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Establishing Correlation between Antibiotic Resistance Patterns and Phylogenetic Groups of Multi Drug Resistant *Escherichia coli*.

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ABSTRACT

Introduction: *Escherichia coli* remains the most common cause of urinary tract infections (UTIs). They account for over 80-90% of all community-acquired and 30-50% of all hospital-acquired UTIs. *E. coli* strains have been found to belong to evolutionary origins known as phylogenetic groups. In 2000, Clermont classified *E. coli* strains into four phylogenetic groups using the triplex PCR method. The aim of this study was to identify the distribution of the phylogenetic groups amongst *E. coli*strains using triplex PCR method and to ascertain the antibiotic resistance profile amongst the phylogenetic groups.

Methods: In this study, 108 stored *E.coli* isolates from Dr. Joshi's Central Clinical Microbiology Laboratory, Vashi, Navi Mumbai were subjected to phylogenetic grouping by a triplex PCR method. Antimicrobial susceptibility testing (AST) was performed by disk diffusion method according to Clinical & Laboratory Standards Institute (CLSI) guidelines. Phenotypic detection of multi-drug resistant strains of *E. coli* based on AST data was performed.

Results: All 108 *E. coli* isolates were found to be multi-drug resistant (MDR). Phylogenetic group B2 (66.67%) was the most predominant, followed by A(15.74%), B1 (12.96%) and D (3.7%). Antibiotic resistance was mainly detected in phylogenetic group B2 (54%). All E. coliphylogenetic groups (B2, A, B1 and D) showed highest sensitivity to carbapenems (imipenem and meropenem) followed by members of aminoglycosides. Phylogenetic groups B2 and B1 recorded sensitivity to some members of fluoroquinolones and cotrimoxazole which was completely absent in group A and D. Majority of the groups exhibited complete resistance to beta-lactams and cephalosporins.

Conclusion: Our findings showed the high prevalence of MDR *E.coli* isolates, with the dominance of phylogenetic group B2. Phylogenetic groups B2 and B1 and A and D showed similar antibioticresistant profiles.

Key Words: MDR, AST, AMR, Phylogenetic, ExPEC, PCR

INTRODUCTION

Escherichia coli, a normal inhabitant of the human intestine, have been known for ages to cause infections of areas within and outside (extra-intestinal) of the gastro-intestinal system. Extra-intestinal pathogenic *E. coli* (ExPEC) strains, unlike the commensal strains, are harmful. ExPEC strains are highly virulent and are involved as the etiology in urinary tract infections (UTIs), bloodstream infections, pneumonia, skin and soft tissue infections and neonatal sepsis.[1]

UTIs are the most common infections along with after respiratory tract infection. Although many bacteria can cause UTIs, *E. coli* is the most common amongst them all.[2] Urinary pathogenic *E. coli* (UPEC) strains are deemed responsible for80% of all uncomplicated UTIs, with constant risein multi-drug resistance (resistant to ≥3 antibiotic classes) strains complicating the treatment options for UTIs.[3] Emergence in bacterial antimicrobial resistance (AMR) is one of the leading public health threats of the 21st century. It is estimated that this phenomenon could be responsible for 10 million deaths per year by 2050. This is a serious situation, alarming us notto neglect the growing AMR in microorganisms.[4]

Owing to the spectrum of disease caused by pathotypes of *E. coli*, Clermont devised a novel phylogenetic grouping scheme. The scheme was a dichotomous classification system based on the presence or absence of three genes using

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end-point PCR and categorizing *E. coli* into four phylogenetic groups namely A, B1, B2 and D.[5] Commensal *E. coli* strains mostly segregate into groups A and B1 whereas ExPEC strains into groups B2 and D, respectively.[1,2,5]

Therefore, the aim of the present study was to evaluate the phylogenetic groups of *E. coli* and their antibiotic resistance profile. The intent was also to understand the correlation between the phylogenetic groups and their antibiotic resistance profilethat can aid in selection of an appropriate antibiotic for the treatment of infections. This might also play a pivotal role in reducing the burden of AMR.

