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ANTIMICROBIAL RESISTANCE PROFILE AND GENDER WISE PREVALENCE OF *ESCHRICHIA COLI* IN CLINICAL SPECIMEN, AT KHAIRPUR SINDH, PAKISTAN

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ABSTRACT

The present study aimed at surveillance of multidrug-resistant *E. coli* strains in clinical specimens, at district Khairpur Mir's, Pakistan. A total of 280 clinical samples of different origins were collected from the tertiary care hospitals of Khairpur and Sukkur cities of Pakistan. *Eschrichia coli* strains were separated and identified using regular microbiological techniques and molecular biography using the 16S rRNA sequence-based homology. Antimicrobial sensitivity was determined using Kirby-Bauer's disc-diffusion assay and penicillin zone-edge test. Overall, ninety nine (99) strains of *E. coli* were isolated with maximum from pus 46 (46.6%) followed by urine 40 (40.4%), HVS 7 (7%) and stool 6 (6%). The *E. coli* in gender wise prevalence, was 100% prevalent in HVS samples, followed by pus (52%), urine (40%) and stool (33%) in female where as it was 67% prevalent in stool followed by urine (60%) and pus (48%) in male patient. The results of antibiotic sensitivity profiling revealed that Moxifloxacin was observed as most effective (78%) against all strains of *E. coli* while rest of the tested antibiotics were ineffective. Phylogenetic correlation of amplified 16S rRNA gene sequence of *E. coli* isolate shared 99% similarity with *E. coli* strain *AS15*. Prevalence of multidrug-resistant pathogen *E. coli* in clinical specimens calls for timely control measures to reduce health care cost and increasing resistance.

Key-words: Multiple Drug Resistance, *Eschrichia coli*, Khairpur, Pakistan.

INTRODUCTION

Resistance to bacteria triggered by broad and frequent use of antimicrobial drugs is currently one of the biggest medical problems, which has a significant impact on the outcome of patients' treatment, as well as on health care cost. Resistance to antimicrobial therapy is associated with failure of treatment, prolonged or additional hospitalisation, increased cost of treatment and increased mortality (Aldžić *et al.*, 2019). The most commonly reported Multiple Drug Resistance (MDR) pathogenic bacteria include *Escherichia coli*, *Proteus* spp., *Klebsiella* spp., *Pseudomonas* spp., *Enterococcus* spp., *Staphylococcus aureus* and coagulase-negative *Staphylococci* (Aldžić *et al.*, 2019; Z. Ali *et al.*, 2015; Brzychczy-Wloch *et al.*, 2013). *E. coli* is the most common facultative bacterium of intestinal normal flora of humans and many animals, and is highly distinguished for acquiring and transferring antimicrobial resistance genes (Jouini *et al.*, 2009; Ryu *et al.*, 2012).

Eschrichia coli is frequently associated with bacterial sepsis, neo natal meningitis, nephritis, cystitis, and gastroenteritis to infants and travellers to countries with poor hygiene. Pathogenic strains of *E. coli* have been kept into following six groups based on their virulence factors i.e., Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enterotoxogenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), diffusely adhering *E. coli* (DAEC) (Rappelli *et al.*, 2005). It is the most frequent microorganisms sharing increased multidrug resistance (Riu *et al.*, 2016). *E. coli* is still by far the most common uropathogen in more than 80% of the positive urine cultures and causing an acute uncomplicated cystitis (AUC) in women (Kahlmeter, 2003).

Resistance pattern in pathogenic bacteria is mobile and vary from area to area like country to country, state to state, large hospital to small hospital and hospital to community. Multi drug resistance issue in Pakistan is due to overuse and misuse of available antibiotics (Tanvir *et al.*, 2012). There is no proper national surveillance and sufficient data of antibiotic resistance to quantify the real issue in hour of need (Abdul *et al.*, 2008). In Pakistan the multiple antibiotic-resistant (MAR) bacteria have been isolated from agriculture resulting from the inappropriate use of antibiotics in agriculture to aim at increase crop yield. The faecal contaminated drinking and agri-water due to lack of proper management, lack of proper spill ways, and water storage capabilities are some of the factors linked with dissemination of MDR pathogens. Thus, nosocomial infections caused by the MDR

bacterial strains are also reported from the consumption of contaminated foods served in health care units (McDermott *et al.*, 2002).

