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Original Article

Microbiology Section

Characterisation of Plasmids in Multidrug Resistant Uropathogenic Gram-negative Bacterial Isolates

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ABSTRACT

Introduction: Urinary Tract Infections (UTI) are one of the commonest conditions for which people seek medical care with an estimated 150 million episodes per annum worldwide. An unprecedented upsurge in the rate of development of antimicrobial resistance has reduced the therapeutic options leading to increased morbidity, prolonged hospital stays, development of complications. Majority of these infections are attributable to Gram negative bacteria which have now acquired resistance to almost all classes of antibiotics.

Aim: To analyse the plasmid-mediated drug resistance and characterise the major plasmid families that are in circulation.

Materials and Methods: A cross-sectional study comprising of a total of 95 non consecutive multidrug-resistant gram-negative bacterial isolates were subjected to Plasmid based replicon typing from January 2017 to June 2018. The 18 major replicons were divided in five multiplex and three uniplex Polymerase Chain Reaction (PCR) formats and the samples were subjected for plasmid characterisation and further sequencing of the plasmid Deoxyribonucleic Acid (DNA). The data obtained was analysed by Microsoft Excel software.

Results: *Escherichia coli*, accounted for maximum n=51 (53.7%), *Klebsiella pneumoniae* n=19 (20%), Citrobacter sp n=11 (11.6%), miscellaneous gram negative n=14 (14.7%) The isolates exhibited a high degree of resistance to almost all tested antibiotics, sparing a few like Fosfomycin, Chloramphenicol, Imipenem, Amikacin. A total of 154 different plasmid families were detected from the 95 isolates. FIB replicon (24%), FIA (21%), F, W (20%), FIC, B/O (14%), Y (12%), I1 replicon (10.5%) were the major plasmid families detected in the present study.

Conclusion: Many isolates exhibited the presence of more than one Incompatibility (Inc.) group plasmids, conferring multidrug resistance to the isolates. The study highlights the need for further research to study the association between plasmid families and their respective antibiotic resistance profiles for a given geographical niche and the need to devise further methods to target these epidemic plasmids.

Keywords: Conjugation, Polymerase chain reaction, Replicon typing, Urinary tract infection

INTRODUCTION

Garret Hardin's famous essay "In a world that is limited, ruin is the destination towards which all men rush" can aptly be put in the context of the ever increasing menace of antimicrobial resistance [1]. According to the CDC, 2.8 million antibiotic-resistant infections occur in the US each year, and more than 35,000 people die as a direct consequence of these infections [2]. Globally, World Health Organisation (WHO) estimates deaths due to drug-resistant organisms to be around 10 million annually by 2050 and may lead up to 24 million people into extreme poverty by end of this decade [3]. The era of discovery of antibiotics had ushered in a ray of hope of eliminating the bacterial diseases from the face of the world, but the hopes were shattered sooner than expected with the emergence of bacterial strains with resistance to these therapeutic agents.

A major contributor to the acquisition and subsequent horizontal transfer of resistance are the presence of extra chromosomal heritable determinants known as Plasmids. The role of plasmids in development of antibiotic resistance was first studied in Japan when it was found that susceptible strains were converting into multidrug-resistant ones, not as a result of mutations under selection pressure but by the acquisition of certain resistance determinant factors [4]. Both intergeneric and intrageneric transfer of resistance has well been documented in several studies [5-7]. Plasmids have been classified into various classes based on their replication controls and are termed as Incompatibility (Inc) groups. Plasmids which possess same replication control are incompatible whilst plasmids with different replication controls are termed as compatible plasmids and can co-exist within the same bacterium [8-10].

Bacteria have developed resistance to all classes of antibiotics including the ones which are often referred to as last resort antibiotics. The occurrence of resistance to Penicillins, Cephalosporins, Fluoroquinolones, Folate synthase Inhibitors, Carbapenems, Polymyxins are all known to exist in Plasmids and the list keeps on increasing day by day [11-14].