MATERIALS AND METHODS

Study Design and Setting

This doctoral research study was carried out at Department of Microbiology, VES College of Arts, Science and Commerce, Chembur, Mumbai and in collaboration with Dr Joshi's Central Clinical Microbiology Laboratory, Vashi, Navi Mumbai and Nexgen Molecular Lab LLP, Vashi, Navi Mumbai.

Re-isolation of Bacterial Strains

A total of 108 *E. coli* isolates thatwere stored at Dr Joshi's Central Clinical Microbiology Laboratory, Vashi, Navi Mumbai were studied. These isolates had been stored at -20°C for a period of 6-12 months. These isolates were recovered from urine samples suspected of UTI. All isolates for research purposes were re-purified on Mac Conkey agar and Sheep blood agar. Pure colonies of the isolates were used for antimicrobial susceptibility testing and phylogenetic grouping.[6]

Colony Morphology

The isolated colony growth of all *E. coli*isolates on Mac Conkeys agar plates were thoroughly studied for their visual presentation and their colony colour, size, shape, margins elevation and consistency data were recorded.[7]

Gram's staining

Microscopy based identification was carried out using Gram Staining. Smears of the isolates from purified colonies prepared on grease-free slides were air dried and heat fixed. The smears were then stained sequentially with crystal violet (primary stain), Gram's iodine (mordant), 95% ethanol (decolourizer) and safranin (secondary stain), followed by brief rinses. The slides were air dried and examined under oil immersion objective lens at a resolution of 100X lens by applying cedar wood oil on it.[7]

Biochemical Identification

a. Indole Test

This test was performed by inoculation of bacteria into peptone water and incubation at37°C for 24 h. After incubation, addition of Kovac's reagent leading to the formation of a cherry-red ring, indicates indole production thereby confirming tryptophan metabolism.[6]

b. Methyl Red (MR) Test

This particular test, involves inoculation of MR broth and incubating it at 37°C for 24-48 h. Addition of methyl red indicator resulting in a red color, signals the production of strong acids via acid fermentation pathways.[6]

c. Voges Proskauer (VP) Test

In the VP test, α-naphthol and potassium hydroxide addition to the inoculated and overnight incubated (37°C for 24-48 h) MR-VP broth indicating a red colour after 10-15 min confirms the presence of acetoin. Acetoin detected is a key intermediate in the butylene glycol fermentation pathway.[6]

d. Citrate Utilization Test

For the citrate utilization test, streaking onto Simmons citrate agar is followed by incubation at 37°C for 24 h. Colour of the slant turning blue indicates the ability of the bacteria under test to use citrate as a carbon source.[6]

e. Triple Sugar Iron (TSI) Test

The TSI agar is inoculated with stab and streak methods and incubated for 37°C for 24 h. Results were then observed for sugar fermentation, gas production, and hydrogen sulfide (H_2S) formation, with black precipitate signifying H_2S production.[6]

Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing for all *E. coli* isolates was done using the Kirby Bauer disk-diffusion method. Isolated colonies from the Sheep blood agar were suspended in saline to obtain turbidity of 0.5 McFarland standard. A sterile cotton swab on a stick was dipped in the colony-saline mixture, excess saline was then squeezed out by pressing the swab against the test tube, and the cotton swab gently applied onto the surface of the Mueller-Hinton (MH) agar (HiMedia Laboratories, Mumbai, India) to obtain a uniform lawn. Up to six antibiotic impregnated discs were gently placed on the agar surface, at a minimum distance of 25 mm from each other, and the plates incubated at 37°C aerobically for 18-24 h. The zones of inhibition diameters around each disc were measured using a ruler and compared against the zone diameter interpretative standards according to CLSI. A panel of 19 antibiotics discs were used: ampicillin (10µg), amoxicillin/clavulanic acid (20/10µg), cefaperazone-sublactam (75/35µg), amikacin (30µg), gentamycin (10µg), tobramycin (10µg), cefipime(30µg), cefotaxime (30µg), ceftriaxone (30µg), ceftazidime (30µg), trimethoprim/sulphamethoxazole (1.25/23.75µg), ciprofloxacin (5µg), gatifloxacin (5µg), levofloxacin (5µg), ofloxacin (5µg), norfloxacin (10µg), az-

teronam (30µg), imipenem (10µg) and meropenem (10µg). *E. coli* ATCC 25922 was used as a control strain[6,8,9](HiMedia Laboratories, Mumbai, India).

