that found by Hoori in this city (16). A study conducted in Kerman, Iran reported that the frequency of blaTEM and blaSHV are respectively equal to 2.4% and 5.5%, whose frequency distributions are significantly lower than those associated with the present study E. coli isolates (17). Another study conducted in Shiraz, Iran found the frequency of TEM to be 83.3% and that of SHV to be 20.4%, the frequency distribution of which were higher than those associated with the present E. coli isolates. They also estimated the frequency of CTX-M at 31.5% (18). A study conducted in Tehran estimated the frequency of TEM and SHV respectively at 60% and 24%, whereas the frequency distribution of SHV was significantly higher than that of the present E. coli isolates (19). Another study performed in Thailand suggested significantly high frequencies for these genes, although the SHV frequency was inconsistent with the present study finding (20). A study conducted in Isfahan, Iran also found the frequency of CTX to be 37.9%, TEM to be 72.4% and blaSHV to be 11.9%, the last two of which were higher than the corresponding frequencies obtained in the present study (21).

The study conducted by Emamghoreishi et al. in Jahrom, Iran reported an MIC of 0.84

for ciprofloxacin compared to the 0.26 found in the present study (22). In Tehran, Toosi et al. investigated the ability of Staphylococcus aureus to form biofilms. They examined all the isolates and found 87.7% of them to carry icaA and icaD using PCR. Moreover, the phenotypic analysis of biofilm formation using microtiter plates showed that 4.4% of the isolates are strong biofilm producers, 40% are moderate and 43.3% are weak biofilm producers, whereas only 12.2% were unable of forming in-vitro biofilms. The results of this study suggested a high prevalence and a relatively high phenotypic expression in biofilm producing genes (23).

The present study showed that biofilm is formed in lower concentrations of MIC in the presence of penicillin compared to ciprofloxacin and that resistance to ciprofloxacin is still lower than to penicillin in isolates carrying ESBLs. This is consistent with the results of previously-conducted studies, indicating the excessive uptake of penicillin in urinary tract infections. Higher levels of biofilms formed in the presence of penicillin compared to ciprofloxacin were also reported in literature (24). In addition, the present findings suggest that most of the samples are resistant to penicillin and penicillin is unable of eliminating biofilms compared to ciprofloxacin, and thus stronger biofilms are produced in the presence of penicillin.

## Conclusion

The present results suggested high levels of ESBLs, including CTX and TEM in this geographical region of Kerman. Moreover, adequate doses of antibiotics such as penicillin and ciprofloxacin are recommended to be taken in urinary tract infections. Given that the ability to form biofilms plays a key role in the virulence of these bacteria, the higher resistance to this antibiotic caused by ESBLs should be taken into account, and isolates with the ability to form biofilms are recommended to be quickly detected to help make more proper therapeutic and managerial decisions.

## Acknowledgements

The authors would like to express their gratitude to the esteemed authorities and experts in the Microbiology Laboratory. The present article was extracted from a research project approved by Islamic Azad University of Kerman, Iran, and sponsored from the fund dedicated to research projects.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the compilation and publication of this article.

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