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Isolation and Identification of Extended Spectrum B-Lactamase *E. coli* from Retail Chicken Meat and the Effect of *Azadirachta indica* (Neem) Plant on Them

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Abstract

Extended Spectrum β-Lactamase (ESBL) producing *Escherichia coli* is an evolving pathogen globally seen as contamination in chicken meat and poultry, contributing to the increased chances of infections in humans. This study analyzes the occurrence of ESBL *E. Coli* in chicken and *Azadirachta Indica* (Neem)'s effect on them. Samples from chicken caeca were taken from different local broiler retail shops of Islamabad.

ESBL producing isolates were identified by culturing on MacConkey & chromogenic media. Phenotypic testing revealed that among the 20 isolates taken out from 20 samples, 10 isolates were ESBL positive *Enterobacteriaceae* isolates. Phenotypic detection of ESBL positive *E. coli* was analyzed by performing the double-disc synergic test using the antibiotics such as; Amoxicillin-Clavulanate, Ceftriaxone, Cefotaxime, and Ceftazidime discs placed 20 mm to 30 mm away from Amoxicillin-Clavulanate (AMC). Antibiotic susceptibility testing was done to analyze the MDR of the isolates that were producing ESBL.

Double-disc synergy showed positive results giving the SD values; AMC (2.024846), CRO (2.250926), CTX (0.971825), and CAZ (0.994429) but the Cefotaxime disc or the Ceftazidime disk in combination with Clavulanic acid was giving results of 5 mm or greater (SD value =2.02 and 1.71 respectively). The antibiotic sensitivity test showed the SD values; CFM (2.7160207), CN (3.88158), CE (2.162817), LEV (2.170509), SXT (2.54733), FEP (2.406011), ATM (2.162817), and CIP (2.796824), but IPM (5.116422).

This means pathogens were resistant to all antibiotics except the Imipenem (ZOI>22 mm) according to CLSI guidelines the effect of different dilutions of *Azadirachta indica* (NEEM) plant extract on isolated ESBL producing strains was observed considering its therapeutic properties. *Azadirachta indica* did not show any zone of inhibition for all the tested strains of bacteria. This showed high susceptibility of ESBL producing pathogenic organisms against antibiotics as well as the plant having antibacterial properties.

Keywords: ***Azadirachta indica, Enterobacteriaceae,*** **Spectrum- β-lactamases, Anti-bacterial, Anti-inflammatory; Extended Spectrum β–lactamase**

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Introduction

ESBL producing microbes belongs to the family *Enterobacteriaceae* [1]. *Enterobacteriacea* is a large gram negative bacteria family, responsible for several types of infections including respiratory, intestinal, urinary tract, bloodstream, and intra-abdominal infections. For treating these types of infections in humans as well as in animals, β-lactam antibiotics, like penicillin's, cephalosporins and carbapenems are widely used β-lactamases. They are commonly used against the resistance mechanisms of Gram-negative bacteria against antibiotics [2].

These bacteria belong to the *Enterobacteriaceae* such as food-borne pathogens like *Escherichia coli* and *Salmonella enterica*, responsible for causing infections like wound infections that leads to meningitis, and also well-known pathogens causing nosocomial infections [3]. These organisms are producing Extended-Spectrum β-Lactamases (ESBLs) resulting in infections. These bacteria have blowout rapidly globally as an outcome of their mobile genetic basics and clonal spreading thus resulting in a clinical and economical challenge worldwide. Therefore, infections and demises

triggered by these bacteria have increased healthcare costs [4].

Enterobacteriaceae responsible for producing ESBL were first reported in Germany in 1983 and since then, the frequency has been informed to be increasing rapidly worldwide. Extended spectrum β-lactamases are group of β-lactamases, that hydrolyzes a broad range of β-lactam antibiotics like oxyimino-cephalosporins and aztreonam, but are repressed by β-Lactam inhibitory substances that can be the clavulanic acid, tazobactam and sulbactam. These enzymes are encoded by the genes mediated by plasmid and are common amongst the species of *Enterobacteriaceae*.