Genomic DNA Extraction

1.5 ml of cultured broth for all bacterial isolates in 2 ml eppendorf tube was centrifuged for 5 min at 8000 rpm to obtain a cell pellet. The collected pellet was suspended in 200µL of 1X TE buffer. Qiagen Blood Mini kit with spin column protocol was employed for the isolation of genomic DNA. The integrity of extracted DNA was evaluated by electrophoresis on a 1% agarose gel. On confirming the presence of genomic DNA on 1% agarose gel the rest of the DNA was stored at minus 20°C until further use.[10]

Clermont's triplex PCR Phylotyping

The distribution of phylogenetic groups amongst *E. coli* isolates was determined by the Clermont original triplex PCR phylogenetic groupingof 2000.[5] Briefly, a single reaction mixture contained 2 µL of 10x buffer (supplied with Taq polymerase), 2 µL of DNA (approximately 100 ng), 20 pmol of each appropriate primer (Eurofins India Pvt Ltd), 2mM of each dNTP (Bioron), and 2U of Taq DNA polymerase (Bioron) in a total volume of 20 µL. Primer sequences for the Clermont'striplex PCR phylogenetic groupingassignment method are as shown in (Table 1). PCR amplifications were carried out on aVeriti thermal cycler (Thermo Fisher) under the following conditions: initial denaturation at 94°C for 5 min and 30 cycles for each denaturation at 94°C for 30 sec, annealing at 54°C-60°C for 30 sec (groups A, B1, B2 & D) amplification at 72°C for 30 sec, and final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis on 2% agarose gel (HiMedia Laboratories, Mumbai, India) stained with ethidium bromide (Himedia Laboratories, Mumbai, India) and visualized using Chemidoc (Biorad, India). A molecular weight standard (100bp ladder, Fermentas) was used.[6,8]

RESULTS

Purification and Gram Staining of Isolates

A total of 108 isolates were re-purified on Mac Conkey's agar for analysis from Dr Joshi's Central Clinical Microbiology Laboratory, Vashi, Navi Mumbai. All the isolates subjected to Gram staining revealed the presence of Gram-negative rods characteristics of *E. coli*. (Table 2)

Biochemical Identification

Biochemical tests identified that the entire set of 108 isolates were *E. coli*. Entire set of isolates fermented lactose, formed indole and strong acids for positive Indole and MR test. These isolates failed VP test and citrate utilization test. TSI test was positive with acidity and yellowing in the slant and butt with gas production dislodging the entire medium with absence of blackening due to H_2S formation (Table 3, Figure 1).

Antibiotic Susceptibility Testing& Multi-Drug Resistance (MDR)

All *E. coli* (108) isolates were subjected to antibiotic susceptibility tests against 19 antibiotics belonging to 7 classes of antimicrobial agents.The entire set (108) of isolates were found to be multi-drug resistant based on their non-susceptibility to≥1 agent in ≥3 antimicrobial categories (Figure 2).

Highest resistant was recorded against all members of cephalosprins, starting with ceftazidime (100%), cefotaxim (100%), ceftriaxzone (100%) followed by cefipime (99.07%). Other antimicrobials with high resistance that followed were aztreonam (100%), amoxicillin-clavulanate (98.14%), ampicillin (98.14%), cefaperazone-sublactam (98.14%) and levofloxacin (93.51%), ciprofloxacin (93.51%), norfloxacin (92.59%), ofloxacin (91.66%), gatifloxacin (89.81%) and cotrimoxazole (82.40%). E. coli isolates failed in mounting any resistance against imipenem and meropenem and showed 100% sensitivity. Members of aminoglycoside group were the least resisted after imipenem and meropenem, amongst whom amikacin (29.62%) ranked first followed by gentamicin (47.22%) and tobramycin (60.18%) (Table 4).

