

Purification, Extraction and Visualization of Lipopolysaccharide of *Escherichia coli* From Urine Samples of Patients With Urinary Tract Infection

Fariha Akhter Chowdhury,¹ Mohammad Nurul Islam,¹ Anamika Saha,¹ Sabrina Mahboob,¹ Abu Syed Md. Mosaddek,² Md Omar Faruque,³ Most. Fahmida Begum,⁴ and Rajib Bhattacharjee^{1*}

¹Department of Pharmaceutical Sciences, North South University, Dhaka, Bangladesh

²Department of Pharmacology, Uttara Adhunik Medical College, Dhaka, Bangladesh

³Department of Physiology and Molecular Biology, Bangladesh University of Health Sciences, Dhaka, Bangladesh

⁴Department of Microbiology, Uttara Adhunik Medical College, Dhaka, Bangladesh

*Corresponding author: Rajib Bhattacharjee, Department of Pharmaceutical Sciences, North South University, Dhaka, Bangladesh. Tel: +1-7807079527, Fax: +880-255668202, E-mail: rajibbhattacharjee@yahoo.com

Received 2015 August 9; Revised 2015 August 22; Accepted 2015 September 19.

Abstract

Background: Urinary tract infection (UTI) is one of the most common infectious diseases in Bangladesh where *Escherichia coli* is the prevalent organism and responsible for most of the infections. Lipopolysaccharide (LPS) is known to act as a major virulence factor of *E. coli*.

Objectives: The present study aimed to purify, extract and visualize LPS of *E. coli* clinical isolates from urine samples of patients with UTI.

Patients and Methods: The *E. coli* strain was isolated from the urine samples of 10 patients with UTI and then the antibiotic sensitivity pattern of the isolates was determined. The purification of LPS was carried out using the hot aqueous-phenol method and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, which was directly stained using the modified silver staining method and Coomassie blue.

Results: The silver-stained gel demonstrated both smooth and rough type LPS by showing trail-like band patterns with the presence and lacking O-antigen region, respectively. Coomassie blue staining showed no band assuring the absence of any contaminating protein.

Conclusions: Our successful extraction of purified LPS from *E. coli* isolates of UTI patients' urine samples can be an important step to understand the UTI disease conditions.

Keywords: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Electrophoresis, Polyacrylamide Gel, Silver Staining, *Escherichia coli*

1. Background

Urinary tract infection (UTI) is one of the frequently encountered infectious diseases, having an estimated number of 150 million cases per annum all over the globe (1). The disease majorly caused by Gram-negative bacteria and *Escherichia coli* is the most prevalent causative agent that is responsible for more than 90% cases of UTI (2). Like all other Gram-negative bacteria, *E. coli* also possess lipopolysaccharide (LPS) as an integral component of their cell wall. Earlier studies by others have shown that LPS can enhance recruitment of leukocytes to the infection site and mucosal inflammation by which LPS plays a role as one of the virulence factors in UTI (3). Hence, purification and visualization of *E. coli* LPS obtained from patients with UTI has great potential to be a study of interest that may reveal ways to better understanding and measurement to treat UTI.

Lipopolysaccharide is the main outer membrane component of Gram-negative bacteria, which consists of major three parts: lipid A, hydrophilic core oligosaccharide and repetitive polysaccharide entitled as O-antigen. Hydrophobic lipid A is highly preserved and mainly respon-

sible for the toxicity of the molecule, while the O-antigen is repeating hydrophilic oligosaccharide side chain that is specific to bacterial serotype (4). Lipopolysaccharide is a common virulence factor and binds to toll-like receptor 4 (TLR4), subsequently triggers the production of pro-inflammatory cytokines, initiating the innate response against uropathogenic *E. coli* (UPEC) (5).

Owing to the virulent role of LPS in infectious diseases, many studies have been conducted and have introduced several methods and protocols for the purification and characterization of LPS from bacteria. However, many of these methods have failed to extract pure LPS because of common problems like nucleic acid and protein contaminations, which is critical for some delicate molecular and immunological experiments. A hot aqueous-phenol method for the purification and isolation of LPS from Gram-negative bacterial cells is a protocol that allows the extraction of LPS away from nucleic acids and proteins that can interfere with visualization of LPS that occurs with shorter, less intensive extraction methods (6).