The extensive utilization of broad-spectrum antibiotics is the reason for the entrance of ESBL enzymes. Transferable enzymes show resistance against different antibiotics such as penicillin, cephalosporins, and monobactams, but are unable to hydrolyze the cephamycin or carbapenem. Resistance against the cephalosporin is because of the release of one or more different β-lactamase enzymes that are said to be as Extended Spectrum-β-Lactamases (ESBLs) [2-4]. ESBL positive strains of *E. coli* are evolving major pathogens worldwide. Different from other anti-microbial resistant pathogens, ESBL producing. *E. coli* is mostly communal-onset pathogens [5]. *E. coli* are mostly present in the intestine of human being and in warm blooded organisms. *E. coli* can result in numerous infections in human as well as in animals. In humans it causes various types of infections. Like UTI, gastroenteritis, food poisoning, peritonitis mastitis, hemolytic-uremic syndrome, septicemia, diarrhea, and neonatal meningitis. Its transmission is mainly through fecal oral pathway [6,7].

In Pakistan few studies have been published related to the propagation of ESBL positive strains of *E. coli* in diverse settings like infected people, affected roles, cattle, chickens, manure sludge as well as raw meat. Mostly in developing countries such as Pakistan Antimicrobial Resistance (AMR) is becoming a developing risk to public health sector due to the extreme antimicrobial contact in human and veterinary medicine. The high occurrence of the ESBL and the Carbapenemase enzymes in the isolates of *Escherichia coli* taken from the retail chicken in Peshawar is observed [8]. Extended spectrum β-lactamase releasing *Enterobacteriaceae* are commonly found in human beings [8], several farm animals, wild animals and companion animals 10 to 12 as well as on surfaces, in hospitals and in waste-water. Out of them, the highest figures of ESBL- positive *Enterobacteriaceae* are testified in broiler chickens [9-13].

Chicken meat act as a habitat for the extended spectrum β-lactamase carrying *Enterobacteriaceae* that inhabit and infect human beings. ESBL- producing *E. coli* taken from chicken samples are pointedly showing resistance against different substances like the aminoglycosides, fluoroquinolones, and trimethoprim-sulfa meth oxazole [14]. The presence of Carbapenemases-Producing *Enterobacteriaceae* (CPE) in Broiler Chicken Fattening Farms is isolated as well as also isolated from stool sample [15].

Azadirachta indica (Neem Plant)

Azadirachta indica belongs to the family *Meliaceae*, generally known as "neem". According to the United Stated National academy of science, the neem tree is known to treat infections globally. Neem plant is cultivated in the sub-continent of India and other areas of minimum thirty countries from the Asian continents to the African continents, and as distant as the United States of America. All the portions of this plant counting the flowers, leaves, seeds, fruits and the barks are broadly utilized in old medication to cure several human related diseases because of being the economical bases of the secondary metabolites that are biologically functioning, predominantly the limonoids or the tetranortriterpenoids, like the *gedunin, salannin, nimbidin, nimbin, azadirachtin*, and nimbolide [16]. The neem tree consists of chemical constituents like 17-hydroxyazadiradione, 7-desacetyl-7-benzoylazadiradione, nimbin, polyphenolic flavonoids, nimbiol, 7-desacetyl-7-benzoylgedunin, azadirachtin etc [17].

Because of the chemical constituency of *Azadirachta indica*, this tree has increased its importance in medicinal field as well as in pharmaceutical industry and is used in extensive range of biological activities such as anti-mutagenic, anti-malarial, anti-carcinogenic, anti-ulcer, anti-bacterial, anti-inflammatory, anti-viral, anti-oxidant and anti-fungal [18,19].