Phylogenetic grouping & Antimicrobial Resistance

Using the Clermont triplex PCR scheme, phylogenetic group B2 (66.59%; 73/108) was the most prevalent, followed by phylogenetic group A (15.74%; 17/108), B1 (12.96%; 14/108) and D (3.7%; 4/108). (Figure 3, Table 4)

Patterns in resistance to antibiotics were seen across all phylogenetic groups. An overwhelming lack of resistance towards imipenem and meropenem resonated consistently across all phylogroups (B2, A, B1, and D). However deep-rooted was the cephalosporin resistance in A, B1, and D, it skipped one member from phylogenetic group B2. Amikacin stood steadfast as the least resisted aminoglycoside across all groups, trailed by gentamicin and tobramycin. Resistance to five fluoroquinolones members was well-established in phylogenetic group A and D, with a spark of sensitivity in only two members of B1whilst members of phylogenetic group B2 did showcase notable sensitivity to various fluoroquinolones. Intriguingly, high antibiotic resistance encircling all phylogenetic groups for aztreonam, amoxicillin-clavulanate and cefaperazone-sublactam was noticed strikingly. (Figure 4)

DISCUSSION

Escherichia coli is the leading cause of urinary tract infections worldwide. The global spread of multi-drug resistance in UPEC strains pose a significant public health threat by

limiting treatment options and increasing UTI associated morbidity and mortality.[8] The stored resistant *E. coli* isolates from Dr. Joshi's Central Clinical Microbiology Laboratory involved in this study exhibited a multidrug-resistant phenotype, with resistance levels remarkably higher than those reported in independent studies from Slovakia (62.6%), Turkey (52.7%), and Thailand (62.6%).[1] In India, detection of MDR UPEC strains was widespread across all geographical regions, ranging from 56.5% in Northern India to 88.9% in Western India.[9] However the notion of lower MDR UPEC in Northern India was challenged by a study in Haryana (Sonipat), a North Indian state, reporting a rate of 83.1%.[11] Our study on isolates from Maharashtra (Navi Mumbai), a West Indian state, reported an impression coherent with that reported for this region and is as aforementioned.[9]

This study employed Clermont's rapid triplex PCR scheme for phylogenetic classification of *E. coli* isolates, which since inception has gained wide acceptance and has been employed for two decades successfully.[1, 2, 5, 8, 12, 13, 14, 15, 16] Our study involved phylogenetic grouping of 108 *E. coli* isolates suspected in UTI, demonstrating that 67.69% isolates belonging to B2 followed by 15.74% to A, 12.96% to B1 and 3.71% to D. One more study from Lucknow, India with 184 *E. coli* strains from women with acute cystitis showed 47.1% strains belonging to B2, 29.1% to A, 12.7% to B1 and 11.1% to D.[17]A study from another continent on 86 *E. coli* strains from UTIs in Algeria demonstrated 48.9% strains belonging to B2, 8.1% to A, 4.7% to B1 and 1.2% to D group respectively.[8] A study on 105 *E. coli* isolates from Slovenian patients with bacteremia of the urinary tract showed that group B2 was followed by D, A and B1 (51%, 20%, 15% and 13% respectively).[18] Similarly, when studying 190 urinary *E. coli* isolates, a Colombian study demonstrated dominance of group B2 followed by D, A and B1 (46.8%, 25.3%, 11.1% and 4.7%).[19] A research on 113 *E. coli* isolates from urinary tract infections in Iran reported predominance of B2 (44.2%) group followed by D (31%), B1 (20.4%) and A (4.4%).Though extremely clear is the predominance of phylogenetic group B2, there are reports wherein D, B1 and A have been seen to rank the first as well.[12, 20-22] Most of the ExPEC strains are seen to predominantly fall into B2 and D phylogenetic groups, whilst the commensals falling into groups A and B1.[12, 23] Therefore, amongst the phylogenetic groups, group B2 deserves more focus than the others. This group with high evolution rate of its virulence and antimicrobial resistance might have caused extra-intestinal infection like UTI a major public health concern.[8]