2. Objectives

In the present study, we have isolated and identified *E. coli* from the urine sample of patients with UTI from a tertiary hospital in Bangladesh and tested the antibiotic susceptibility pattern. The aim of the study was to carry out hot aqueous-phenol extraction complemented with proteinase K digestion and nuclease elimination to extract LPS from *E. coli* prior to separation and visualization by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and standard silver staining methods, respectively. This study could be important to understand the pathogenesis of UTI caused by *E. coli*.

3. Patients and Methods

3.1. Sample Collection

The study population was considered to be inpatients and outpatients at Uttara Adhunik Medical College Hospital, Uttara, Dhaka, with clinically diagnosed UTI. The diagnosis of UTI was based on microscopic findings of more than 5 white blood cells per high power field (1000× for high power) and a colony count of 10⁵ colony forming units/mL of a single pathogen. Patients with UTI were selected randomly between ages 1 month to 80 years, comprising of both male and female, either out- or in patients over a period from August to September 2014. We excluded the patients who were already taking antibiotic for treatment. The urine of 58 patients was sampled by clean catch of midstream urine. Urine samples were delivered to the laboratory within 1 hour of collection and processed within 2 - 4 hours. Fifty-one samples have presented *E. coli* upon isolation and identification as UTI causing organism, among which 10 *E. coli* isolates were randomly selected for LPS extraction.

3.2. Isolation and Identification of *Escherichia coli*

Isolation and identification were done according to the previous reference method (7) with modification. A loopful 0.01 mL of urine sample was cultured on blood agar (Sisco Research Laboratories Pvt. Ltd.) and MacConkey agar (Oxoid, USA) and incubated for 24 hours at 37°C aerobically. Organisms were selected for inclusion in further study when they occurred as pure culture and in concentrations greater than 10⁵ CFU/mL. Selection of isolates was executed on bacterial colonies with different morphology and identified by their biochemical profiles.

3.3. Antibiotic Susceptibility Test

Antibiotic sensitivity pattern of *E. coli* isolates was determined on Mueller-Hinton agar (Sisco Research Laboratories Pvt. Ltd.) with the Kirby Bauer's disk diffusion method (8) using the following antimicrobial drugs (Mast, UK): Amoxiclav (10 µg), ceftriaxone (5 µg), ceftazidime (30 µg), cefpodoxime (10 µg), aztreonam (30 µg), ciprofloxacin (5

µg), trimethoprim-sulfamethoxazole (25 µg), cefuroxime (5 µg), cefixime (5 µg), netilmicin (10 µg), erythromycin (5 µg), mecillinum (30 µg), imipenem (10 µg), meropenem (10 µg), azithromycin (15 µg), gentamicin (10 µg), amoxicillin (10 µg), and cefradine (30 µg). Isolates were stated as sensitive or resistant on the basis of zone of inhibition according to the guideline of Clinical Laboratory Standards Institute (9).

3.4. Bacterial Strains and Growth Conditions

The *E. coli* strain was grown in the Luria-Bertani broth (LB) medium (Sisco Research Laboratories Pvt. Ltd.) at 37°C in a shaker incubator (Shel Lab) overnight. After that the culture was diluted with LB and OD₆₀₀ reading was taken. Based on the OD₆₀₀ reading, a 1.5 mL suspension of *E. coli* was made to an OD₆₀₀ of 0.5. Bacteria were pelleted in a microcentrifuge (Humanlab Instrument Co.) at 10,600 × g for 10 minutes. After centrifugation of culture suspension, sedimented bacteria were harvested and used for LPS extraction and purification.

3.5. Lipopolysaccharide Extraction and Purification

LPS was extracted by the hot phenol-water method. The pelleted bacteria were resuspended in 200 µL of 1× SDS-buffer. Then the suspended bacteria were boiled in a water bath (Shel Lab) for 15 minutes. Treatment with Proteinase K, DNase I and RNase was performed prior to extraction step. For this purpose, DNase I (Thermo Scientific, USA) and RNase (10 µg/mL) (Favorgen Biotech Corp, Taiwan) were added to the cell mixture and the tubes were kept at 37°C for 30 minutes. Mixture was subsequently treated with Proteinase K (10 µg/mL) (Favorgen Biotech Corp, Taiwan) and incubation was continued at 59°C for 3 hours. At the next step, to each sample 200 µL of ice-cold Tris-saturated phenol was added and vortex for approximately 5 to 10 seconds. After that the samples were incubated at 65°C for 15 minutes and 1 mL of room-temperature diethyl ether was added to each sample and vortex for 5 to 10 seconds. Samples were then centrifuged at 20, 600 × g 4°C for 10 minutes. After centrifugation the blue bottom layer was extracted and 200 µL of 2× SDS-buffer was added each of the extracted samples before separating by SDS-PAGE.