Material and Methodology

Study design

The research was planned to isolates of *E. coli* that are producing ESBL from different chicken cecal samples collected from different areas and to determine the effects of neem extract on ESBL producing *E. coli* [20]. Cecal samples were collected. The cecal samples were collected from different regions of Fatehjang, Attock, and Tarnol and the project was accomplished at bacteriology laboratory of National Veterinary Laboratory (NVL) Islamabad.

Sampling area

Twenty samples were collected from Fateh jang, Attock, and Tarnol. The samples were collected from different shops for isolation of ESBL producing *E. coli*.

Sampling materials

Gloves, zip lock bags, storage box containing ice packs, scissor, lab coats, seventy percent ethanol, cotton.

Materials

Hot air oven, Autoclave, Incubator, Water bath, Weighing balance, Laminar flow, Hot stirrer, Tube shaker, different medias, Biochemical's, Antibiotic disc, Conical flask, Petri dishes, Media culture tubes, and test tubes.

Isolation of ESBL *E. coli* from chicken ceca

Intact cecal specimen was kept on a sheet of aluminum foil and weighed as 1 gram, using analytical weighing balance. After weighing 1 gram cecal sample to 9 ml of Buffered Peptone Water (BPW) was added. The tubes were incubated at the temperature of 37 ± 1°C for 18 h to 22 h. After incubation we inoculated one loop-full (10 µl loop) of incubated BPW on to an ESBL selective MacConkey agar plate/ MAC-CEF plate (Containing 4 mg/L Cefotaxime (CTX), following inoculation of agar plate, 4-streak technique was performed to isolate individual colony. MacConkey agar plate was incubated at the temperature of 37°C for 18 h to 22 h. On MAC-CEF plate, presumptive ESBL *E. coli* colonies were of purple/red color. Subculturing was done, any 3 colonies were sub cultured by streaking 3 different ESBL selective MacConkey agar plate (one for each colony) and incubated the plates at 37°C for 18 h to 22 h. We screened culture in all three plates, for biochemical confirmation as *E. coli*. Culture which was confirmed as *E. coli* was subjected to screening and confirmation as ESBL strain by utilizing Disk Diffusion Test (DFT), Combination Disk Test and Double Disk Synergic Test.

MacConkey with cefotaxime was selected for our experiment

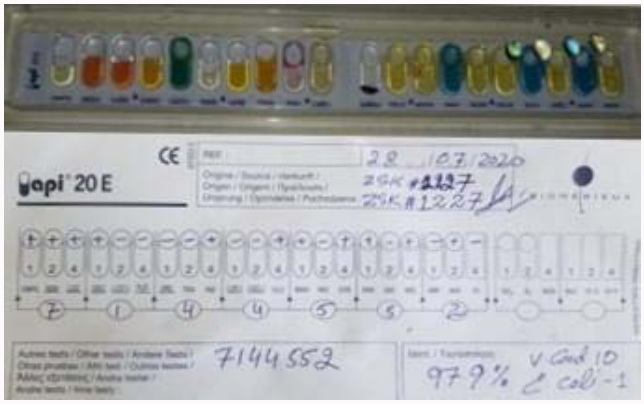

Figure 1:

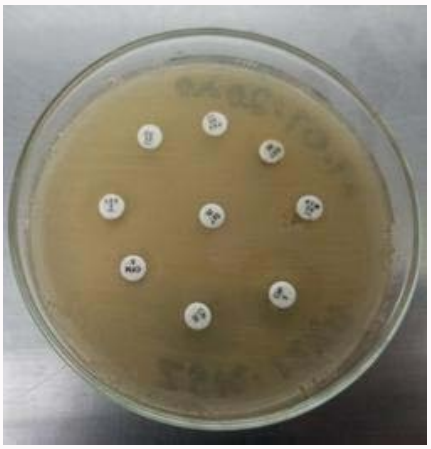

Figure 2:

Figure 3:

Figure 4:

as it was both differential and selective media 50 g powder was then suspended in 1 liter of the distilled water then heated on hot plate to dissolve powder and applied autoclave tape on Flask. The solution was then autoclaved at the temperature of 121°C for 15 min. Cefotaxime (CTX) stock solution was prepared with concentration (5 mg/ml). This was done by dissolving 10 mg powder from Cefotaxime sodium salt in 2 ml of sterile water for injection or 0.1 g (100 mg) Cefotaxime sodium salt powder in 20 ml sterile water for injection. The media was cooled down up to 50°C and removed 1 ml from media and added 1 ml of CTX stock solution in 1 L media. Shaking of media flask was done to dissolve it completely. Media was in plates, 20 ml to 25 ml in 80 mm sterile Petri plates.