This present study also focused on understanding the AST patterns demonstrated by different phylogenetic groups of *E. coli*. All phylogenetic groups cumulatively recorded high percentage resistance of 82.40% towards trimethoprime/sulfamethoxazole. This resistance value was extremely high surpassing the values recorded in Algeria (51.16%) and Iran (53.1%).[2, 8]A similarly high percentage of resistance to all cephalosporins (98–100%) was observed in these stored resistant *E. coli* isolates, in contrast to the low resistance reported in studies from Algeria, Uganda, and Iran.However, our results regarding high resistance to β-lactam group resonated (98-100%) with the same studies conducted in Algeria, Uganda and Iran.[2, 8, 16] Addition of β-lactamase inhibitor clavulanic acid did not reduce the resistant strains to amoxicillin in this study and so was similar to that observed by Algerian research group but not by the Iranian group who noticed significant susceptibility to amoxicillin-clavulanic acid in all phylogenetic groups except phylogenetic group A.[2, 8] It was observed that all the strains irrespective of their phylogenetic groups were totally susceptible to imipenem (100%) and meropenem (100%). This outcome matched exactly with two independent studies, one from India and other from Iran.[2, 24] Also, the studies carried out in Uganda and Columbia reported low resistance rates of 1.43% towards imipenem and 9.5% towards meropenem respectively.[16, 19] Following carbapenems, lowest resistance in this study was recorded in all phylogenetic groups for amikacin, gentamicin and tobramycin (29.62 %, 47.22% and 60.18%) respectively. In a study in Algeria, most isolates showed lowest resistance in a slightly different order beginning with gentamicin, amikacin and tobramycin (13.95%, 18.6% and 23.25%).[8] In an Iranian study, only amikacin recorded 0.9% of resistance against all *E.coli* isolates whereas in a study in Uganda gentamicin alone recorded 37.14% of resistance respectively.[2, 16] In an Algerian study, moderate resistance rates ranging from 32% to 37.1% were reported for the fluoroquinolone group of antimicrobials. In contrast, our study showed extremely high resistance rates for all fluoroquinolone agents, ranging from 89% to 93%. Similarly, when comparing aztreonam resistance between the two studies, our study recorded significantly higher resistance rates (92.59%) than the Algerian study (41.56%).[8]

The present study when compared with other studies showed differences in the presentation of *E. coli* phylogenetic groups with variable resistance patterns. It can be assumed that mutation and pathogenicity in multiple genes can be instrumental in altering the distribution of phylogenetic groups. Consequently, studying and evaluating the distribution of phylogenetic groups in a particular region along with their antibiotic susceptibility patterns could be critical towards antimicrobial stewardship. This in a longer run will aid in appropriate and cost-effective treatment of UTIs and reduce the burden of AMR.

CONCLUSION

In conclusion, we observed the antibiotic resistance profiles between *E. coli* phylogenetic groups B2, D, A and B1 and the predominance phylogenetic group B2 over the rest. It is

strongly felt by us that routine surveillance of antimicrobial resistance amongst phylogenetic groups must be undertaken. Further studies on a large data set involving phylogenetic grouping to elucidate the changing trends in antimicrobial resistance is of paramount significance. This will enlighten us in executing antimicrobial stewardship, reducing indiscriminate use of antibiotics and diminishing the escalating burden of AMR.

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Authors' Contribution:

Conceptualization: M.K. and N.M.; writing—original draft preparation: M.K.; writing—review and editing: N.M. and S.J.; article communication: M.K. and N.M.; All authors have read and agreed to the published version of the manuscript.

