Caspian Journal of Environmental Sciences
Online ISSN: 1735-3866
Print ISSN: 1735-3033

Prevalence of *SHV* gene and antibiotic resistance of extended-spectrum β-lactamase-producing *Escherichia coli* strains isolated from abattoir wastewater in Mazandaran Province, north of Iran

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ABSTRACT

Abattoir wastewater is a major source of pollution burden and life-treating human pathogenic bacteria. The aim of this study was to determine the extended-spectrum β-lactamase-producing (ESBL) producing *E. coli* isolates and their antibiotic resistances in wastewater samples. In this experimental study, abattoir wastewater samples were collected and identified from 32 different regions in Mazandaran Province, North Iran. Disk agar diffusion test was applied for testing antibiotic resistance. Phenotypic detection of ESBL-producing isolates was performed using combined disk method. The presence of bla_{SHV} gene was investigated using PCR method. The prevalence of *E. coli* in wastewater samples was 10%. The ESBL test analysis was positive for 14 (93.33%) isolates. The prevalence of bla_{SHV} gene in ESBL-producing *E. coli* isolates was 93.34%. The highest antibiotic resistances in ESBL-producing *E. coli* were found concerning to ceftizoxime and gentamicin (100%), nalidixic acid, ceftazidime and ciprofloxacin (93.34%). Nitrofurantoin was the most effective antibiotic against *E. coli* isolate exhibiting the highest sensitivity (73.34%). In conclusion, the prevalence of ESBL-producing *E. coli* is high and being increased. The high prevalence of bla_{SHV} gene in these isolates may be a reason for their pathogenesis and ability in transferring the resistance genes to multiple antibiotics. Therefore, there is a need to develop appropriate treatment and safer disposing abattoir wastes in this province.

Keywords: *Escherichia coli*, bla_{SHV} gene, abattoir water, antibiotic resistance.

INTRODUCTION

Environmental contaminations have been increased over the last decades due to improper management of pollution and also uncontrolled flow of wastes into the surrounding waters (Tijani AA *et al*. 2017). Abattoir wastewater is now considered as one of the major sources of environmental pollution and human pathogenic bacteria (Adesemoye AO *et al.* 2006). The animal blood and wastes can be penetrated into the water pools and environment and cause the growth of life-treating bacterial strains, including *Salmonella*, *Escherichia coli*, *Shigella*, and *Klebsiella* (Onuoha SC *et al.* 2016). Contamination with these bacterial isolates can be associated with different problems such as diarrhea, typhoid and dysentery (Picozzi SCM *et al*. 2014). Therefore, there is a need to consider the prevalence of bacterial isolates in abattoir wastewater from each geographical region.

E. coli isolates are one of the most important global pathogenic bacteria because of their existence in human and animal intestine as normal flora (Makhdoumi 2018). They can be easily penetrated from wastes and sewages to human water sources and consequently cause various problems such as enteric, diarrhogenic or extraintestinal infections, as well as sepsis or meningitis (Asadi 2016). Recent studies have demonstrated an increase in the prevalence of the multi-drug resistant *E. coli* isolates worldwide (Lu PL *et al.* 2012, Picozzi SCM *et al.* 2014). Extended-spectrum β-lactamases (ESBLs) producing *E. coli* strains have now become a major life-treating

Caspian J. Environ. Sci. Vol. 19 No. 1 pp. 11~17
DOI:

Received: April 28. 2020 Accepted: Sep. 10. 2020
Article type: Research

strain among the antibiotic resistance bacteria (Behrooozi A *et al*. 2010, Lu PL *et al*. 2012). ESBL are plasmid-mediated enzymes mediating resistance to a wide range of antibiotics such as penicillins, cephalosporins, and clavulanic acid (Keynan Y & Rubinstein E 2007). So, these bacterial strains are resistant to a wide range of antibiotics such as penicillins, cephalosporins and aztreonam (M 2001). The bla_{SHV} gene, located on a family of related β-lactamase plasmids, is a significant cause for antibiotic resistance and pathogenesis of ESBL-producing *E. coli* isolates (Mansouri M & R 2009, Picozzi S *et al.* 2013). This implicates the importance of considering these isolates in each region and also the need to adopt appropriate strategies for their control.