Nowadays, the people of district Khairpur Mir's Pakistan have been suffering from various diseases caused by MDR pathogens, especially enteropathogens like *E. coli*. These pathogenic bacteria are mostly invading humans through contaminated drinking water resulting from improper sewage treatment, sewage spills, unhygienic practices, and contaminated food. Moreover, there has been an extensive and empirical use of antibiotics that also plays major role in the development of antibiotic resistance. Therefore, the current study focuses on the surveillance of multidrug-resistant *E. coli* strains in various clinical specimen causing human diseases in district Khairpur Mir's, Pakistan.

MATERIALS AND METHODS

The present study was carried out in the Postgraduates Research Laboratory (PGRL), Department of Microbiology, Shah Abdul Latif University Khairpur Mir's Sindh Pakistan.

Materials (media, test reagent and chemicals)

The growth media such as Nutrient agar, Mac Conkey agar, Eosin Methylene Blue (EMB), Mueller Hinton Agar (MHA), Simon Citrate Agar, and commercial antimicrobial disks including Gentamycin, Amoxicillin clavunic acid, Sparfloxacin, Fosfomycin, Moxifloxacin, Fusidic acid, Enoxacin, Azomax, Piperacillin-Tazobactam and Sulbactam were purchased from Oxoid (Oxoid, UK). The chemicals, test reagents and sugars were purchased from Sigma Aldrich (Sigma-Aldrich, USA). All the glassware used in this study was purchased from Borosil (Borosil, USA).

Sample collection and isolation of *E. coli* strains

The clinical specimen like urine, pus, blood, high vaginal swab (HVS), stool, ear, throat, cerebrospinal fluid, ascitic fluid and pleural fluid from different patients of age and gender (male and female) were collected aseptically and processed for isolation of pathogenic bacteria. Each different bacterial pathogen was assigned to a different group viz. NS1, NS2, NS-3, NS-4 and NS-5 based on their similar morpho-microscopic and biochemical characteristics. Among these groups, the *E. coli* strains were assigned to NS1 group. The streaking plate technique was used for the isolation of *E. coli* on the surface of nutrient agar and further sub cultured on MacConkey agar and EMB agar in order to confirm their cultural features.

Characterization of *E. coli*

Isolates were characterised according to the society of American Bacteriologists and Nasreen *et al.* (Nasreen *et al.*, 2015), pink colony on MacConkey agar were observed for morphological characteristics and were further selected for identification. Several biochemical tests such as Citrate Utilization, Oxidase, Catalase, Methyl Red (MR), Voges-Proskauer (VP), Indole , sugar fermentation test and morpho-microscopic (Gram staining and capsule, Hanging drop technique), examination were carried out for *E. coli*.

Antimicrobial susceptibility testing

Antimicrobial resistance profile of *E. coli* against panel of antibiotics (commercial antimicrobial disk) was determined in vitro by disc diffusion method. This test was performed using Kirby-Bauer method as mentioned previously (Mangi *et al.*, 2016) and results were interpreted according to the twenty fourth supplement of Clinical Laboratory Standard Institute guidelines (CLSI, 2014).

16s rRNA gene sequencing and phylogenetic correlation

For molecular identification, the pure culture stock of selected bacterial isolate, i.e. *E. coli* strain EC33_NS1, was sent to Genomic Division, Macrogen Inc., Seoul, Korea for amplification and partial sequencing of 16S rRNA gene using set of universal amplification of primers, i.e. 27F and 1492R primers (5'AGAGTTTGATCMTGGCTCAG-3' and 5'- TACGGYTACCTTGTTACGA CTT-3', respectively). After amplification, the resulting amplicons were subjected to partial sequencing of 16S rRNA gene using ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystem, USA) by using universal sequencing primers, i.e. 518F (5' CCAGCAGCCGCGGTAATACG-3') and 800R (5'TACCAGGG TATCTAATCC-3'). Finally, the obtained partial sequences by each primer were then assembled using an online CAP3 sequence assembly program (Huang and Madan, 1999). Resulting contiguous sequences were then analysed and compared with existing GenBank nucleotide sequence databases at National Centre for