Chromatic ESBL Agar

Peptones provide vitamins, carbon, nitrogen, minerals, amino acids and other various nutritious substances which help in sustenance of the growth and development of microorganisms. Chromogenic mixture of different substances helps in the microorganism identification based on the color and morphology of the colonies. Selective mix inhibits ESBL non-producing organisms. Agar is the solidifying agent.

Gram staining biochemical API 20E test were used for confirmation of ESBL *E. coli.*

Analytical profile index 20E test

Principle: API 20E test was used for the identification and the differentiation of *Enterobacteriaceae* family members. This test usually detects enzymatic activity that is mostly related to the carbohydrate fermentation or the amino acids or the proteins. Each of the wells can be rehydrated by bacterial suspensions and strips are incubated. During incubation, color changes are produced due to metabolism that is either spontaneous or revealed by the addition of reagents. A profile number is obtained by compilation of all of the positive and negative tests, which is then compared with the give numbers of profile in a codebook that is available commercially or online for the indication of the identification of different species of bacteria.

Antimicrobial Sensitivity Testing Of ESBL Producing *E. coli*

Disc diffusion method: Disc diffusion is used for testing the antimicrobial susceptibility of bacterial isolates (i.e. antibiogram determination). This method indicates susceptibility of ESBL against the tested antibiotic by forming a clear zone of inhibited growth around the impregnated filter.

Mueller Hinton agar (MHA): MHA media was used for disk diffusion antimicrobial vulnerability testing of frequent, quickly growing bacteria by the help of Kirby-Bauer methodology as a CLSI.

Phenotypic confirmation of ESBL *E. coli*

Combination disk test (CDT): A Cefotaxime and Ceftazidime disk alone as well as in blend or combination with clavulanic acid (Cefotaxime-Clavulanatethat is 30/10 and Ceftazidime Clavulanate that is 30/10) was applied on MH plate. After incubation, test was positive.

Double disk synergy test (DDST): A cephalosporin

Figure 5:

(Cefotaxime-30 OR Ceftazidime 30) disk was applied on a MH plate. Then Amoxicillin- Clavulanic Acid Disk (AMC 30) disk along with the previous disk was applied by keeping 20 mm center to center distance between the two disks. After incubation, positive results were observed when the inhibitory zones round any of the disk of cephalosporin were augmented towards the disc of Amoxicillin-Clavulanic Acid.

Azadirachta indica (Neem Plant) test

Fresh leaves and bark of neem (*A. indica*) were collected from local area at Islamabad. The collected leaves and bark were then air dried separately in 10 gram of dried leaves and bark of plant was dipped in 100 ml of ethanol for 72 h and filtered thereafter in Soxhlet apparatus.

Dilution in series: Serial dilution is a widely used method that is applied in many events in immunology. A little quantity of a solute can be diluted in series by transferring the specific quantity of the diluent. When it gets transferred in the series, the factor of dilution turns doubles with every transfer (1:10, 1:100, 1:1000 ...). The dilutions are performed either in micro titer plates or test tubes which depend on the sample volumes and the used diluent.