Recent studies have demonstrated an increase in the prevalence and antibiotic resistance of ESBL-producing *E. coli* strains in different wastewater samples, especially in hospital and abattoir wastewater (Onuoha SC *et al*. 2016, Tijani AA *et al*. 2017). However, the incidence of ESBL-producing isolates varies from a geographical locality to another. Since these isolates can be transferred into the human water and nutritional sources, it is essential to consider and control the spread of these isolates in each area. Currently, there is a little information about the ESBL-producing *E. coli* isolates from abattoir wastewater samples in Mazandaran Province, north of Iran. Thus, a molecular characterization experiment was performed on wastewater samples of ESBL-producing *E. coli* collecting from 32 abattoirs in this province. This study is a primer report on high prevalence of bla_{SHV} gene in ESBL-producing isolates of *E. coli* and denotes the need to conduct more extensive studies on this gene to determine the magnitude of the problem of antibiotic resistance existing in these regions.

MATERIALS AND METHODS

Sample collection and isolate identification

In this experimental study, wastewater samples were collected from 32 abattoirs in different parts of Mazandaran Province, north of Iran. Samples were collected using sterile Bijou bottles and then placed on ice during transport to the laboratory for analysis. Samples were collected four times per month at an interval of one week over a period of four months from each abattoir and labeled appropriately. There were a total of 4-5 replicates for each sample. Most probable number (MPN) test was performed to estimate the concentration of viable coliform bacteria in wastewater samples (Highsmith AK & Abshire RL 1975).

Wastewater samples were diluted serially and inoculated in lactose broth. The presence of coliforms was identified through color change of the medium and also the presence of gas bubbles collected in the inverted durham tube present in the medium. Finally, the number of total coliforms was calculated by counting the number of tubes giving positive reaction and comparing the pattern of positive results with standard statistical tables (Highsmith AK & Abshire RL 1975).

Wastewater samples with positive coliforms were cultured on eosin methylene blue (EMB) medium for 24 h at 37 ºC. Thereafter, standard biochemical and microbiological tests, including gram staining, catalase, oxidase, Simmons Citrate agar, SIM (sulfide, indole, and motility), triple sugar iron agar (TSI), and Methyl Red - Voges-Proskauer (MR-VP) tests were performed for the identification of *E. coli* isolates.

Antibiotic susceptibility test

After *E. coli* isolates confirmation, disk agar diffusion test using Kirby- Bauer method according to CLSI procedure was used to assess antibacterial effects of different antibiotics. The *E. coli* strains (1.5×10^8 CFU mL^{-1}) were spread onto the surface of the Muller Hinton Agar (MHA) with a sterile swab. Amoxicillin (20 µg), amikacin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), tetracycline (30 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), gentamycin (10 µg), ciprofloxacin (5 µg), cotrimoxazole (10 µg), cefalotin (30 µg), cefixime (5 µg), norfloxacin (10 µg), ceftriaxone (30 µg), and ceftizoxime (10 µg) disks were used as antibiotics. All antibiotics were purchased from the PADTAN TEB Company (Tehran, Iran). The agar plates were incubated for 24 h at 37 ºC followed by measuring the zone of inhibition diameter for each microorganism. All tests were performed as triplicate.

Phenotypic detection of ESBL-positive isolates

To identify the extended-spectrum beta-lactamase (ESBL) positive isolates, bacteria were initially cultured in MHA, then ESBL production was examined by the CLSI confirmatory test using both ceftazidime (30 mg) and cefotaxime (30 mg) disks alone and in combination with clavulanic acid (10 mg). Plates were incubated at 37 º C for 24 h and the zone of inhibition diameter was measured. Observing an increased growth-inhibitory zone

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DOI:

Received: April 28. 2020 Accepted: Sep. 10. 2020
Article type: Research

around either the ceftazidime disk or that containing cefotaxime in combination with clavulanic acid was 5 mm in diameter or greater than those around the disk containing cefotaxime or ceftazidime alone, the isolates were considered positive for ESBL production (Freedman, D & Et, AL 2005).