Biotechnology Information (NCBI) website using Basic Local Alignment Search Tool (BLAST) program in order to confer the percentage sequence similarities. The phylogenetic correlations were obtained according to the Kumar *et al*., (Kumar *et al.*, 2016) using MEGA7 software using the Maximum Likelihood method based on the model of Tamura (Tamura *et al.*, 2004).

RESULTS

Study site and samples collection in order to fight bacterial infections successfully, the rapid recognition of proper treatment modalities are critical. The determination of antibiotic susceptibility and resistance are keys to this process. In present study 280 clinical samples were successfully collected in order to investigate the prevalence and antimicrobial resistance profile of *E. coli*. The clinical samples (162 from male and 118 from female) were randomly collected at different health care facilities of Khairpur and Sukkur cities of Pakistan. The uniqueness of present study was the very high samples number in order to accomplish the promising results in the area of Khairpur and Sukkur.

Isolation and Identification of *E. coli*

Total ninety nine (n=99) strains of *E. coli* were isolated with the maximum number of isolates from the pus 46 (46.6%) followed by urine 40 (40.4%), HVS 7 (7%) and stool 6 (6%) while it was complete absent in blood, pleural fluid, CSF, ascetic, throat and ear. In particular, the prevalence *E. coli* beside other pathogenic isolates in different samples was maximum in urine, followed by HVS, stool, and pus (Fig. 1A). The isolates were identified by the cultural characteristics as Smooth, shiny, low convex, entire edges colonies.

Morphologically, all the members of NS-1 group isolates were Gram negative bacilli, non-spore former, motile and non-capsulated. Based on their biochemical characteristics, the isolates were tested positive for catalase, indole, methyl red and nitrate reductase assays, while negative for the oxidase, citrate ultilization, urease and VP tests (Table 1). The isolate ferment glucose, lactose, mannose, sucrose and maltose. The based on cultural, morphological and biochemical basis, the isolates were presumed as *E. coli.*

Table 1. Morphological, Biochemical and Sugar fermentation profile of the bacterial isolates belonging to NS1 group of pathogens.

Test type	Assay/Test	Group NS1 bacterial isolates
Microscopy	Gram's staining	Gram negative
	Shape	*Bacilli*
	Spore staining	Non-sporulating
	Capsule staining	Non-capsulated
	Motility	+
	Flagella staining	Peritrichous arrangement
Biochemical	Catalase	+
	Oxidase	-
	Nitrate reductase	+
	Indole	+
	Methyl red	+
	Voges Proskauer	-
	Urease	-
	Citrate Utilization	-
Sugar fermentation	Glucose	+, AG
	Lactose	+, AG
	Maltose	+, AG(±)
	Sucrose	±, AG
Tentatively Identified as:		*E. coli*

Note: +, positive result; -, negative result; ±, variable; AG, positive for both Acid and Gas; AG (±), variable for Acid and Gas.

Gender wise prevalence of *E. coli*

The overall gender-wise prevalence (%) of *E. coli* was found to be approximately 50.5% and 49.5% in male and female patients, respectively. The *E. coli* was found 100% prevalent in female samples of HVS, followed by pus (52%), urine (40%) and stool (33%) where as in male patient it was 67% prevalent in stool followed by urine (60%) and pus (48%) as displayed in Fig. 1B.

Antimicrobial sensitivity profile (ASP) of *E. coli*

According to the CLSI manuals (CLSI, 2012, 2014), the results were interpreted and it was found that antimicrobial panel used was almost ineffective and did not display any desired results (zone of inhibition) against *E. coli* isolates. The NS1 (isolates) were mostly resistant to GM, followed by FD, AMC, FOS and other antibiotics while Moxifloxacin was observed as most effective (78%) against all strains Fig 2A. Keeping in view the ASP, strain EC33_NS1 was found completely resistant against panel of tested antibiotics such as AMC, SPX, GM, TZP, FOS, MXF, AZM, FD and SCP (Fig. 2B).