3.6. SDS-PAGE for LPS

The sodium dodecyl sulfate-polyacrylamide gel (Sisco Research Laboratories Pvt. Ltd.) containing 4% and 12% acrylamide for the stacking and separating gel respectively, were prepared and electrophoresis (Bio-Rad) was done at 30 mA until the tracking dye bromophenol blue front is a few mm from the bottom.

3.7. Gel Staining

SDS-PAGE fractionated LPS preparations were stained by the silver and Coomassie blue staining. SDS-PAGE fractionated LPS preparations were stained according to the previously described methods of silver staining (10) and standard Coomassie blue staining (11).

4. Results

4.1. Antibiotic Susceptibility

After successful isolation and identification, *E. coli* samples demonstrated highest antimicrobial susceptibility towards meropenem followed by mecillinum and ceftazidime. A higher percentage of samples showed resistance to amoxicillin and cefradine (Table 1).

4.2. Lipopolysaccharide Extraction and Purification

After the LPS extracts from *E. coli* were run by SDS-PAGE, the gels were stained by Coomassie blue (Figure 1) and silver nitrate (Figure 2). Bands below 20 kDa of molecular weight

marker were considered to be significant for *E. coli* LPS.

To signify the effectiveness of the contaminating protein elimination treatment, the gel was stained by Coomassie blue. In Figure 1, the Coomassie blue stained gel showed no banding pattern verifies the absence of protein contaminations.

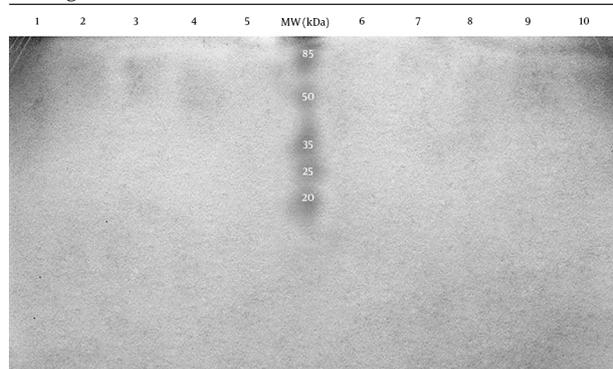
LPS was visualized and characterized upon staining the gel by silver staining. As illustrated in Figure 2, LPS showed dark trail-like bands where lanes 2, 3, 10 and lanes 4, 5, 7 showed similar positions of O-antigen region, respectively. Rest of the four lanes exhibited different patterns of LPS banding that lacked O-antigen (Table 2). At the bottom of the gel, darker regions of all the bands indicate the migration of Lipid A-core core that happened to be nearby the dye-front and therefore got stained intensely.

Table 1. Antibiotic Susceptibility and Resistance Pattern of *Escherichia coli* Strains Isolated From Patients With Urinary Tract Infection^a

Antimicrobial Agent	Resistant Isolates	Susceptible Isolates
Amoxiclav	25.49	74.51
Ceftriaxone	39.22	60.78
Ceftazidime	9.80	90.20
Cefpodoxime	41.18	58.82
Aztreonam	21.57	78.43
Ciprofloxacin	50.98	49.02
Trimethoprim-sulfamethoxazole	49.02	50.98
Cefuroxime	54.90	45.10
Cefixime	43.14	56.86
Netilmicin	15.69	84.31
Erythromycin	35.29	64.71
Mecillinum	7.84	92.16
Imipenem	11.76	88.24
Meropenem	0	100
Azithromycin	45.10	54.90
Gentamicin	21.57	78.43
Amoxicillin	94.12	5.88
Cefradine	78.43	21.57

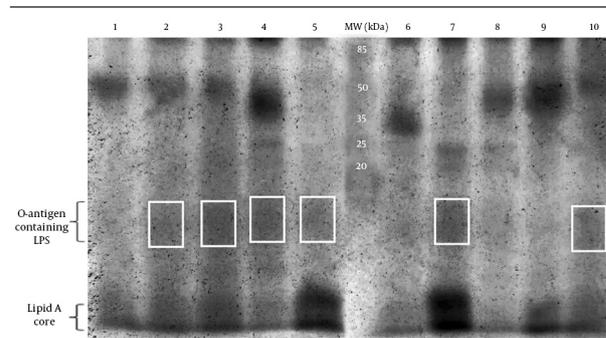
^aValues are presented based on percent.