Molecular detection of *SHV* gene by PCR

The SHV gene was investigated in ESBL-producing isolates by PCR method using specific primers (Table 1). *E. coli* isolates were initially cultured in nutrient broth medium at 37 ºC for 24 h. Cultured isolates were then centrifuged at 4000 rpm for 10 min. Supernatants were removed and the pellets were used for DNA extraction. DNA was extracted using a specific commercial kit provided from CinnaGen Company (Tehran, Iran). The quantity and quality of extracted DNA were evaluated using Nanodrop (Thermo 2000) and agarose gel electrophoresis methods, respectively (Ramazanzadeh R *et al.* 2015). The supernatants containing the DNA were stored at -20 °C for further procedures.

PCR assay

PCR amplification was carried out in a 25 µL reaction mixture with each primer (contained 2.5 µL buffer, 0.75 µL $MgCl_2$, 0.5 µL dNTP, 0.2 µL Taq DNA polymerase, 5 µL template DNA, 1 µL each primer and 14.05 µL dH_2O) as the following steps: an initial denaturation step at 95°C for 5 min, followed by 35 cycles including denaturation at 95°C for 40 sec, annealing at 48 °C for 40 sec, extension at 72 °C for 40 sec and a final extension at 72 °C for 1 min. The PCR products were electrophoresed in a 2% agarose gel for 10-20 min at 70-120 V. The gels were then stained with ethidium bromide and visualized using UV transilluminator.

Statistical analysis

Descriptive statistics was applied for the analysis of frequencies among patients group. Data were analyzed using SPSS software (version 19).

RESULTS

In this study, 150 abattoir wastewater samples were collected from 32 different abattoirs in Mazandaran Province, north of Iran. Fifteen samples (10%) were positive for lactose-fermenting *E. coli* in the MPN test searching for faecal and total coliforms. Positive isolates were confirmed upon gram staining, catalase and oxidase tests, as well as by culturing in Simmons' citrate agar, SIM, TSI, and MR-VP media. The isolated *E. coli* produced a metallic green sheen on EMB agar. After gram staining, the isolates were found as pink coloured bacilli on microscopic examination. They were positive in catalase test, but negative for oxidase one. The Simmons' citrate agar test exhibited negative results for the isolates. The SIM test results were as follows: Sulfide (-), Indole (+) and Motility (+). In TSI medium seemed yellow/yellow with bubbles or gas production due to glucose and lactose fermentation along with acid production and alkaline reduction (A/A). The MR-VP test exhibitd that the strains were positive in MR while negative in VP. The antibiotic susceptibility of the isolates is shown in Table 2. According to the table, the highest antibiotic resistance was found concerning to ceftizoxime (100%), gentamicin (100%), nalidixic acid (93.33%), ceftazidime (93.34%), and ciprofloxacin (93.34%), respectively. Nitrofurantoin was the most effective antibiotic against the isolates (73.34% in sensitivity) (Table 2).

The ESBL test was positive in 14 (93.33%) isolates. Molecular analysis for bla_{SHV} gene in 14 out of 15 isolated *E. coli* (93.34%) were positive, while one isolate (6.66%) was negative (Fig. 1).

DISCUSSION

In this research we considered the prevalence of SHV beta-lactamase producing *E. coli* isolates and also their antibiotic resistance pattern from 32 different abattoirs in Mazandaran Province. Overall, our findings have revealed that the prevalence of *E. coli* in abattoir wastewater samples was 10%. These isolates were resistant to most of examined antibiotics, particularly ceftizoxime (100%), gentamicin (100%), nalidixic acid (93.33%), ceftazidime (93.34%), and ciprofloxacin (93.34%). Nitrofurantoin was the most effective antibiotic against the *E. coli* isolates, exhibiting 73.34% in sensitivity. The phenotype ESBL test indicated that 93.33% of these isolates were beta-lactamase enzyme positive. Furthermore, molecular analysis of *SHV* gene revealed that 14 (93.34%) out of 15 isolates were bla_{SHV}-gene positive . These data indicate that wastewater samples of abattoirs

Caspian J. Environ. Sci. Vol. 19 No. 1 pp. 11~17
DOI:

Received: April 28. 2020 Accepted: Sep. 10. 2020
Article type: Research

from Mazandaran Province are a potential reservoir for ESBL-producing *E. coli*. The mechanism of multiple antibiotic

Table 1. Primers used for *bla*$_{SHV}$ amplification.