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Table 1: Primers used for phylogenetic grouping in this study

Gene Name	5' – 3' Sequence	Amplicon Size (bp)	References
ChuAF	GGACACCTGAATGGCACTTT	434	Adapted from Clermont et al., 2000
ChuAR	GTCTGCGGATTTTTTGGTTC		
TspE4.C2F	ACACTATTCGTAAGGTCATCCC	122	
TspE4.C2R	CCAACAAAGTATTACGCAGC		
yjaAF	ATCGCCAATTTCTTTGTTGC	104	
yjaAR	ATATCCATACGTTTTGCTGGC		

Table 2: Microscopic and cultural characteristics of purified *E. coli* isolates

Isolates (n)	Gram Staining	Motility	Mac Conkeys Agar	Results
108	**-ve, short rod**	**Motile**	**Dry pink,smallcolonies**	**LF**

–ve = Negative, + = Positive, LF -Lactose fermenter

Table 3: Biochemical identification of *E. coli* isolates.

Isolates (n)	Biochemical Tests									Identification
	Lac	I	MR	VP	C	Slant	Butt	Gas	H2S	
108	+	+	+	-	-	**A**	**A**	+	-	***Escherichia coli***

Lac – Lactose Fermentation Test, I- Indole Test, MR – Methyl Red Test, VP - Voges Proskauer Test, Citrate- Citrate utilization Test, H_2S- H_2S production Test, TSI-Triple sugar Iron Agar Test, A – Acid, K – Alkaline, Gas - Gas formation, (+) = Positive, (-) = Negative

Table 4: Antibiotic Resistant Profile of *E. coli* isolates based on their AST response.

Antibiotics	Number of Resistant Isolates of the Phylogenetic Groups				Total Number of Resistant Isolates	% Resistance
	B2 (73)	A (17)	B1 (14)	D (4)		
AMP	71	17	14	4	106	98.14
AMC	71	17	14	4	106	98.14
CFS	71	17	14	4	106	98.14
AK	26	1	4	1	32	29.62
GN	35	9	5	2	51	47.22
TOB	43	12	8	2	65	60.18
CAZ	73	17	14	4	108	100
CEP	72	17	14	4	107	99.07
CTX	73	17	14	4	108	100
CTR	73	17	14	4	108	100
CIP	68	17	12	4	101	93.51
GAT	64	17	12	4	97	89.81
LEV	68	17	14	4	101	93.51
OF	66	17	12	4	99	91.66
NX	67	17	12	4	100	92.59
AT	73	17	14	4	100	92.59
COT	61	14	10	4	89	82.40
IMP	0	0	0	0	0	0
MRP	0	0	0	0	0	0

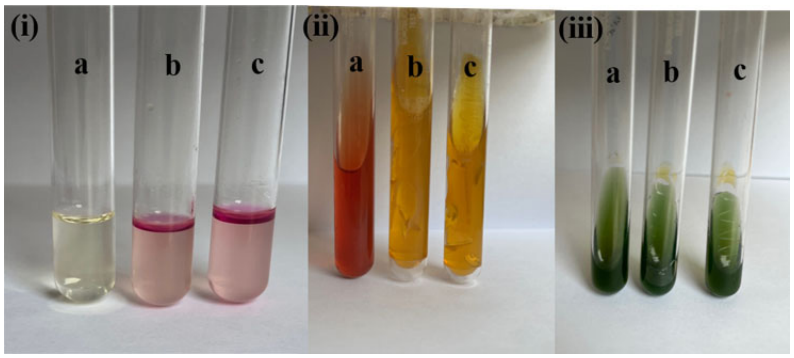

Figure 1: Biochemical tests for *Escherichia coli* isolates.(i)- Indole Test (ii) – Triple Sugar Ion Test (iii) – Citrate Test a – Uninoculated , b – Standard, c – Sample