Phylogenetic correlation analysis

The isolate (NS1-EC 33) from urine has displayed complete resistance against all the tested antibiotics was subjected to molecular characterization using 16S rRNA sequence homology. The phylogenetic correlation studies revealed that the amplified 16S rRNA gene sequence of EC33_NS1 isolate shared 99% similarity with various strains of *E. coli*, but the neighbour-joining tree displayed a distant species in the phylogenetic relationship showing bootstrap value of 62% only (Fig. 3).

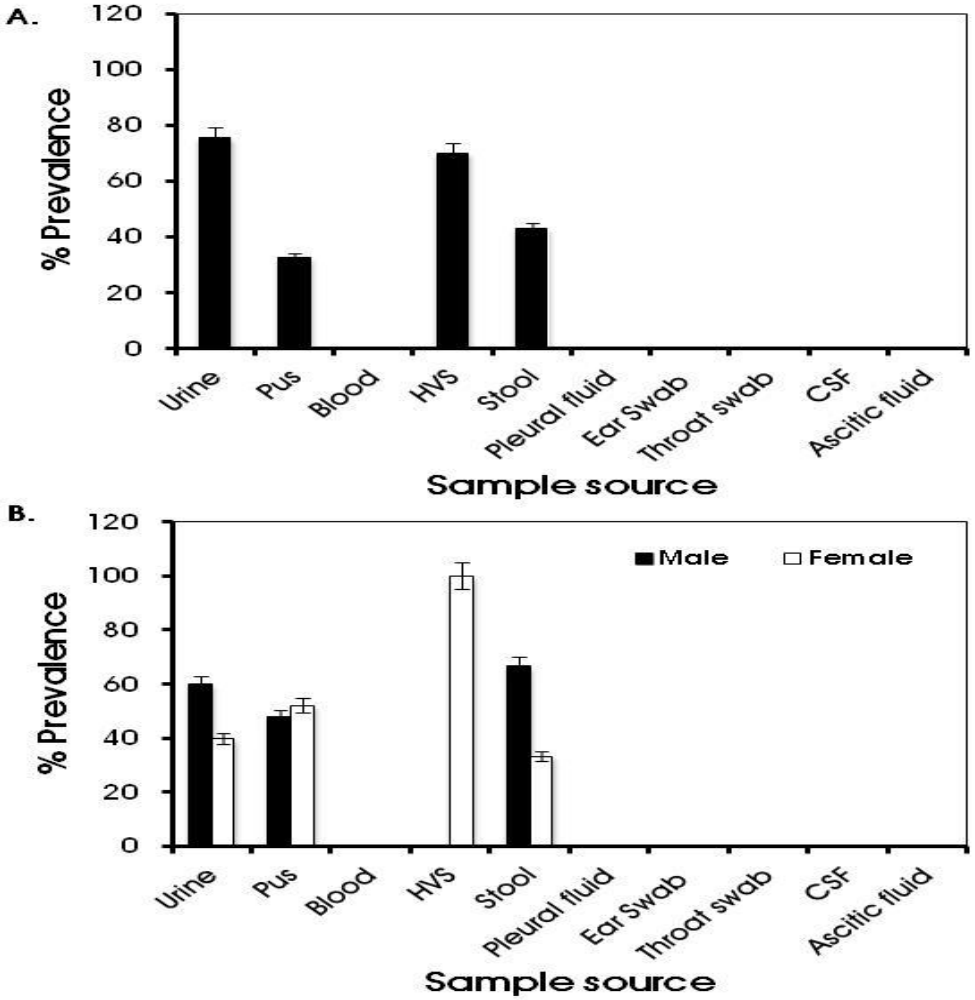

Fig. 1. Sample-wise (A) and Gender-wise (B) prevalence of *E. coli* isolates in clinical samples. The percentage prevalence indicates number of *E. coli* strains isolated from each sample source. The error bars indicate percentage error at 95% confidence interval ($P<0.05$).