Primer	Sequences	Product size
Forward	5'-TCAGCGAAAAACACCTTG-3'	471 bp
Reverse	5'-TCCCGCAGATAAATCACC-3'	

Table 2. Antimicrobial resistance rates of *E. coli* isolates.

Antibiotics	Resistant (R)	Intermediate resistance (I)	Sensitive (S)
Ceftriaxone	9 (60%)	-	6 (40%)
Nalidixic acid	14 (93.34%)	-	1 (6.66%)
Ceftizoxime	15 (100%)	-	-
Nitrofurantoin	3 (20%)	1 (6.66%)	11 (73.34%)
Amikacin	9 (60%)	-	6 (40%)
Gentamicin	15 (100%)	-	-
Ciprofloxacin	14 (93.34%)	-	1 (6.66%)
Tetracycline	12 (80%)	-	3 (20%)
Cotrimoxazole	13 (86.66%)	-	2 (13.34%)
Cefalotin	8 (53.34%)	-	7 (46.66%)
Cefixime	13 (86.66%)	-	2 (13.34%)
Norfloxacin	12 (80%)	-	3 (20%)
Cefotaxime	13 (86.66%)	-	2 (13.34%)
Amoxicillin	9 (60%)	-	6 (40%)
Ceftazidime	14 (93.34%)	-	1 (6.66%)

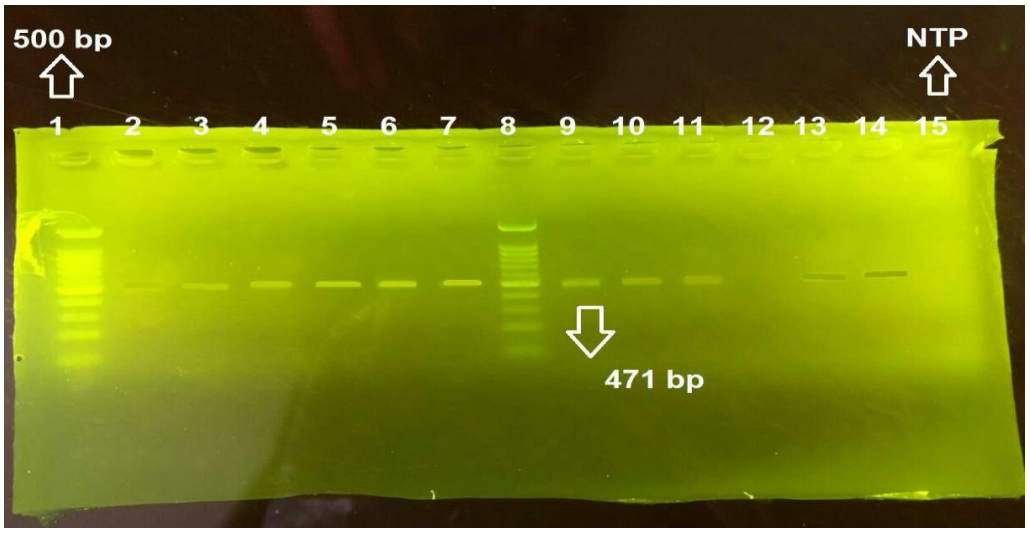

Fig. 1. Agarose gel electrophoresis of PCR-amplified *bla*$_{SHV}$ gene. Lanes 1 and 8: 500-bp ladder; Lanes 2-7 and 9, 10, 11, 13, and 14: *E. coli* isolates showing 471 bp *bla*$_{SHV}$ amplicon. Lane 12: *E. coli* isolate without *bla*$_{SHV}$ amplicon. Lane 15: negative control.