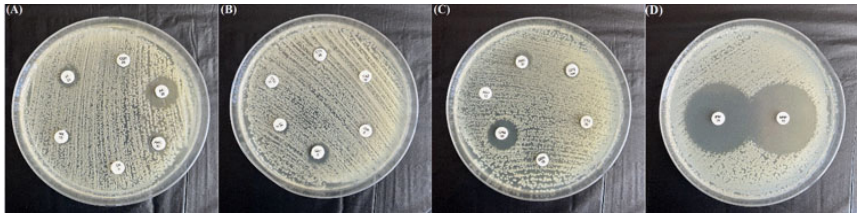

Figure 2: Antimicrobial susceptibility testing of *E. coli* by disc diffusion method exhibiting the presence and absence of zone of clearance around the disc. (A) - AT-azteronam (30µg), COT-trimethoprim/sulphamethoxazole (1.25/23.75 µg), AK-amikacin (30µg), AMC-amoxicillin/clavulanic acid (20/10µg), OF-ofloxacin (5µg), NX-Norfloxacin (10µg);
(B) - CTX-cefotaxime (30µg), CAZ-ceftazidime (30µg),CIP-ciprofloxacin (5 µg), GAT-gatifloxacin (5µg),LE-levofloxacin (5µg),CTR-ceftriaxone (30µg);
(C)- AMC-amoxicillin/clavulanic acid (20/10µg), CFS- cefaperazone-sublactam (30µg), CTX-cefotaxime (30µg), GEN-gentamycin (10 µg), CPM-cefipime (75/35µg), AMP-ampicillin (10µg);
(D)- IMP- imipenem (10 µg), MRP - meropenem (10 µg)

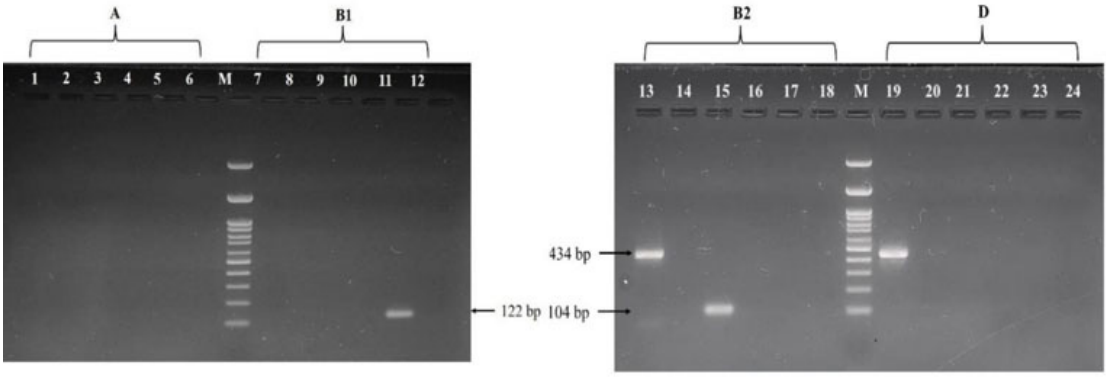

Figure 3: Agarose gel electrophoresis for Single plex PCR profiles of *E. coli* clinical isolates according to Clermont Phylotyping method. Lane M: (100 bp, Fermentas); Representative sample for Phylogroups A (Lane 1-6, chu -, yja -, tsp -), B1 (Lane 7-12, chu -, yja -, tsp +), B2 (Lane 13-18, chu +, yja +, tsp -) and D (Lane 19-24, , chu +, yja -, tsp -). Chu Sample – Lane 1,8,13,19; NTC – 2, 8,14, 20)

