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Isolation of Bacterial Pathogens and Detection of MRSA and ESBL Producers causing Asymptomatic Bacteriuria in Antenatal Women

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A B S T R A C T

To isolate the bacterial pathogens causing asymptomatic bacteriuria in antenatal women,to study the drug resistance pattern and prevalence of MRSA and ESBL producers. Bacterial pathogens were isolated from 1000 urine samples collected from antenatal women following standard procedures. All the isolates were studied for antibiotic susceptibility pattern using modified Kirby bauer disc diffusion method. Resistant isolates were identified and studied for MRSA and ESBL by various methods. Out of 1000 urine samples culture positive were 118(11.8%). *E.coli* was the predominant organism isolated 54 (45.76 %).All Gram negative bacilli were 100% sensitive to Piperacillin –tazobactam and Imipenem. All Gram positive cocci were 100% sensitive to Vancomycin and Linezolid. No MRSA was detected. Among the Enterobacteriaceae isolates, 6(8%) were found to be ESBL producers. As asymptomatic bacteriuria is related to the complications in pregnancy, it is imperative that Urine culture tests should be done routinely for all the pregnant women to find out asymptomatic bacteriuria, and every positive case should be managed with appropriate antibiotics, to prevent any complication related to pregnancy.

Introduction

Urinary tract infections (UTI) are one of the most prevalent bacterial infections and the significant cause of morbidity and mortality (Shruthi *et al.*, 2012; Raksha *et al.*, 2003). Of all uncomplicated urinary tract infections, *Escherichia coli* are responsible for 50%-90% of infections. In pregnancy, urinary tract infections commonly occur, because of the physiological and morphological changes that occur in the genitourinary tract.

There are 2 types of urinary tract infection (UTI). They are asymptomatic and symptomatic urinary tract infection (Sujatha and Manju Nawani, 2014; Jayaseelan *et al.*, 2013, Annie Rajaratnam *et al.*, 2014). The definition of asymptomatic bacteriuria is the occurrence of actively multiplying bacteria, more than 10^5 bacteria per ml of urine inside the urinary tract, exclusive of the distal urethra at a time when the patient has nil

symptoms of UTI (Santogita Jain *et al*., 2013; Chandel Lata *et al*., 2012).

In pregnant women, the occurrence of asymptomatic bacteriuria was found to be 2% to 10% (Sudha Birader *et al*., 2013; Rajshekhar Kerure and Umashanker, 2013). Pregnancy increases the succession from asymptomatic bacteriuria to symptomatic bacteriuria which can cause acute pyelonephritis in 20 to 50% of cases and contributes to adverse perinatal outcomes like post partum hypertensive disease, urinary tract infections, anaemia, prematurity and increased fetal mortality rates if left untreated (Sujatha and Manju Nawani, 2014; Raul Raz, 2003; Graham and Galloway, 2001).

Asymptomatic bacteriuria is a microbial diagnosis. The gold standard test for asymptomatic bacteriuria is the urine culture. The relatively increased occurence of asymptomatic bacteriuria, the consequences faced by the antenatal women, the ability to avoid undesirable outcomes with the management justifies the testing of asymptomatic bacteriuria in pregnancy (Sujatha and Manju Nawani, 2014).

In different geographical regions, the frequency of the pathogen isolated and their antimicrobial resistance patterns can vary. Hence, the common etiological agents of asymptomatic bacteriuria should be investigated and their antimicrobial resistance pattern to be made aware of (Sujatha and Manju Nawani, 2014).

Materials and Methods

This cross sectional study was done for one year and six months from January 2014 to June 2015 to study the bacterial isolates causing asymptomatic bacteriuria in pregnancy at Government Kilpauk Medical College and Hospital, Chennai. Totally 1000 urine samples were collected from 18-40 years of age group among pregnant women and were studied during this period.

Sample Collection and Transport (Sujatha and Manju Nawani, 2014)

From 1000 asymptomatic antenatal women, urine specimens were collected by mid-stream clean catch method in a sterile container, which is wide mouthed and covered with tight fitting lids after obtaining informed consent. Specimens were transported immediately to the microbiology laboratory and processed without delay using standard procedures.