resistances of these strains is likely due to the presence of *SHV* gene which is an alarm for health service in this region. Many studies have reported the prevalence of the *SHV* beta-lactamase producing *E. coli* isolates in various samples from different parts of the world. Onuoha *et al*. (2016) reported the distribution of antibiotic-resistant bacteria from abattoir wastes in Nigeria including *P. aeruginosa* (28.56%), *E. coli* (14.28%), *S. aureus* (7.14%), *Klebsiella* (7.14%), *Shigella* (7.14%), *Enterococcus* (5.57%), *Salmonella* (28.56%), and *Streptococcus* (3.57%). Interestingly, they found that all isolated bacterial strains were completely resistant to tetracycline, cephalothin, penicillin G, cefuroxime, erythromycin, nalidixic acid, sulphamethoxazole, cefpirome, and oxytetracycline antibiotics. Azithromycin and imipenem were the most effective antibiotics (Onuoha *et al*. 2016). Similarly, in our study the prevalence of the ESBL-producing *E. coli* isolates was 10% , completely resistant to ceftizoxime (100%) and gentamicin (100%), and also highly resistant to nalidixic acid (93.33%), ceftazidime (93.34%), and ciprofloxacin (93.34%). In another study, Jørgensen *et al*. (2017) reported the prevalence of ESBL-producing *E. coli* from clinical, recreational water and wastewater samples. These isolates

Caspian J. Environ. Sci. Vol. 19 No. 1 pp. 11~17
DOI:

Received: April 28. 2020 Accepted: Sep. 10. 2020
Article type: Research

were multidrug-resistant and found in 40% of recreational water samples. Gündoğdu *et al*. (2013) also reported 252 ESBL-producing *E. coli* isolates in hospital wastewaters and sewage treatment plants in Australia. These strains were also resistant to up to 9 non-β-lactam antibiotics. Noteworthy, over 73% of the hospital wastewater isolates possessed *SHV*-type ESBL. In our study, 93.34% of isolates (14 out of 15 isolates) were *SHV*-type ESBL. These data suggest the variation in ESBL-producing *E. coli* obtained from different samples. Čornejová *et al*. (2015) reported the ESBL-producing *E. coli* isolates from municipal wastewater, finding ESBL phenotype in 26% of environmental strains. In another study in Lebanon, The prevalence of ESBL-producing *E. coli* isolates in refugee camp and wastewaters was 53.1% and 49.1%, respectively (Tokajian S *et al*. 2018). Diallo *et al*. (2013) examined ESBL-producing *E. coli* isolates in the municipal wastewater treatment plant receiving slaughterhouse wastewater, finding their prevalence in wastewater, slaughterhouse wastewater and in the treated effluent as 0.7%, 0.2% and 0.5%, respectively. In another study in Indonesia, Sudarwanto *et al*. (2017) reported the multi-drug resistance prevalence in the ESBL-producing *E. coli* strains isolated from the environment of Bogor slaughterhouse to be 14.3%. Approximately, 80% of ESBL-producing *E. coli* isolates showed multi-drug resistance phenotypes against several antibiotics. The antibiotic resistances to penicillin G, streptomycin, gentamicin, trimethoprim-sulfamethoxazole, tetracycline, ciprofloxacin, enrofloxacin, and polymyxin B was 100%, 100%, 60%, 60%, 40%, 40%, 20% and 0%, respectively (Sudarwanto MB *et al.* 2017), similar to the results of the present study. We found that the prevalence of ESBL-producing *E. coli* isolates was 10% and they were resistant to most examined antibiotics. Chishimba *et al*. (2016) examined the prevalence of ESBL-producing *E. coli* and their antibiotic resistances in Market-Ready Chickens reporting that 20.1% of total samples were ESBL-producing *E. coli*. Furthermore, 85.7% of these isolates were resistant to beta-lactam and other antimicrobial antibiotics. Therefore, our findings suggest that the prevalence of ESBL-producing *E. coli* from abattoir wastewater in Mazandaran Province is high and can be a life-treatening source for human health. More importantly, these ESBL-producing strains are multi-drug resistant and increasing throughout the world, which raises concerns regarding the treatment and also the antibiotic administering policies.

