



Bacteriological profile of multidrug resistant Gram negative bacilli causing urinary tract infection in paediatric age group in a tertiary care centre

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Abstract

Introduction: Urinary tract infection is among the most common causes of febrile illness in children. It can lead to complications including septicaemia, if appropriate therapy is not given. Drug resistance has emerged as a significant problem in treating UTI. Knowing the pattern of antimicrobial resistance will help the clinicians in prescribing antibiotics.

Aims and Objectives: To determine the proportion of multidrug resistant Gram negative bacilli causing urinary tract infection in paediatric age group, antibiotic susceptibility pattern and detection of genes conferring resistance.

Materials and Methods: This is a descriptive study in which a total of 480 Gram negative urinary isolates from children less than 12 years over a period of 1 year were included. Multidrug resistant isolates were subjected to phenotypic confirmatory tests for ESBL, MBL, AmpC. 104 randomly selected isolates were subjected to PCR for CTX, TEM, SHV and VIM genes.

Results: Majority of isolates were from males. 72.9% of isolates were multidrug resistant. Majority of the isolates were *E coli* followed by *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Proteus mirabilis*, *Klebsiella oxytoca*, *Proteus vulgaris* and *Citrobacter freundii*. *Acinetobacter baumannii* had the largest number (76.9%) of multidrug resistant isolates. 47.2% of the MDR isolates were ESBL producers, 11.6% were AmpC producers and 11.2 % were MBL producers. 85 % of the ESBL producing isolates tested had CTX M gene and 22 % had TEM gene. 1 out of the 4 MBL producing *Pseudomonas aeruginosa* isolates tested had VIM gene.

Conclusion: The study emphasize the need for surveillance and development of local and paediatric specific antibiograms to reduce the growing resistance rates.

Keywords: ESBL, MBL, paediatric UTI, TEM, CTX- M.

Introduction

Urinary tract infection (UTI) is among the most common causes of febrile illness in children and is

an important cause of mortality in developing countries. The prevalence of UTI varies with age. The male:female ratio is 2.8-5.5 :1 in the first year

of age. There is a female preponderance beyond 1-2 years. Factors that influence the development of UTI include female gender, anatomical and physiological abnormalities of urogenital tract, iatrogenic factors such as catheter related infection, low immune status etc.

Most UTIs are caused by Gram negative bacteria, of which *Escherichia coli* is the most common (> 75%) as in adult UTIs. Other organisms include *Klebsiella* spp, *Proteus* spp, *Enterobacter* spp, *Citrobacter* spp, *Pseudomonas aeruginosa*, *Serratia* spp, *Enterococcus* spp. The main consequences of inappropriately treated UTI in children include chronic renal damage, end stage renal insufficiency and arterial hypertension.

Multidrug resistance (MDR) is defined as acquired non susceptibility to at least one agent in three or more antimicrobial classes. Recently, studies on paediatric UTI are showing an increase in drug resistance among uropathogens, with 23.3% of *E.coli*, 23.5% of *Klebsiella* spp, 25% of *Proteus* spp being multidrug resistant¹. This has occurred probably due to empirical therapy and misuse of antibiotics. The commonest mechanism of acquired drug resistance in most *Enterobacteriaceae* is the production of betalactamases. This study was conducted to determine the proportion of MDR Gram negative bacilli causing paediatric UTI. their antibiotic susceptibility pattern and to detect the extended spectrum betalactamase (ESBL) and metallobeta-lactamase (MBL) genes in these organisms.

Materials and Methods

This was a hospital based descriptive study conducted at the Department of Microbiology, SAT hospital, a 1000-bedded tertiary health-care centre, Trivandrum, Kerala, for a period of 1 year from 1st July 2016 – 30th June 2017. A total of 480 Gram negative isolates from urine specimens of children less than 12 years received in SAT microbiology laboratory were included. Midstream urine, suprapubic aspirates and catheter specimens were included. Study was

started after obtaining clearance from institutional ethics committee (IEC.No.06/12/2015/MCT). All samples were processed as per standard protocol² and isolates were identified using standard biochemical reactions. Antibiotic susceptibility testing was done according to CLSI recommended Kirby Bauer disc diffusion testing method for Ampicillin (25µg), Gentamicin (10µg), 1st generation Cephalosporin (30µg), Ciprofloxacin (5µg), Amikacin (10µg), Ceftriaxone (30µg), Ceftazidime (30µg), Piperacillin-tazobactam (100/10µg), Nalidixic acid (30µg), Norfloxacin (10µg), Cotrimoxazole (1.25/23.75 µg), Nitrofurantoin (300 µg), Meropenem (10µg), Imipenem (10 µg), Cefepime (30 µg) discs.