Figure 1. Coomassie Blue Staining of Purified Lipopolysaccharide of UTI Causing *E. coli* Clinical Isolates



Lipopolysaccharide from ten *E. coli* strains from urine samples of patients with UTI was purified by modified hot-aqueous phenol extraction and fractionated by the SDS-PAGE followed by silver staining following the standard protocol. The staining revealed dark trail-like bands of purified LPS where O-antigen positions are boxed with white borders. MW = molecular weight marker.

Figure 2. Silver Staining of Purified Lipopolysaccharide of UTI Causing *E. coli* Clinical Isolates



Lipopolysaccharide from ten *E. coli* strains from urine samples of patients with UTI was purified by modified hot-aqueous phenol extraction and fractionated by the SDS-PAGE followed by silver staining following the standard protocol. The staining revealed dark trail-like bands of purified LPS where O-antigen positions are boxed with white borders. MW = molecular weight marker.

Table 2. Selected Profile of Patients With Urinary Tract Infection

Sample No.	Complaints	Age, y	Lane No. on Gel	LPS Status
1	Burning sensation during micturition, fever, pain in lower abdomen	32	1	Rough
2	Burning sensation during micturition, fever, pain in lower abdomen	51	2	Smooth
3	Burning sensation during micturition, fever, nausea, vomiting, pain in lower abdomen, red color urine, history of diabetes	50	3	Smooth
4	Burning sensation during micturition, fever, nausea, vomiting, pain in lower abdomen	55	4	Smooth
5	Burning sensation during micturition, fever, reddish color urine, pain in lower abdomen	59	5	Smooth
6	Burning sensation during micturition, fever, pain in lower abdomen	24	6	Rough
7	Burning sensation during micturition, fever, pain in lower abdomen	51	7	Smooth
8	Burning sensation during micturition, fever, dysuria	42	8	Rough
9	Burning sensation during micturition, fever, pain in lower abdomen	4	9	Rough
10	Burning sensation during micturition, fever, pain in lower abdomen	50	10	Smooth

Abbreviation: LPS, lipopolysaccharide.

5. Discussion

In the present study, the patients were experiencing symptomatic UTI with symptoms like: burning sensation during micturition, fever, nausea, vomiting, pain in lower abdomen etc. These symptoms are seen to be very commonly encountered by UTI patients of all ages (12). Most of the isolated *E. coli* strains showed resistance to amoxicillin and cefradine, which supports the findings of other related studies that showed *E. coli* demonstrates antimicrobial resistance toward amoxicillin and cefradine almost every time (13-15).

The extraction of LPS was focused mainly by three ideas: fragmentation of the bacterial cell envelope and macromolecular constituents, treatment of the remaining fractionated residue of the cell structure with DNase I, RNase and Proteinase K and finally hot aqueous phenol extraction, a method adapted from that of previous study. SDS, a potent negatively charged detergent, binds tendentiously to the cationic sites and nearby hydrophobic regions of lipid A core (17). Even after treating *E. coli* cell with boiling 4% SDS, researchers have always found remaining insoluble residues tend to be complex macromolecules that are potential contaminants and can interfere with most downstream immunological and biological experiments (18, 19). Nuclease elimination of contaminating RNA and DNA and proteinase K conducting bacterial protein degradation prior to phenol-water extraction provides highly pure LPS free of protein and nucleic acids. The advantage of this method compared to the protease treatment described by Hitchcock and Brown (6) is that this technique leads to pure LPS samples that can be better resolved by the SDS-PAGE and silver-staining. The Coomassie blue staining is most well-known for protein staining but considerably has less sensitivity (20). Thus, this staining method will show bands of higher abundance of proteins. In this study, no visualizing band in the Coomassie blue gel staining revealed absence of con-

taminating bacterial proteins, which suggests the effectiveness of protein elimination by protease treatment. However, silver-staining, having the advantage of its high sensitivity, is competent to detect as low as 1 ng of LPS (21). This method is suitable for analytical procedures of other Gram-negative bacteria; however, it is not applicable to yield LPS for structural analysis and likewise other purposes. Serological evidence of UPEC can be specified by LPS profile typing, evaluating correlation between *E. coli* strains and type of LPS. By this technique characteristic band-patterns for smooth and rough-LPS of isolates within a suspected outbreak will permit to find out the presence and absence of O-antigen side chain and therefore to signify serotypeable and nonserotypeable respectively in terms by this technique.