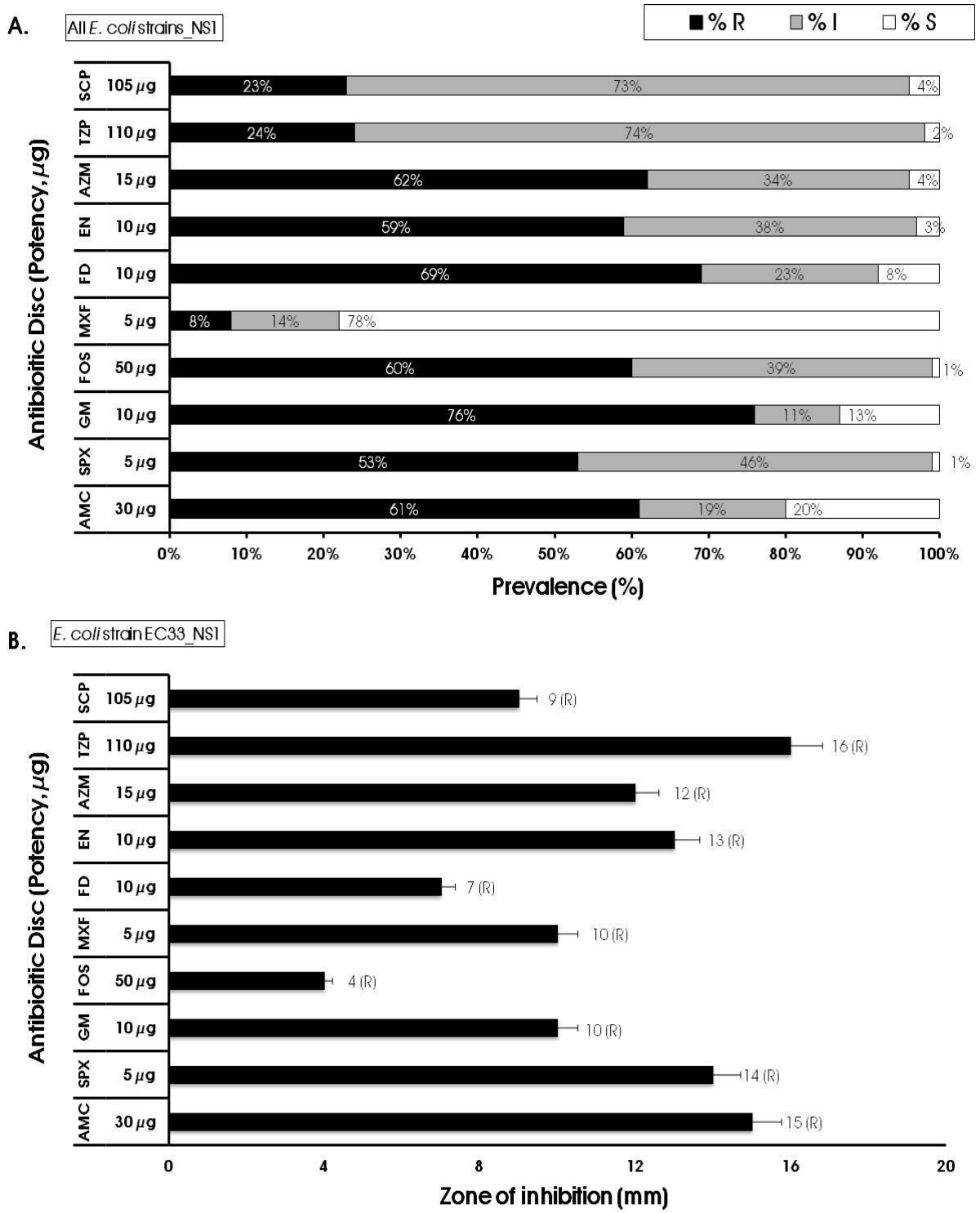

Fig. 2. Cumulative antibiotic-sensitivity profile of all the *E. coli* strains of NS1 group (A.) and multidrug resistant *E. coli* strain E33_NS1 (B.) against panel of test antibiotics.