Until 2011, there was no clear cut definition of what comprised a Multidrug Resistant Organism (MDRO) in the context of bacteria. European Centre for Disease Prevention and Control (ECDC) and (CDC) defined it as an organism non susceptible to ≥1 anti-microbial agent in ≥3 antimicrobial categories [15]. They also defined Extensive Drug Resistance (XDR) and Pan Drug Resistance (PDR) as non susceptible to ≥1 agent in all but ≤2 antimicrobial categories and non susceptible to all antimicrobial agents respectively [15,16]. This study aimed to evaluate the presence of these major plasmid families in circulation amongst gram negative MDROs in our geographical niche and attempt to fulfill the gaps in knowledge about increasing antibiotic resistance in India.

MATERIALS AND METHODS

This cross-sectional study was carried out at Armed Forces Medical College (AFMC), Solarpur, Pune, Maharashtra, India. The study was carried out over a period of 18 months from January 2017 to June 2018. The study was duly approved by Institutional Ethics Committee (AFMC, Solarpur, Pune, IEC meet held in 2015) and written informed consent was obtained from the patients. The main objectives of the study was to isolate, identify and perform antibiotic susceptibility testing on all gram negative isolates from UTI cases in our centre followed by analysing plasmid mediated drug resistance by performing molecular characterisation of these plasmids.

Sample size calculation: The culture positivity rate for urine sample was taken to be 15%. The sample size had been worked out based

on the presumption of ~41% drug resistance in isolates where the power of the study is α=0.05 (CI=95%, d=10%) [17-19]. The total number of urine samples processed in order to obtain these 95 MDROs was 1500. MDRO is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories [15].

Inclusion criteria: Consecutive, non repeat, significant growth >=10^5 CFU/mL positive multi drug resistant gram negative bacterial isolates obtained from both outpatients and inpatients departments. All patients were clinically suspected to have UTI by the treating doctor.

Exclusion criteria: Urine cultures exhibiting Gram positive bacteria, Candida, Mixed growth obtained upon culture were excluded from the study and any growth <10^5 CFU/mL was considered non-significant growth and excluded from the study.

Sample collection: Mid-stream clean catch urine samples from suspected UTI cases were included in the study. For catheterised patients, urine aspirate was drawn aseptically from the catheter.

Microbiological Methods

Isolation and identification and antibiotic susceptibility testing of the samples n=95 were performed as per standard microbiological methods using semi-quantitative culture technique on Cysteine Lactose Electrolyte Deficient medium (CLED agar) (Hi-Media, India). Further identification and susceptibility was performed using automated Vitek2 Compact SL (Bio-Merieux Inc, SA Marcy-l'Etiole, France). Additional testing of antibiotics, not in the panel of Vitek2 was performed by Kirby Bauer disk diffusion method on Mueller-Hinton agar (Hi-Media, India) and results obtained were interpreted as per The Clinical and Laboratory Standards Institute (CLSI) 2017 and 2018 M-100 interpretive criteria [20].

Plasmid Extraction and Analysis

Plasmid DNA was extracted by miniprep spin column method using Hi Pur A™ Plasmid DNA Miniprep Purification Kit (Hi Media Labs, India) as per manufacturer's instructions. The Plasmid DNA was preserved at -80°C until further analysis.

PCR based Replicon typing (PBRT): The PBRT system was initially developed and further validated by Dr. Alessandra Carattoli, at the Italian National Institute of health, Rome which comprises of 18 major plasmid incompatibility groups and replicates genes which were identified and subsequently sequenced and are known to be circulating amongst gram negative bacteria [21-23]. The replicons used were HI1, HI2, I1, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIS, FrepB, K, B/O. All primer sequences were procured from Sigma Aldrich, India [Table/Fig-1]. Primers used in the study were divided into five Multiplex (M1-M5) and three uniplex PCR (S1-S3) on the basis of their amplicon size to avoid overlapping of amplicon bands. Amplicon (Amp) sizes ranged from 139 bp-785 base pairs [22,24,25].

All PCR amplifications were carried out in Gene Amp PCR system 97°C (Applied Biosystems) with the following protocol; one cycle of denaturation at 94°C for five minutes, 30 cycles of Denaturation at 94°C for one minutes, Annealing at 55°C for 30 seconds, Elongation at 72°C for one minute followed by final extension program of one cycle at 72°C for 5 minutes. In Uniplex one PCR carrying F rep plasmid the annealing temperature was kept at 50°C, rest of the PCR protocols were kept same. The post PCR products were electrophoresed on 1% agarose gel and viewed under Ultraviolet (UV) transilluminator.