Phenotypic tests for ESBL, Amp C beta lactamases, MBL were done on multidrug resistant isolates^{3,4}.

For detecting ESBL producers

1) Screening test done was CLSI recommended disc diffusion test using Ceftazidime (30 µg) and Cefotaxime (30 µg). Isolates with a zone size \leq 22mm for Ceftazidime and/ or \leq 27 mm for Cefotaxime were subjected to confirmatory testing.

2) Confirmatory test done was CLSI recommended Cephalosporin/ clavulanate combination disc test in which antibiotic discs of Ceftazidime (30 µg) and Ceftazidime-clavulanic acid (30/10µg), Cefotaxime (30 µg) and Cefotaxime-clavulanic acid (30/10µg) were used. The organism is considered an ESBL producer if Cephalosporin/clavulanate combination disc showed a zone diameter \geq 5mm than Cephalosporin disc alone.

For detecting AmpC producers,

1) Screening test done was CLSI recommended disc diffusion test using Cefoxitin (30 µg). Isolates with a zone size \leq 14mm are subjected to confirmatory testing.

2) Confirmatory test done was combined disc diffusion test using phenyl boronic acid in which antibiotic discs of Cefoxitin (30 µg) alone and Cefoxitin(30 µg) in combination with 400µg phenyl boronic acid were used. The organism is

considered an Amp C producer if Cefoxitin – phenyl boronic acid combination disc showed a zone diameter ≥ 5 mm than Cefoxitin disc alone.

For detecting MBL producers

1) Screening test done was CLSI recommended disc diffusion test using Ertapenem (10 μ g) and Meropenem(10 μ g) . Isolates with a zone size ≤ 18 mm for Ertapenem and/ or ≤ 19 mm for Meropenem are subjected to confirmatory testing.

2) Confirmatory test done was CLSI recommended combined Imipenem EDTA disc test in which antibiotic discs of Imipenem (10 μ g) and Imipenem EDTA (10/750 μ g) were used. The organism is considered an MBL producer if Imipenem EDTA combination disc showed a zone diameter ≥ 7 mm than Imipenem disc alone

Molecular characterisation of ESBL and MBL

Molecular characterisation was done only on 30 % of MDR isolates. Uniplex polymerase chain reaction (PCR) was done for the detection of TEM and CTX M genes in 100 randomly selected ESBL producers and for SHV genes in 5 randomly selected ESBL producers. PCR for detecting VIM gene was done only in 4 MBL producing *Pseudomonas aeruginosa* isolates.

Preparation of DNA template

DNA template was prepared by growing fresh culture of the test organism and control strains overnight in a shaking incubator in Mueller Hinton broth followed by centrifugation. The

pellet is resuspended in sterile milli-Q (double distilled water), heated at 80°C in a water bath for 20 minutes, chilled immediately and stored at -20°C.

PCR amplification of the target DNA was carried out in a thermal cycler (Master cycler Eppendorf) in 0.2ml PCR tubes with a reaction mixture volume of 20 μ l containing 20 Pico moles of forward and reverse primers each (Table 1), 1.5mM MgCl₂ (2 μ l), 200 μ M dNTPs (1.5 μ l), 1X PCR buffer (2 μ l) (Sigma), and 1.5U of Taq DNA polymerase (0.3 μ l) (Sigma), 1 μ l of DNA template and sterile water to a final volume of 20 μ l.

Thermal cycling conditions for CTX –M,TEM and VIM includes initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, extension at 72°C for 1 minute, followed by final extension at 72°C for 10 minute and hold at 4°C. Thermal cycling conditions for SHV includes initial denaturation at 94°C for 4 minutes followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds, extension at 72°C for 1 minute 50 seconds, followed by final extension at 72°C for 10 minute and hold at 4°C.