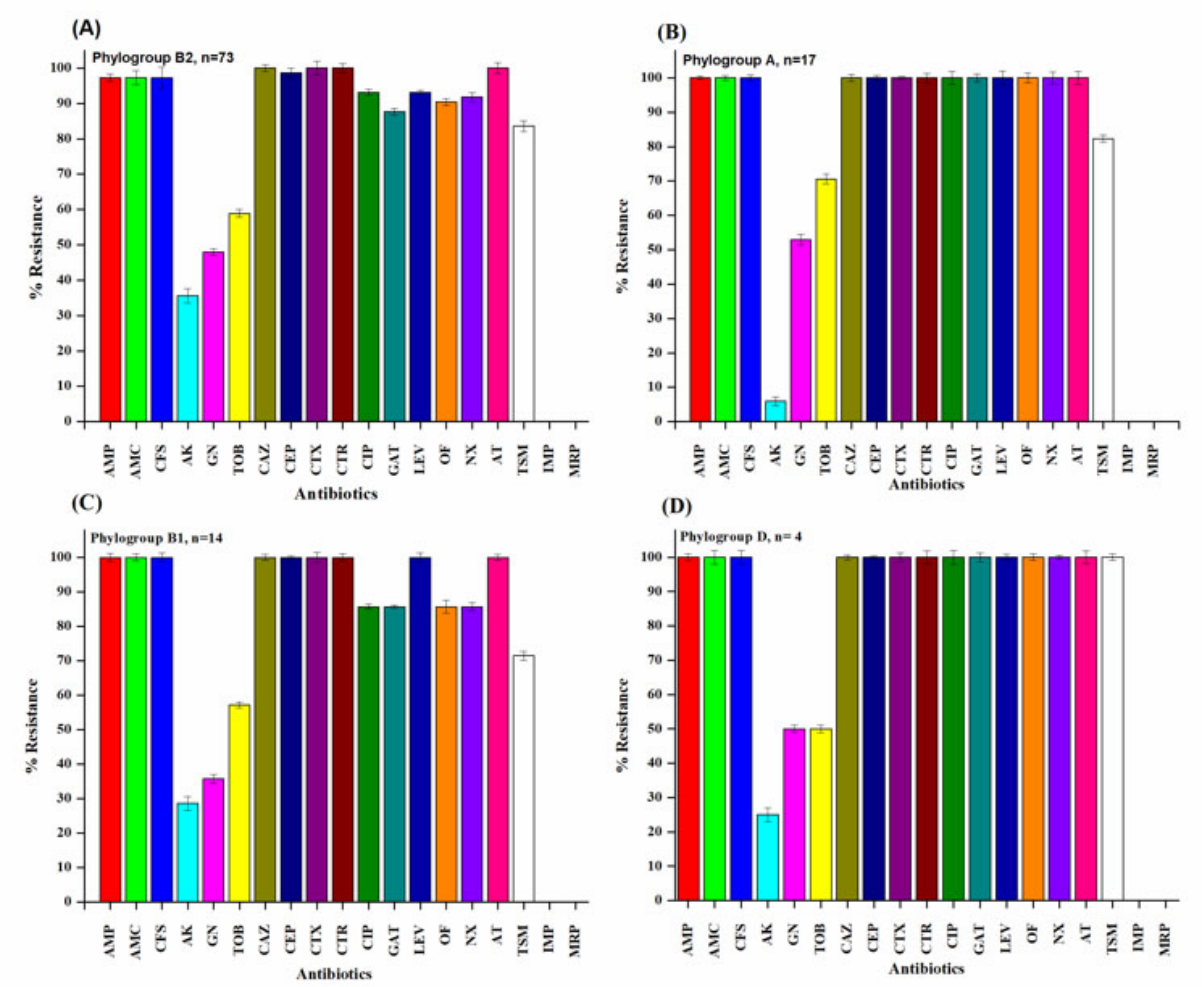

Figure 4: Antibiotic resistance patterns of gram negative uropathogen - *Escherichia coli.*

*AMP-Ampicillin, AMC-Amoxicillin+Clavulanate, CFS-Cefperazone-sublactam, AK-amikacin, GEN-Gentamicin, TOB-Tobramicin, CEP-Cephalothin, CAZ-Ceftazidime, CTX- Cefotaxime, CTR-Ceftriaxone, CIP-Ciprofloxacin, GAT-Gatifloxacin, LEV-Levofloxacin, OF-Oloxacin, NX-Norfloxacin, AT-Azteronam, TSM – trimethoprime/sulfametahoxazole, IMP-Imipenem, MRP-Meropenem.