Assembling of the constituents at the worktable was done. The tubes were then labeled for the dilutions in series as; Tube 1 was 1:10, Tube 2 was 1:100, Tube 3 was 1:1000, Tube 4 was 1:10,000, and Tube 5 was 1:100,000. Using a micropipette, 1 mL of distilled water was poured in all the labeled tubes. Using a micropipette, 1 mL of neem extract was pipette into the tube number 1. Mixed lightly by moving the solution up and down 3 times. Transferred 1 mL of solution from tube 1 into tube 2. Mixed softly and transfer 1 mL of the solution from the tube number 2 into the tube number 3. Mixed mildly and continued to transference and mixed through tube number 5. Discarded the remaining 1 mL of the mixture from tube number 5. Examined the dilutions in the tube. Noted the color of the solution decreased with increasing tube number.

Infections by ESBL producing pathogens affected by these pathogenic organisms have limited the therapeutic options resulting in treatment failures. In this research, we have examined the occurrence of ESBL that are multiple drug resistant present in chicken meat which shows the direct human cause of infections. ESBL enzyme releasing isolates were identified by culturing them on the chromogenic medium that is Hi-Chrome agar for ESBLs on which ESBL-releasing isolates looks as brownish green colonies. The species distributions of the analyzed isolates and the β-lactamases

Figure 6:

Figure 7:

Table 1: The sample isolates and their B-lactamases.

Strain	Total samples	ESBL *E. coli*
Poultry	20	10

are shown in Table 1. Phenotypic testing has revealed that among 20 isolates taken out from 20 samples, 10 isolates were ESBL positive *Enterobacteriaceae* isolates. This showed that 50% of the isolates of chicken meat contain ESBL producing *Enterobacteriaceae*.

Phenotypic detection of ESBL release was initially examined by the help of double-disc synergic test by using the Amoxicillin-Clavulanate (AMC) as an antibiotic that was positioned at the middle of the Petri plate and Ceftriaxone (CRO), Cefotaxime (CTX), and Ceftazidime (CAZ) were positioned 20 mm to 30 mm far from the antibiotic dislocated in center. This test was interpreted on the basis of CLSI guidelines confirmatory test applying both Cefotaxime and Ceftazidime disks of 30 mg solely and in combination with Clavulanic acid of 10 mg. The double-disc synergy test data for ESBL-releasing isolates of the research are given in Table 3. The analysis showed positive results when an expansion in the zone of inhibition was observed around either the Cefotaxime disk or the Ceftazidime disk in combination with Clavulanic acid was approximately 5 mm or more (SD value =2.02 and 1.71 respectively) as compared to the distance around the disks containing Cefotaxime (SD value =0.97) or Ceftazidime (SD value =0.99) alone. It means that antibiotics showed a synergic effect because of clavulanic acid combined with CAZ and CTX.

Table 2: Synergy & DDT, zone of inhibition (mm).

STRAIN	CTX	CTX/CA	CAZ	CAZ/CA	CRO	AMC
ESBL *E. coli*	2	9	0	9	4	0
ESBL *E. coli*	3	7	3	7	6	0
ESBL *E. coli*	4	10	0	11	3	6
ESBL *E. coli*	3	8	1	8	0	0
ESBL *E. coli*	5	9	2	6	0	3
ESBL *E. coli*	3	6	1	9	2	0
ESBL *E. coli*	4	4	0	10	0	0
ESBL *E. coli*	5	5	2	11	5	0
ESBL *E. coli*	3	5	1	7	0	0
ESBL *E. coli*	3	8	1	8	2	0
SD Value	0.97182	2.024846	0.994429	1.712698	2.250926	2.024846

Table 3: Antibiotic susceptibility test of ESBL *E. Coli* poultry, zone of inhibition (mm).