Amplified products were separated by gel electrophoresis on 1% agarose gel, stained with ethidium bromide and visualized by using gel documentation system, Fluro S multi imager (Bio rad, USA).(Figure 1, 2,3,4)

Table 1: Primer sequences used in the study^{5,6,7}

Primer	Sequence 5'.....3'	Amplicon size
TEM	ATA AAA TTC TTG AAG ACG AAA GAC AGT TAC CAA TGC TTA ATC	1080 bp
CTX-M	SCS ATG TGC AGY ACC AGT AA CCG CRA TAT GRT TGG TGG TG S = G or C, R = A or T	544 bp
VIM	GTT TGG TCG CAT ATC GCA AC AAT GCG CAG CAC CAG GAT AG	382 bp
SHV	ATT TGT CGC TTC TTT ACT CGC TTT ATG GCG TTA CCT TTG ACC	1018bp

Results

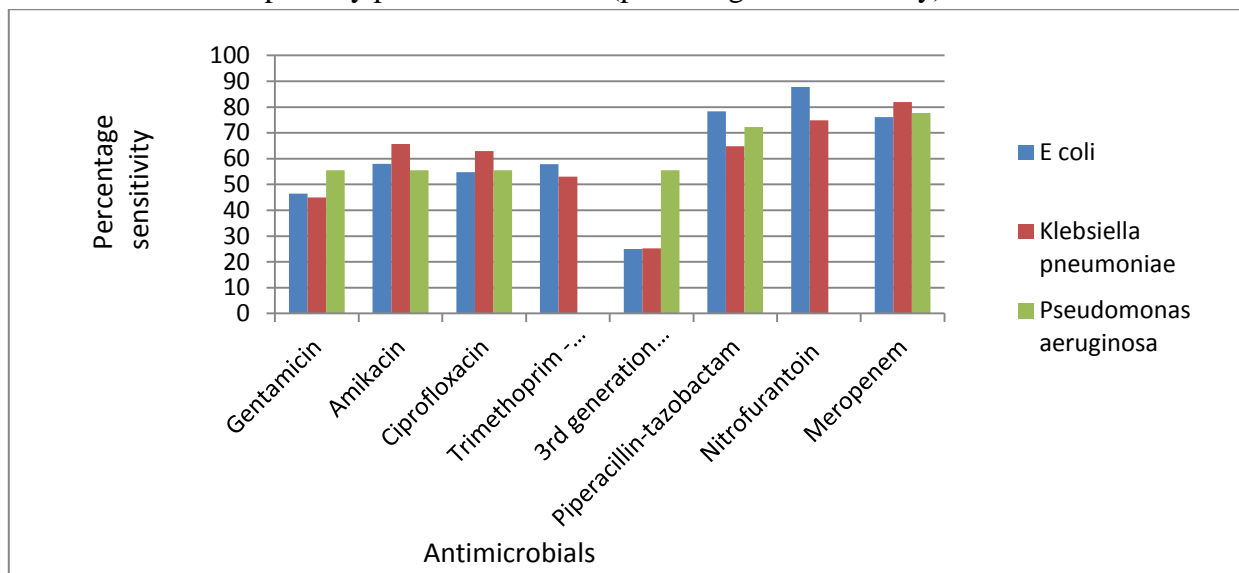
In the study population, 58% were males and 42% were females. Maximum number of isolates were from the age group of 28 days to 1 year, (42%),

followed by 1- 3 year age group (22.5%) and 5-12 year age group (19%). Remaining 16.5 % of isolates were from the age group of 3-5 years and < 28 days, of which least number were from <28

days age group. Majority of the isolates were *E coli* (66%), followed by *Klebsiella pneumoniae* (23%), *Pseudomonas aeruginosa* (3.8%), *Acinetobacter baumannii* (2.8%). The remaining 4.6 % were constituted by *Proteus mirabilis*, *Klebsiella oxytoca*, *Proteus vulgaris* and *Citrobacter freundii*.

Susceptibility pattern of *E coli*(largest number of isolates) shows a higher rate of susceptibility to Nitrofurantoin (87.7%), Piperacillin- tazobactam (78.3 %), Meropenem (76.1%) and Amikacin (58%) compared to other antibiotics. (Chart 1)

Chart 1: Antibiotic susceptibility pattern of isolates (percentage of sensitivity)



72.9% of total isolates were resistant to 3 or more classes of antibiotics (MDR). Considering the number of MDR isolates among the individual organisms, *Acinetobacter baumannii* had the largest number (76.9%) (Table 2).