CONCLUSION

Our findings indicated that the prevalence of ESBL-producing *E. coli* from abattoir wastewater in Mazandaran Province is high and emerging which can lead to various health problems. More importantly, the frequency of bla_{SHV} gene in these isolates, particularly in MDR isolates, is very high which may be a reason for their pathogenesis and multiple antibiotic resistant. Therefore, there is a need to develop the strategies on the antibiotic administering policies in these isolates.

ACKNOWLEDGMENT

This study was supported by grant received from Department of Biology, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran. We would also like to appreciate the staffs of the Department of Biology, Sari Branch, Islamic Azad University, Sari, Iran.

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Received: April 28. 2020 Accepted: Sep. 10. 2020
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Caspian J. Environ. Sci. Vol. 19 No. 1 pp. 11~17
DOI:

Received: April 28. 2020 Accepted: Sep. 10. 2020
Article type: Research

شیوع مقاومت آنتی بیوتیکی و ژن بتالاکتاماز *SHV* از *شریشیا کلی* جدا شده از پساب کشتارگاه در استان مازندران، شمال ایران

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(تاریخ دریافت: ۹۹/۰۲/۰۹ تاریخ پذیرش: ۹۹/۰۶/۲۰)

چکیده

فاضلاب کشتارگاه‌ها یکی از مهمترین منبع آلودگی به باکتری‌های بیماری زا و تهدید کننده حیات انسان هاست. هدف این پژوهش، تعیین میزان مقاومت آنتی بیوتیکی و ژن بتالاکتاماز SHV از *اشریشیا کلی* جدا شده از نمونه‌های پساب کشتارگاه است. در این تحقیق تجربی، نمونه‌های پساب کشتارگاه از ۳۲ ناحیه مختلف در استان مازندران، شمال ایران، جمع‌آوری شد. سنجش انتشار از دیسک برای بررسی مقاومت به آنتی بیوتیک استفاده شد. شناسایی فنوتیپی سویه‌های تولید کننده ESBL به روش دیسک ترکیبی انجام شد. حضور ژن blaSHV به روش PCR بررسی شد. نتایج شیوع *E. coli* در نمونه‌های پساب ۱۰٪ بود. نتیجه تست ESBL برای ۱۴ سویه (۹۳/۳۳٪) مثبت بود. شیوع ژن blaSHV در سویه‌های *E. coli* تولید کننده ESBL ۹۳/۳۴ ٪. بیشترین مقاومت آنتی بیوتیکی در سویه‌های *E. coli* تولید کننده ESBL برای سفتیزوکسیم و جنتامایسین (۱۰۰٪)، نالیدیکسیک اسید، سفتازیدیم و سیپروفلوکسازین (۹۳,۳۴٪) مشاهده شد. نیتروفورانتونین مؤثرترین آنتی بیوتیک علیه سویه *E. coli* بود که ۷۳/۳۴٪ حساسیت نشان داد. در نتیجه، شیوع سویه‌های *E. coli* تولید کننده ESBL بالا و در حال افزایش است. افزایش فراوانی ژن blaSHV در این جدایه‌ها احتمالاً یکی از دلایل بیماری زایی و همچنین قابلیت انتقال ژن‌های مسئول مقاومت به چندین آنتی بیوتیک است. بنابراین، تیمار مناسب و دسترسی سالم نمونه-های پساب در مازندران بسیار حائز اهمیت است.

*مولف مسئول

Bibliographic information of this paper for citing:

Tonekabony, N, Izadi Amoli**,** R, Gholami, A, Oskoueiyan, R 2021, Prevalence of *SHV* gene and antibiotic resistance of extended-spectrum β-lactamases-producing *Escherichia coli* strains isolated from abattoir wastewater in Mazandaran, North of Iran. Caspian Journal of Environmental Sciences, 19: 11 - 17

Caspian J. Environ. Sci. Vol. 19 No. 1 pp. 11~17
DOI:
Received: April 28. 2020 Accepted: Sep. 10. 2020
Article type: Research