CFM	CN	CE	LEV	SXT	FEP	ATM	IPM	CIP
7	10	5	5	0	6	5	30	14
0	8	0	0	0	0	9	30	17
4	5	0	3	2	0	8	29	11
0	0	0	0	5	0	10	30	15
5	0	5	0	0	5	9	22	19
0	5	3	0	0	0	6	22	17
3	0	0	0	0	2	10	22	13
0	0	0	3	7	0	6	22	10
0	0	0	5	0	0	8	30	14
5	0	0	0	0	4	12	30	16
2.7160207	3.88158	2.162817	2.170509	2.54733	2.406011	2.162817	4.056545	2.796824

Antibiotic susceptibility testing was done to analyze the multi-drug resistance of the ESBL producing isolates. The isolates were further tested against different antibiotics given; Trimethoprim/ Sulfamethoxazole/Cotrimoxazole (25 mg) antibiotic disk was positioned at the midpoint of the plate and Ciprofloxacin (5 mg), Cefixime (5 mg), Gentamicin (30 mg), Aztreonam (30 mg), Levofloxacin (5 mg), Cefepime (30 mg), Doxycycline (30 mg), and Cephradine (30 mg) were placed around SXT. The growth inhibitory zones were observed and then interpreted as showing resistance or are sensitive as per the given standards of CLSI guidelines. The multiple antibiotic resistance indexes then was obtained as the ratio of the total antibiotics used to the total numbers of antibiotics to which the bacterium was observed as resistant. In the antibiotic sensitivity test, imipenem belonging to carbapenem class of antibiotic was also tested, the bacterial sensitivity against carbapenem was observed showing greater zone of inhibition according to CLSI.

The effect of different dilutions of *Azadirachta indica* (Neem) plant extract on isolated ESBL producing strains was observed considering its therapeutic properties. *Azadirachta indica* did not show any zone of inhibition against all the tested bacterial strains. Different dilution (1:10, 1:100, 1:1000, 1:10,000, and 1:100,000) showed different results because of the different volume of ethanol used. This showed the high susceptibility of ESBL producing pathogenic organisms against antibiotics as well as the plant having antibacterial properties.

Discussion

The escalating frequency of infections has displayed an extended downturn in health as well economic area globally. This global crisis has become an alarming condition causing severe menaces to human health, the safety of food, the environment, economy, and development. This is because of the antibiotic resistance development in the microorganisms over the past half century. WHO has reported resistance against antibiotic is one of the tremendous threats to global health as it costs numbers of victims per month resulting in about 7 lack deaths per annum. These remarkable genetic potentials of microbes to be resistant to drugs are because of overuse of antibiotics [20]. This leads to change in chromosome resulting in developing the resistance against all older antibiotics in *Streptococcus pneumonia*, *Streptococcus pyogenes*, and *Staphylococci*, and members of the *Enterobacteriaceae* organisms causing diarrhea, urinary tract infection, and sepsis [21,22]. *Escherichia coli* is a well-known occupy human GIT as well as animals and is additionally the most significant reasons of nosocomial-acquired infections in the humans and can effortlessly become resistant to antibiotics utilized by humans and animals. Extended-spectrum β-lactamases are the hydrolytic enzymes that are released by Gram-negative bacteria and the most utmost strains producing them related to the family of *Enterobacteriaceae*. These enzymes hydrolyze a broad range of β-lactam antibiotics nonetheless are hindered by the β-lactam inhibitors that can be tazobactam, clavulanic acid, and sulbactam [23]. ESBL positive *E. coli* is an evolving leading pathogen globally. In humans, it can cause many types of diseases like food poisoning, pneumonia, hemolytic-uremic syndrome, neonatal meningitis, gastroenteritis, peritonitis mastitis, urinary tract infections, septicemia, diarrhea

and transmission of *E. coli* infection is mainly through the fecal-oral route. Transmission source can also be the food and travelling [23,24]. Flies are seen as a basis of transmission of ESBL producing *E. coli* from the farm environment (poultry) to the human population. In Pakistan few researches have been published related to the propagation of ESBL producing *E. coli* in different environments: Fit individuals, affected people, sewage sludge, livestock, chickens, and uncooked meat. In the current study ESBL positive e coli is seen as a contamination in chicken meat and poultry contributing to the increase in the proportion of infections with these bacteria in humans. β-lactam antibiotics were the most frequently used antibiotics include penicillin, cephalosporins, cephamycin, and carbapenems. But, it has been observed that ESBLs started hydrolyzing several types of antibiotics that are β-lactam, includes the cephalosporins of third generation and the monobactams, and make bacteria resistant against these antibiotics as we detected in our study. Carbapenem class of antibiotics has been last resort for the cure of the infections produced by such pathogens [25,26].