Note: Commercial Antibiotic disc codes: AMC = Amoxicillin-Clavulanate, SPX = Sparfloxacin, GM = Gentamicin, FOS = Fosfomycin, MXF = Moxifloxacin, FD = Fusidic acid, EN = Enoxacin, AZM = Azithromycin, TZP = Piperacillin-Tazobactam, SCP = Sulbactam. Alphabetical letter "R" in each data label stands for "Resistant". The error bars indicate percentage error at 95% confidence interval *(P<0.05)*.

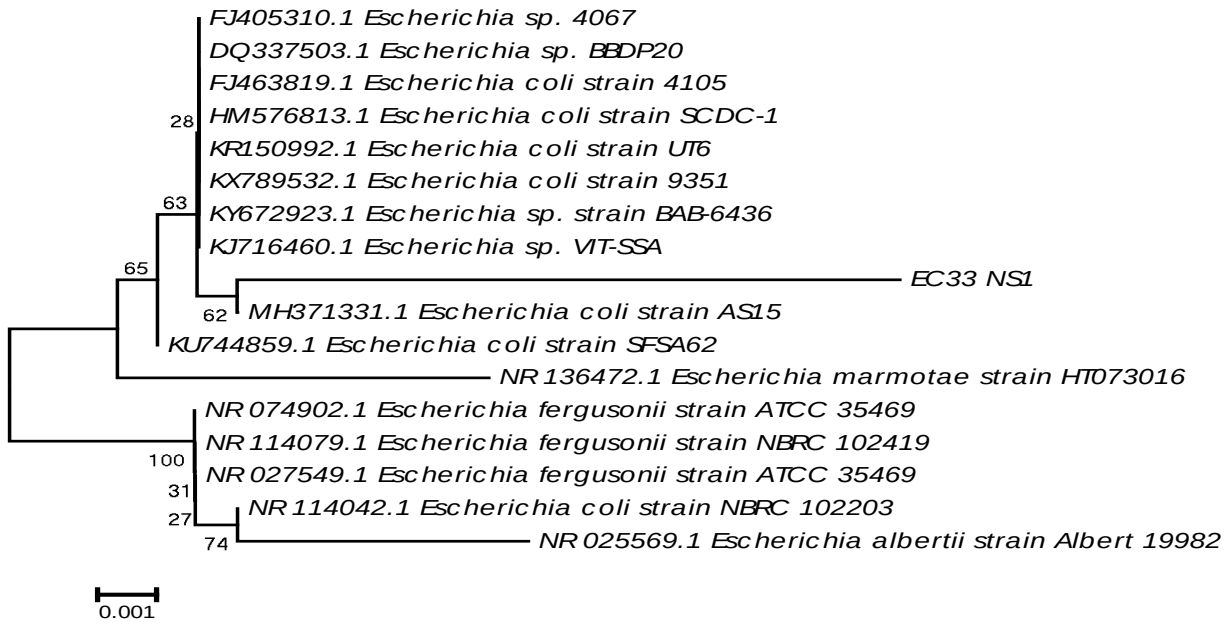

Fig. 3. Neighbour-joining tree showing evolutionary relationship of EC33_NS1 isolate with closely related taxa. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.09211806 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1441 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

DISCUSSION

The antibiotic susceptibility profile (ASP) determination is key to success for the recognition of significant treatment modalities and fighting against MDR bacterial pathogen. The emergence of multidrug resistance among *E. coli* has made it very difficult to control with available antimicrobial agents. The factors like resistance to multiple drugs among currently effective drug regimen and ability to acquire rapid mutations have enabled this microbe more infectious and increased their frequent involvement in serious infections associated with medical devices for example, urinary catheters, intravascular medical devices, endotracheal tubes etc. (Z. Ali *et al.*, 2015). By far, the *E. coli* is still the most common uropathogen with more than 80% positive urine cultures and causing an acute uncomplicated cystitis (AUC) in women (Kahlmeter, 2003). It is the most frequent microorganism causing bacteraemia, whether hospital- or community-acquired infection and has an increasing share of multidrug resistance (Riu *et al.*, 2016). Therefore, the present study was aimed to determine the gender wise prevalence and antimicrobial resistance profile of *E. coli* isolated from public and private health care units. The current study also highlighted the resistance of *E. coli* to most commonly used antibiotics.