In the present study, lanes with the presence of O-antigen portion are characteristics of smooth-form and lanes lacking O-antigen are typical for rough-form of LPS in Gram-negative bacteria (22). One of the significant findings of the study is that *E. coli* isolated from the urine samples of patients who were more than 50 years of age were found to possess smooth LPS as seen from our data (Table 2). On the other hand, *E. coli* from the patients below 50 years showed rough type LPS lacking O-antigen. The absence of O-antigen specifies that the strains were with rough LPS, which is more hydrophobic than the smooth type (23). Considering the limitation of the number of samples of our study, the connection between patient's age and the type of LPS involved in pathogenesis, is difficult to infer. However, it can be speculated that as smooth LPS with O-antigen is comparatively hydrophilic and as patients over 50 years predominantly were infected by *E. coli* with smooth LPS, hydrophilic antibiotic could be a better option to manage this type of UTI patients. On the other hand, selection of hydrophobic antibiotics could be a better treatment option for the younger patients.

Targeting not the appropriate strains of organisms by appropriate antibiotics can be a reason of therapeutic failure and development of drug resistance (24). There have not been enough studies establishing a relationship between age of patients and *E. coli* strain characteristics, but several studies on other animal species have discovered a relationship. Studies have shown that calves shed *E. coli* O157:H7 longer than adult cattle given the same level of *E. coli* O157:H7 inoculums (25). Moreover, different sexes or ages have influence on the distribution of the *E. coli* genotypes (26). Further study testing this hypothesis could be very exhilarating.

In conclusion, this study could be vital to understand UTI disease conditions and further work on LPS profiling and serotyping UPEC strains, which can contribute to more effective diagnosis and treatment.

Acknowledgments

Authors wish to thank all the staffs and the Pharmaceutical Sciences laboratory technicians of North South University, Bangladesh, who helped and cooperated during the research work.

Footnotes

Authors' Contribution:All persons who meet authorship criteria are listed as authors. They have participated in the research work and took responsibility for the content, including participation in the concept, design, analysis and writing of the manuscript. Fariha Akhter Chowdhury, Anamika Saha, and Sabrina Mahboob have performed the research works. Fariha Akhter Chowdhury has written manuscript. Mohammad Nurul Islam has checked the manuscript and helped in lab experiments. Abu Syed Md. Mosaddek, Md Omar Faruque, Most. Fahmida Begum were the collaborators of the work and helped by collecting patient samples, and determined their clinical profile. Rajib Bhattacharjee designed the experiment, checked the manuscripts and supervised the overall works.

Funding/Support:This study was supported by the Faculty of Pharmaceutical Sciences, North South University, Dhaka, Bangladesh.