Sequencing study: Pure LinkTM Quick Gel Extraction, Invitrogen by Life technologies was used as per manufacturer's instructions for DNA purification and extraction. ABI 3730 XL Analyser (Applied Biosystems, CA) based on Sanger's dideoxy DNA Capillary sequencing was used. The sequence was analysed using ABI Portal Resources for Indiana Science and Mathematics (PRISM) SeqScape version2.0 software.

STATISTICAL ANALYSIS

The data were entered in Microsoft Excel and analysed. Frequency (n) and percentages (%) were analysed for the collected data. The sequence got in all the samples was analysed using ABI (PRISM) SeqScape vers.

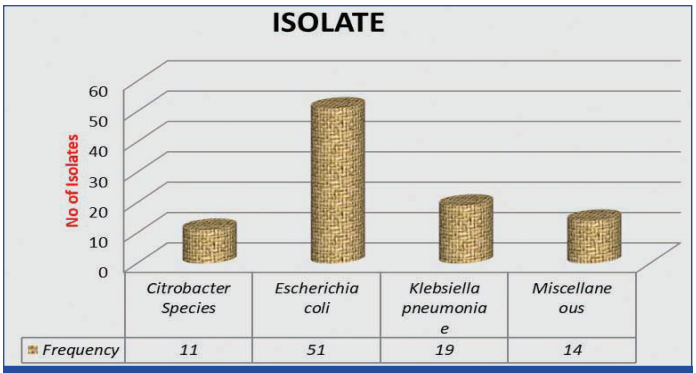
Plasmid family nomenclature	Sequence 5'-3' [21]	Amplicon size (bp)
HI1 FW	GGA GCG ATG GAT TAC TTC AGT AC	471
HI1 RV	TGC CGT TTC ACC TCG TGA GTA	
HI2 FW	TTT CTC CTG AGT CAC CTG TTA ACA C	644
HI2 RV	GGC TCA CTA CCG TTG TCA TCC T	
I1 FW	CGA AAG CCG GAC GGC AGA A	139
I1 RV	TCG TCG TTC CGC CAA GTT CGT	
X FW	AAC CTT AGA GGC TAT TTA AGT TGC TGA T	376
X RV	TGA GAG TCA ATT TTT ATC TCA TGT TTT AGC	
L/M FW	GGA TGA AAA CTA TCA GCA TCT GAA G	785
L/M RV	CTG CAG GGG CGA TTC TTT AGG	
N FW	GTC TAA CGA GCT TAC CGA AG	559
N RV	GTT TCA ACT CTG CCA AGT TC	
FIA FW	CCA TGC TGG TTC TAG AGA AGG TG	462
FIA RV	GTA TAT CCT TAC TGG CTT CCG CAG	
FIB FW	GGA GTT CTG ACA CAC GAT TTT CTG	702
FIB RV	CTC CCG TCG CTT CAG GGC ATT	
W FW	CCT AAG AAC AAC AAA GCC CCC G	242
W RV	GGT GCG CGG CAT AGA ACC GT	
Y FW	AAT TCA AAC AAC ACT GTG CAG CCT G	765
Y RV	GCG AGA ATG GAC GAT TAC AAA ACT TT	
P FW	CTA TGG CCC TGC AAA CGC GCC AGA AA	534
P RV	TCA CGC GCC AGG GCG CAG CC	
FIC FW	GTG AAC TGG CAG ATG AGG AAG G	262
FIC RV	TTC TCC TCG TCG CCA AAC TAG AT	
A/C FW	GAG AAC CAA AGA CAA AGA CCT GGA	465
A/C RV	ACG ACA AAC CTG AAT TGC CTC CTT	
T FW	TTG GCC TGT TTG TGC CTA AAC CAT	750
T RV	CGT TGA TTA CAC TTA GCT TTG GAC	
FIIS FW	CTG TCG TAA GCT GAT GGC	270
FIIS RV	CTC TGC CAC AAA CTT CAG C	
FrepB FW	TGA TCG TTT AAG GAA TTT TG	270
FrepB RV	GAA GAT CAG TCA CAC CAT CC	
K/B FW	GCG GTC CGG AAA GCC AGA AAA C	160
K RV	TCT TTC ACG AGC CCG CCA AA	
B/O RV	TCT GCG TTC CGC CAA GTT CGA	159

[Table/Fig-1]: Sequences of primers used in the study.