Conclusion

As the ESBL has rapidly become resistant to all the antibiotics except carbapenem, it is thought not too far that they would get resistant to carbapenem as well due to overuse and the only option for treatment because some studies have shown resistance against carbapenem depending on the sample load. In order to find out an alternative way for treating ESBL causing infection, this study was conducted to determine the effect of different dilutions of *Azadirachta indica* (Neem) plant considering their antimicrobial activities. *Azadirachta indica* did not show any zone of inhibition against all the tested bacterial strains. Different dilutions showed different results because of the volume of ethanol used. This showed the high susceptibility of ESBL producing pathogenic organisms against antibiotics as well as the plant having antibacterial properties. There is a need, therefore, for an alternative way for treating infections caused by ESBL producing *E. coli* either by further modification in application of Neem plant extracts on these pathogenic isolates or by the production of new class of antibiotics.

References

1. Mahamat OO, Lounnas M, Hide M, Dumont Y, Tidjani A, Kamougam K, et al. High prevalence and characterization of extended-spectrum ß-lactamase producing Enterobacteriaceae in Chadian hospitals. BMC Infect Dis. 2019;19(1):1-7.
2. Haller L, Chen H, Ng C, Le TH, Koh TH, Barkham T, et al. Occurrence and characteristics of extended-spectrum β-lactamase-and carbapenemase-producing bacteria from hospital effluents in Singapore. Sci Total Environ. 2018;615:1119-25.
3. Sanjit Singh A, Lekshmi M, Prakasan S, Nayak BB, Kumar S. Multiple antibiotic-resistant, Extended Spectrum-β-Lactamase (ESBL)-producing enterobacteria in fresh seafood. Microorganisms. 2017;5(3):53.
4. Kaarme J, Riedel H, Schaal W, Yin H, Nevéus T, Melhus Å. Rapid increase in carriage rates of Enterobacteriaceae producing extended-spectrum β-lactamases in healthy preschool children, Sweden. Emerg Infect Dis. 2018;24(10):1874-81.
5. Laupland KB, Church DL, Vidakovich J, Mucenski M, Pitout JD. Community-onset Extended-Spectrum β-Lactamase (ESBL) producing *Escherichia coli*: Importance of international travel. J Infect. 2008;57(6):441-8.
6. Blaak H, Hamidjaja RA, van Hoek AH, de Heer L, de Roda Husman AM, Schets FM. Detection of Extended-Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli* on flies at poultry farms. Appl Environ Microbiol. 2014;80(1):239-46.
7. Saleem R, Ejaz H, Zafar A, Younas S, Rathore AW. Phenotypic characterization of extended-spectrum-beta-lactamase producing *E. coli* from healthy individuals, patients, sewage sludge, cattle, chickens and raw meat. Pak J Med Sci . 2017;33(4):886-90.
8. Umair M, Mohsin M, Ali Q, Qamar MU, Raza S, Ali A, et al. Prevalence and genetic relatedness of extended spectrum-β-lactamase-producing *Escherichia coli* among humans, cattle, and poultry in Pakistan. Microb Drug Resist. 2019;25(9):1374-81.
9. Coque T, Baquero F, Canton R. Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. Euro Surveill. 2008;13(47):19044.
10. Ewers C, Bethe A, Semmler T, Guenther S, Wieler L. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: A global perspective. Clin Microbiol Infect. 2012;18(7):646-55.