References

- Bashir MF, Qazi JI, Ahmad N, Riaz S. Diversity of urinary tract pathogens and drug resistant isolates of *Escherichia coli* in different age and gender groups of Pakistanis. *Trop j pharm res.* 2008;7(3):1025-31. doi:10.4314/tjpr.v7i3.14687.
- Behzadi P, Behzadi E. The microbial agents of urinary tract infections at Central Laboratory of Dr. Shariati Hospital, Tehran, Iran. *Turk Klin Tip Bilim.* 2008;28(4):445.
- Elgavish A. Effects of *Escherichia coli* and *E. coli* lipopolysaccharides on the function of human ureteral epithelial cells cultured in serum-free medium. *Infect Immun.* 1993;61(8):3304-12. [PubMed: 8101508]
- Erridge C, Bennett-Guerrero E, Poxton IR. Structure and function of lipopolysaccharides. *Microbes Infect.* 2002;4(8):837-51. [PubMed: 12270731]
- Rudick CN, Billips BK, Pavlov VI, Yaggie RE, Schaeffer AJ, Klumpp DJ. Host-pathogen interactions mediating pain of urinary tract infection. *J Infect Dis.* 2010;201(8):1240-9. doi: 10.1086/651275. [PubMed: 20225955]
- Hitchcock PJ, Brown TM. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol.* 1983;154(1):269-77. [PubMed: 6187729]
- Nerurkar A, Solanky P, Naik SS. Bacterial pathogen in urinary tract infection and antimicrobial susceptibility pattern. *J Pharm Biomed Sci.* 2012;21:1-3.
- Apu AS, Chowdhury FA, Khatun F, Jamaluddin ATM, Pathan AH, Pal A. Phytochemical screening and in vitro evaluation of pharmacological activities of *Aphanamixis polystachya* (Wall) Parker fruit extracts. *Trop J Pharm Res.* 2013;12(1):11-6. doi: 10.4314/tjpr.v12i1.18.
- Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial disk susceptibility tests: approved standard-CLSI document M02-A11. 11; 2012.
- Tsai CM, Frasch CE. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal biochem.* 1982;119(1):15-9. [PubMed: 6176137]
- Turk Center for Biotechnology. *Coomassie Blue Staining*. 2009. Available from: http://www.btk.fj/fileadmin/Page_files/proteomics/PDF-files/Protocols/Coomassie_Blue_staining_version_W.01.pdf.
- Komala M, Kumar KPS. Urinary Tract Infection: Causes, Symptoms, Diagnosis and It's Management. *Ind J Res Pharma Biotech.* 2013;1(2):226-33.
- Farrell DJ, Morrissey I, De Rubeis D, Robbins M, Felmingham D. A UK multicentre study of the antimicrobial susceptibility of bacterial pathogens causing urinary tract infection. *J Infect Dis.* 2003;46(2):94-100.
- Shahina Z, Islam MJ, Abedin J, Chowdhury AHMI, Arifuzzaman MD. A Study of Antibacterial Susceptibility and Resistance Pattern of *E. coli* Causing Urinary Tract Infection in Chittagong, Bangladesh. *Asian J Biol Sci.* 2011;4:548-55. doi: 10.3923/ajbs.2011.548.555.
- Saeed MA, Haque A, Ali A, Mohsin M, Bashir S, Tariq A, et al. A profile of drug resistance genes and integrons in *E. coli* causing surgical wound infections in the Faisalabad region of Pakistan. *J Antibiot (Tokyo).* 2009;62(6):319-23. doi: 10.1038/ja.2009.37. [PubMed: 19444298]
- Davis Jr MR, Goldberg JB. Purification and visualization of lipopolysaccharide from Gram-negative bacteria by hot aqueous-phenol extraction. *J Vis Exp.* 2012;(63):3916. doi: 10.3791/3916. [PubMed: 22688346]
- Hitchcock PJ. Aberrant migration of lipopolysaccharide in sodium dodecyl sulfate/polyacrylamide gel electrophoresis. *Eur J Biochem.* 1983;133(3):685-8. [PubMed: 6190650]
- Wang X, Zhang C, Shi F, Hu X. Purification and characterization of lipopolysaccharides. In: Wang X, Quinn PJ, editors. *Endotoxins: Structure, Function and Recognition. Subcellular Biochemistry.* Netherlands: Springer; 2010. pp. 27-51.
- Wu MC, Heath EC. Isolation and characterization of lipopolysaccharide protein from *Escherichia coli*. *Proceedings of the National Academy of Sciences.* 1973;70(9):2572-6.
- Weiss W, Weiland F, Görg A. Protein detection and quantitation technologies for gel-based proteome analysis. In: Reinders J, Sickmann A, editors. *Proteomics. Methods in Molecular Biology™.* Humana Press; 2009. pp. 59-82.
- Rezania S, Amirmozaffari N, Tabarraei B, Jeddi-Tehrani M, Zarei O, Alizadeh R, et al. Extraction, Purification and Characterization of Lipopolysaccharide from *Escherichia coli* and *Salmonella typhi*. *Avicenna J Med Biotechnol.* 2011;3(1):3-9. [PubMed: 23407691]
- Hendrick CA, Sequeira L. Lipopolysaccharide-Defective Mutants of the Wilt Pathogen *Pseudomonas solanacearum*. *Appl Environ Microbiol.* 1984;48(1):94-101. [PubMed: 16346603]
- Bantoch S, Buhler T, Lam JS. Appropriate coating methods and other conditions for enzyme-linked immunosorbent assay of smooth, rough, and neutral lipopolysaccharides of *Pseudomonas aeruginosa*. *Clin Diagn Lab Immunol.* 1994;1(1):55-62. [PubMed: 7496923]

24. Gomaa AA, Klumpe HE, Luo ML, Selle K, Barrangou R, Beisel CL. Programmable removal of bacterial strains by use of genome-targeting CRISPR-Cas systems. *MBio*. 2014;**5**(1):e00928-13. doi: 10.1128/mBio.00928-13. [PubMed: 24473129]
25. Cray WC, Moon HW. Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Appl Environ Microbiol*. 1995;**61**(4):1586-90. [PubMed: 7747972]
26. Tenaillon O, Skurnik D, Picard B, Denamur E. The population genetics of commensal *Escherichia coli*. *Nat Rev Microbiol*. 2010;**8**(3):207-17. doi: 10.1038/nrmicro2298. [PubMed: 20157339]