RESULTS

The total number of urine samples processed was 1500, out of which 95 culture positive MDROs were selected for the study. Majority of the present study population was in the age group of 15-45 years n=57 (60%), followed by above 45 years n=30 (31.6%) and below 15 years of age n=8 (8.4%). Frequency distribution of gender profile had no major difference with almost equal male n=49 (51.6%) to female ratio n=46 (49.4%). Outpatients accounted for n=30 (31.6%) of the study size whilst inpatients were n=65 (68.4%). Amongst frequency distribution of isolates [Table/Fig-2]; *Escherichia coli* n=51 (53.7%), *Klebsiella pneumoniae* n=19 (20%), *Citrobacter* sp n=11 (11.6%), Miscellaneous n=14 (14.7%) were the other gram negative isolates obtained. Amongst the Miscellaneous group *Pseudomonas* spp n=6 (6.2%), *Proteus* spp n=4 (4.2%), and one isolates each of *Myroides odoratimimus* and *Serratia marcescens, Enterobacter aerogenes*, and *Stenotrophomonas maltophila* were obtained. Analysis of the antibiogram of the MDR isolates [Table/Fig-3] showed a very high resistance to Ampicillin, Amoxycillin-clavulanate and third generation Cephalosporins. However, Fosfomycin, Chloramphenicol

were the only few antibiotics to which resistance was found to be relatively low.



[Table/Fig-2]: Bar graph depicting frequency distribution of Isolates (n=95).

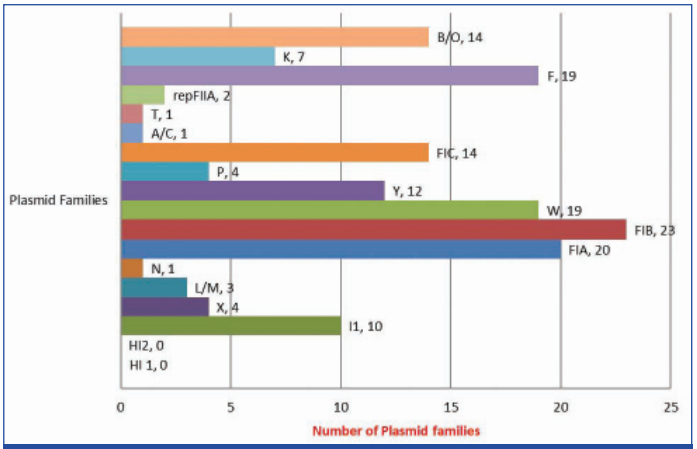
Antibiotic	Percentage resistance n (%)
AMP	84 (88.4)
PI	82 (86.3)
CPM	54 (57.5)
CTX	68 (71.5)
CTR	53 (55.7)
CAZ	45 (47.8)
GEN	23 (24.7)
AK	18 (18.9)
SAM	76 (80.6)
PIT	48 (50.5)
CIP	63 (66.3)
LE	63 (66.3)
OF	44 (46.9)
NOR	68 (71.5)
ETP	22 (23.2)
IMP	15 (16.1)
MRP	37 (38.6)
COT	53 (56.4)
NIT	48 (50.5)
AMC	86 (90.5)
C	4 (4.2)
AZT	73 (77.0)
NAL	83 (87.3)
FOS	9 (9.5)

[Table/Fig-3]: Resistance pattern of the isolates for the tested antibiotics (n=95).
AMP: Ampicillin; PI: Piperacillin; CPM: Cefepime; CTX: Cefotaxim; CTR: Ceftriaxone; CAZ: Ceftazidime; GEN: Gentamicin; AK: Amikacin; SAM: Ampicillin-sulbactam; PIT: Piperacillin-tazobactam; CIP: Ciprofloxacin; LE: Levofloxacin; OF: Ofloxacin; NOR: Norfloxacin; ETP: Ertapenem; IMP: Imipenem; MRP: Meropenem; COT: Cotrimoxazole; NIT: Nitrofurantoin; AMC: Amoxicillin clavulanate; C: Chloramphenicol; AZT: Aztreonam; NAL: Nalidixic acid; FOS: Fosfomycin