Out of the total 480 isolates, 47.2% were ESBL producers. 11.6% were AmpC producers and 11.2 % were MBL producers. In all organisms except *Pseudomonas aeruginosa* and *Acinetobacter*

baumannii, among the total isolates ESBL producers were found to be more, followed by AmpC and MBL producers. In *Pseudomonas aeruginosa* isolates, MBL production was high (22.2%) as compared to ESBL (16.7%) and AmpC (5.5%) production. Among *Acinetobacter* isolates, ESBL production was high (38.4%) followed by MBL (30.7%) and AmpC (7.6%) production.

Table 2: Distribution of MDR organisms among the isolates

Organism	Total number of isolates	Number of MDR organisms	Percentage
<i>E coli</i>	318	238	74.8%
<i>Klebsiella pneumoniae</i>	111	83	74.7%
<i>Klebsiella oxytoca</i>	7	5	71.4%
<i>Proteus vulgaris</i>	3	2	66.6%
<i>Proteus mirabilis</i>	8	3	37.5%
<i>Pseudomonas aeruginosa</i>	18	8	44.4%
<i>Acinetobacter baumannii</i>	13	10	76.9%
<i>Citrobacter freundii</i>	2	1	50%
Total	480	350	72.9%

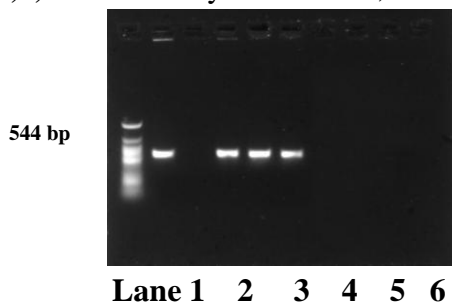
Table 3: Distribution of ESBL genes among the MDR isolates tested

Organism	Isolates tested for CTX M & TEM	Isolates tested for SHV	CTX M present	TEM present	SHV present
<i>E. coli</i>	73	3	63(86.3%)	10(13.6%)	3(100%)
<i>Klebsiella pneumoniae</i>	25	1	22(88%)	11(44%)	0
<i>Klebsiella oxytoca</i>	1	1	0	0	0
<i>Proteus mirabilis</i>	1	0	0	1(100%)	0
Total	100	5	85(85%)	22(22%)	3(60%)

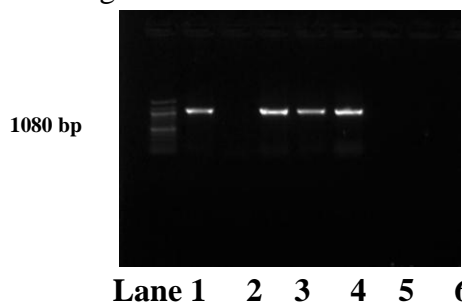
CTX M gene was detected in 85% of the isolates tested, TEM gene in 22%, SHV gene in 60% (Table 3). CTX M + TEM genes were detected in 17% of isolates tested. CTX M + SHV genes were detected in 40% of isolates tested (Table 3).

Among the total 54 MBL producing organisms, there were 4 *Pseudomonas aeruginosa* isolates. All the 4 isolates were subjected to PCR for detection of VIM gene. Only 1(25%) isolate showed presence of VIM gene.

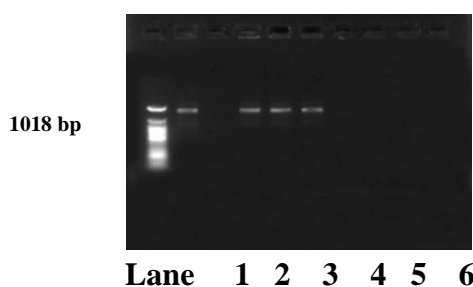
Figure 1,2,3,4: PCR assay for CTX M, TEM, SHV and VIM genes



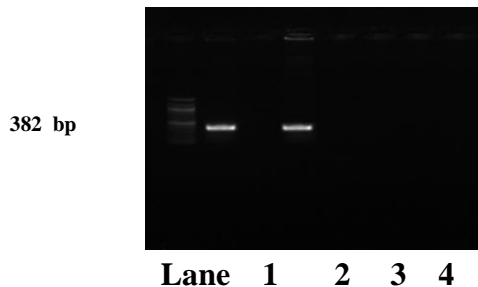
PCR assay for CTX M gene showing :-
Lane 1- 100 b p ladder (NEB), Lane 3 – negative control,
Lane 2 – positive control ,Lane 4,5,6 – CTX M positive isolates.