11. Rubin JE, Pitout JD. Extended-spectrum β-lactamase, carbapenemase and AmpC producing Enterobacteriaceae in companion animals. Vet Microbiol. 2014;170(1-2):10-8.
12. Mohsin M, Raza S, Schaufler K, Roschanski N, Sarwar F, Semmler T, et al. High prevalence of CTX-M-15-Type ESBL-producing E. coli from migratory avian species in Pakistan. Front Microbiol. 2017;8:2476.
13. Bortolaia V, Guardabassi L, Trevisani M, Bisgaard M, Venturi L, Bojesen AM. High diversity of extended-spectrum β-lactamases in *Escherichia coli* isolates from Italian broiler flocks. Antimicrob Agents Chemother. 2010;54(4):1623-6.
14. Campos PH, Merlot E, Damon M, Noblet J, Le Floc'h N. High ambient temperature alleviates the inflammatory response and growth depression in pigs challenged with *Escherichia coli* lipopolysaccharide. Vet J. 2014;200(3):404-9.
15. Abdallah H, Reuland E, Wintermans B, Al Naiemi N, Koek A, Abdelwahab A, et al. Extended-spectrum β-lactamases and/or carbapenemases-producing Enterobacteriaceae isolated from retail chicken meat in Zagazig, Egypt. PLoS One. 2015;10(8):e0136052.
16. Suttiarporn P, Choommongkol V. Microwave-assisted improved extraction and purification of anticancer nimbolide from *Azadirachta indica* (Neem) Leaves. Molecules. 2020;25(12):2913.
17. Borkotoky S, Banerjee M. A computational prediction of SARS-CoV-2 structural protein inhibitors from *Azadirachta indica* (Neem). J Biomol Struct Dyn. 2021;39(11):4111-21.
18. Ali A, Shahid MA, Hossain MD, Islam MN. Antibacterial bi-layered Polyvinyl Alcohol (PVA)-chitosan blend nanofibrous mat loaded with *Azadirachta indica* (neem) extract. Int J Biol Macromol. 2019;138:13-20.
19. Qiu Z, Andrijauskaite K, Morris J, Wargovich MJ. Disruption of epigenetic silencing in human colon cancer cells lines utilizing a novel supercritical CO2 extract of neem leaf (*Azadirachta indica*). Anticancer Res. 2019;39(10):5473-81.
20. Jayaweera JAAS, Reyes M. Antimicrobial misuse in pediatric urinary tract infections: Recurrences and renal scarring. Ann Clin Microbiol Antimicrob. 2018;17(1):27.
21. Schroeder MR, Stephens DS. Macrolide resistance in *Streptococcus pneumoniae*. Front Cell Infect Microbiol. 2016;6:98.
22. Cattoir V, Pourbaix A, Magnan M, Chau F, de Lastours V, Felden B, et al. Novel chromosomal mutations responsible for fosfomycin resistance in *Escherichia coli*. Front Microbiol. 2020;11:575031.
23. Medernach RL, Domitrovic TN, Rispens JR, Hujer AM, Qureshi NK, Marshall SH, et al. 2336. Resistance mechanisms and factors associated with CTX-M-9 group Extended-Spectrum β-Lactamase (ESBL)-producing Enterobacteriaceae infections in children. Open Forum Infectious

Diseases: Oxford University Press US. 2018;S694-S694.

24. Schaumburg F, Correa-Martinez CL, Niemann S, Köck R, Becker K. Aetiology of traveler's diarrhea: A nested case-control study. Travel Medicine and Infectious Disease. 2020:101696.

25. Potter RF, D'Souza AW, Dantas G. The rapid spread of carbapenem-resistant Enterobacteriaceae. Drug Resist Updat. 2016;29:30-46.

26. Sinha DJ, Nandha KD, Jaiswal N, Vasudeva A, Tyagi SP, Singh UP. Antibacterial effect of *Azadirachta indica* (neem) or *Curcuma longa* (turmeric) against *Enterococcus faecalis* compared with that of 5% sodium hypochlorite or 2% chlorhexidine *in vitro*. Bull Tokyo Dent Coll. 2017;58(2):103-9.