A total of 154 Plasmid replicon families were detected from the total isolates (n=95) confirming presence of multiple plasmid families within a single MDR isolate. Of the 18 gene targets, the indepth study found the presence of 16 plasmid rep families. FIB replicon was the commonest plasmid family detected in 23 out of 95 (24.2%) samples [Table/Fig-4]. Of the 16 Plasmid families, only eight products gave sequences that were subsequently [Table/Fig-5] converted in FASTA files and were analysed using the NCBI BLAST software program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

DISCUSSION

The UTIs are one of the commonest presentations in any hospital setting and various studies conducted worldwide have suggested prevalence of UTI being much higher in females as compared to males [26-28]. The study had a slight male preponderance with 51.6% of males as compared to 48.4 % females. Most of the isolates were obtained from



[Table/Fig-4]: Bar graph depicting frequency distribution of plasmid families (n=154).

[Table/Fig-5]: MW-100 bp molecular weight ladder.
PC: Positive control; NC: Negative control; Lane 1- 19: Plasmid DNA (from left to right: Lane 4 FIB replicon (702 bp), Lane 7 FIA replicon (462 bp), Lane 13 containing both FIA and FIB replicons, Uniplex 1 PCR Frep plasmids seen in Lanes 1,3,9,15 and 17)

the age group of 15-45 years, which accounted for 60% followed by >45 year of age (31.6%). Only 8.4% of the samples were from the paediatric age group. This variation in the gender distribution among the adult population is likely due to our patient pool comprising mainly of serving soldiers, veterans and their families and dependents. The results from the Study for Monitoring Antimicrobial Resistance Trends (SMART) study concluded similar findings in which the authors found that family Enterobacteriaceae comprised 86.0% of all the isolates out of which Escherichia coli accounted for 56.5% and Klebsiella pneumoniae was found in 13.8%, occuring as the two most common species isolated [29]. Antimicrobial susceptibility data commensurated with findings of various multi centric studies [16,30-34]. The overall high prevalence of resistance to oral antimicrobials is also explained by the indiscriminate use of these antibiotics in the present setting. Amongst the injectable agents, the susceptibility rates were better than the oral as their usage is somewhat regulated and antimicrobial stewardship is more in these cases. Cross-resistance or Co-Selection may also be a reason for higher resistance amongst the studied uropathogens. In various studies of Caratolli A, the presence of IncFII, IncA/C, IncL/M, and IncI1 plasmids showed the highest occurrence among typed resistance plasmids [10,21,22,35]. Presence of these plasmids can be rightfully termed as Epidemic plasmids as they accounted for majority in these isolates and the resistance genes they harbor. Association studies between Plasmid families and their respective antibiotic resistance profiles were attempted but could not be interpreted by Chi-square test because of very small frequencies. The significant families that were associated most commonly with resistance were found to be located in FIB, FIA [Table/Fig-5], Frep, W, FIC, I1 and B/O replicons in order of decreasing frequency. However, since the bacteria has multiple resistance mechanisms to exhibit for the same antibiotic, finding a definite plasmid coding for a particular antibiotic resistance would not be entirely correct and more studies with larger sample sizes would be required to have an accurate correlation between the same. The information provided here further adds to the understanding of the mechanisms of drug resistance in this specific region and their evolutionary relationship with other parts of world [23].

Limitation(s)

A larger sample size may have delineated the association between the Plasmid families and antibiotic resistance. The study did not characterise Chromosome mediated drug resistance.

CONCLUSION(S)

Having an insight into the major plasmid families circulating in the present setting can help us understand the resistance profiles and also help us in specifically targeting those. This would go a long way in our efforts to implement antimicrobial stewardship in a more targeted and efficient manner. The study aimed at understanding the transferable drug resistance amongst gram negative bacterial isolates and molecular characterisation of the plasmids that were found in the study. The study highlights the importance to know the plasmid families that are prevalent in a given geographical niche and understand the resistance genes that they carry and devise further methods to target such epidemic plasmids using novel techniques.

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