PCR assay for TEM gene showing :-
Lane 1 – 100 b p ladder (NEB), Lane 3 – negative control
Lane 2 – positive control, Lane 4, 5,6 – TEM positive isolates



PCR assay for SHV gene showing:-
Lane 1- 100 bp ladder (NEB), Lane 3 – negative control,
Lane 2 – positive control, Lane 4, 5, 6 – SHV positive isolates



PCR assay for VIM gene showing:-
Lane 1 – 100 bp ladder (NEB), Lane 3 – negative control
Lane 2 – positive control, Lane 4 – VIM positive isolate

Discussion

The study shows a male predominance which correlates well with another study conducted in children upto 12 years of age by Krishnan et al in a tertiary care centre in Kerala¹⁰. Majority of the isolates were from the age group of 28 days to 1 year ; but a similar study from northern Kerala shows increased prevalence of UTI in 1-5 year age

group⁸. Considering the etiological agents, similar findings as that of the present study were observed in other studies conducted by Taneja et al and Krishnan et al from different geographical area^{8,9}. A higher percentage of susceptibility of *E. coli* to Nitrofurantoin, Meropenem, Piperacillin-tazobactam was seen in the present study. Studies conducted by Nagaraj et al and Nisha K.V et al on

paediatric UTI also show similar antimicrobial susceptibility pattern^{10,11}.

The percentage of multidrug resistant isolates identified in this study is much higher than that found in other studies¹². ESBL producers were found to outnumber AmpC and MBL producers in all isolates except *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in this study; whereas varying results were obtained in other studies. In a study conducted in South Kerala by Anitha Madhavan et al, AmpC producers were more than ESBL and MBL producers¹³. But the present study shows a reduced prevalence of MBLs among *Enterobacteriaceae* compared to other studies¹⁴ probably because of the judicious use of carbapenems. However, among *Pseudomonas aeruginosa* isolates, MBL producers were seen more than ESBL and AmpC producers in this study, which is in parallel with the findings of a similar study conducted by Umadevi et al¹⁵.

In the study CTX M gene was the most common ESBL gene detected, followed by SHV and TEM gene. In other studies also CTX gene was the most prevalent gene. However TEM gene followed next and SHV gene came only as the third prevalent gene¹¹. CTX M + TEM genes were detected in 17% of isolates tested and CTX M + SHV in 40%. A study on ESBL producing *Enterobacteriaceae* by Kaur M et al also had similar results¹⁶. 25 % of *Pseudomonas aeruginosa* isolates tested had VIM gene. A study conducted in Kerala on carbapenem resistant isolates by Anjali Swaminathan et al in 2016 showed that 50% of MBL producing *Pseudomonas aeruginosa* isolates had VIM gene¹⁷.

Tests to detect inducible AmpC producers, ESBL and AmpC co-producers were not done.

Conclusion

Majority of the isolates were from males. Maximum number of isolates were from age group of 28 days to 1 year. Largest number of isolates were *E coli*. 72.9 % of the total isolates showed multidrug resistance. *Acinetobacter baumannii* had the largest number of multidrug

resistant isolates. Largest number of isolates were ESBL producers followed by AmpC producers and MBL producers. CTX M was the most prevalent ESBL coding gene detected followed by SHV and TEM. Among the multiple gene combinations present in ESBL producers, CTX M + SHV was found to be more compared to CTX M + TEM. The study emphasises the need for periodic surveillance to identify the antibiotic resistance and formulation of local antibiograms especially paediatric specific antibiograms to guide clinicians in treating paediatric UTI, so that the emergence of MDR organisms can be effectively controlled.

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Conflicts of interest: There are no conflicts of interest

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