The prevalence of multidrug-resistant *E. coli* isolates in diverse clinical specimen like pus, urine, HVS, and stool has confirmed their highly infectious nature. In order to get optimistic results, total 280 samples (118 from female and 162 from male) were collected from the private and public health care hospitals and their associated laboratories. Antibiotic resistance among bacterial isolates has been known as a growing clinical problem and a serious threat to public health. In this study, similar threat was evident from extremely high antibiotic resistance among *E. coli* strains, which showed complete resistance to all the tested antibiotics, although it has been around 94% in Bangladesh and China (Matin *et al.*, 2017). Similarly, Subedi and co-workers tested nine antibiotics, none of the antibiotics showed 100% effectiveness against the *E. coli* strains (Subedi *et al.*, 2018). They reported that maximally 98% of *E. coli* isolates were resistant to Ampicillin minimally 16% of *E. coli* isolates were resistant to amikacin and cotrimoxazole, doxycycline hydrochloride, and ciprofloxacin account more than 60% resistivity among the tested *E. coli* isolates, where as we found 61% resistance against amoxicillin, (76%) gentamicin, (60%) fosfomycin and (59 %) against enoxacin in our *E. coli* strains. According to the present study Moxifloxacin

seemed to be drug of choice against *E. coli* strains with its effectiveness (78%) while Ali his and co-authors (I. Ali *et al.*, 2017) have mentioned 83% effectives against E. *coli* under the age of fifteen years.

An attention-grabbing study was carried out lately in China that indicated the presence of two strains with resistance against 10 different antibiotics (Calderón *et al.*, 2006), Similarly, in our study EC33_NS1 strain from urine showed 100% to all ten antibiotics which is an Enterotoxigenic *E. coli* (ETEC) as already reported in Bangladesh, where Enterotoxigenic *E. coli* (ETEC) is a common cause of acute watery diarrhea in infants and young children, prevalence of MDR strains were observed among ETEC isolated from surface water samples (Campbell, 2005). In Vietnam, it is the most commonly isolated pathogen from patients having diarrhea and has displayed a significant prevalence of resistance to antibiotics in use (Brzychczy-Wloch *et al.*, 2013).

Resistance to multiple drugs is an emerging problem in clinical settings and has been reported worldwide (Chowdhary *et al.*, 2017; Hawkey *et al.*, 2018; Logan and Weinstein, 2017; Miyoshi-Akiyama *et al.*, 2017; Roca *et al.*, 2015). The emergence of CTX-M types of ESBLs producing *E. coli* isolated from a tertiary care urology setting in Pakistan have also been reported (Bouzari *et al.*, 2007). These strains may be a result of the emergence of multiple mechanisms of resistance after exposure to a number of different anti *E. coli* drugs and cross-resistance between these drugs.

Conclusion

The present study indicates that *E. coli* can be regularly isolated from clinical samples with substantial prevalence and emergence of MDR *E. coli* as well as its frequent transmission is out of debate. In addition, isolated *E. coli* was resistant to a whole panel of antibiotics used against it including front-line anti *E. coli* drugs. Antimicrobial sensitivity profile research is not significant to maintain pace with the problems of MDR clinical pathogen. Due to insufficient drug discovery, increasing antimicrobial resistance, and other issues like inadequate treatment are leaving dangerous consequences on the public health. To overcome these issues, the new novel antibiotics from natural sources with significant efficacy, minimum toxicity, and cost effective are need of hour. The policy makers have suggested to make policies, to limit the self medication, unnecessary usage and to take steps for sewage management (a source of enteric pathogen).

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