Urine Culture

Semi-quantitative method (Kheya Mukherjee *et al*., 2014; Sushama Thakre *et al*., 2012; Gayathree *et al*., 2010)

To isolate the organism, a semi-quantitative calibrated loop technique was used. One loopful of properly mixed urine that was not centrifuged was inoculated onto the surface of Nutrient agar, 5% sheep Blood agar, Mac Conkey agar and Cysteine Lactose Electrolyte Deficient agar using a calibrated loop that delivers 0.01 ml of urine sample. The culture plates were incubated under aerobic conditions at 37^0 C for 18-24 hours. The colonies were counted using colony counter and the number of colony forming units were multiplied by 100 to find out the number of microorganisms present per millilitre of urine.

$\geq 10^5$ colony forming units/ml - significant bacteriuria.

The diagnostic criteria for asymptomatic bacteriuria was considered when atleast two consecutive urine samples showed more than or equal to 10^5 colony forming units in 1ml of urine of the single species without any UTI symptoms (Lindsay Nicolle, 2003). To differentiate pathogens from commensals,

standard microbiological methods were followed.

Antibiotic Susceptibility Testing (Niranjan and Malini, 2014; Bauer *et al*., 1996)

Antibiotic sensitivity testing was performed by the Kirby-Bauer's disc diffusion method using Mueller Hinton agar as per CLSI guidelines 2014(M100-S24).

The quality control strains used were

Staphylococcus aureus - ATCC 25923
Escherichia coli - ATCC 25922
Pseudomonas aeruginosa - ATCC 27853

Test to detect methicillin resistant *Staphylococcus aureus* Cefoxitin disc diffusion test (Anand *et al*., 2009)

The test was performed by placing 30µg of cefoxitin disc in the Mueller Hinton Agar plate without NaCl supplementation inoculated with test organism. The plate was kept in the incubator at a temperature of 37˚C. The zone of inhibition was determined after 24 hrs and the zone size was interpreted as

Susceptible – ≥ 20 mm

Resistant – ≤ 19 mm

Methods to Detect Extended Spectrum Beta Lactamases (Taneja and Sharma, 2008; Ankur Goyal, 2008; CLSI, 2014)

Quality control

Quality controls were performed using
Escherichia coli ATCC 25922 - Negative control
Klebsiella pneumoniae ATCC 700603 - Positive control

Disk Diffusion Methods

Disk diffusion test was done for all Enterobacteriaceae isolates against cefotaxime (30 µg), ceftriaxone (30 µg), cefpodoxime (10 µg) and ceftazidime (30 µg) antibiotic disks for the screening of the isolates for potential ESBL production. Overnight incubation was done at 37˚C after which the zone size was read as per CLSI recommendations for ESBL screening criteria (CLSI, 2014).

Phenotypic confirmatory tests or disc potentiation test

This test was done for all enterobacteriaceae isolates against Ceftazidime (30 µg) antibiotic discs with and without clavunalic acid (10 µg). These discs were placed on a Mueller – Hinton agar plate inoculated with bacterial suspension equivalent to 0.5 McFarland standards. Overnight incubation was done at 37°C after which the result was interpreted as follows.If the zone diameter of ceftazidime / clavulanic acid was augmented by ≥ 5 mm in comparison with ceftazidime alone was taken as ESBL positive (Nandagopal *et al*., 2015; Mandira Mukherjee *et al*., 2013; Umadevi *et al*., 2011; Babypadmini and Appalaraju, 2004).

Detection of ESBL Producers using Etest ESBL strip (Anandkumar Harwalkar *et al*., 2013; Revathi, 1997)

The ESBL E-strip was based on two gradients. One end of the strip contained ceftazidime (0.5-32 µ/ml), and the opposite end was impregnated with ceftazidime (0.125-8µg/ml) and clavulanate (4µg/ml). An overnight culture of the test organism on brain – heart infusion agar was suspended in saline to match the 0.5 McFarland standard turbidity. Then the suspension was used to inoculate a Mueller Hinton agar plate by swabbing the plate using a sterile cotton swab. The E-strip was placed on the plate after the plate was dried and it was incubated at 37^0 C overnight.

For both the ends of the strip, the point of intersection between the inhibition eclipse and the edge of the E-strip was considered the minimum inhibitory concentration (MIC). A ceftazidime MIC / ceftazidime-clavulanate MIC ratio ≥ 8 indicates the presence of ESBL enzymes as per the manufacturer's instruction manual.

Detection of ESBL gene (TEM, SHV and CTX-M) was done by using polymerase chain reaction (PCR) (Dallene *et al*., 2010).

Results and Discussion

Urine samples collected from 1000 Antenatal women without signs and symptoms of infection of the urinary tract were tested and 118 bacterial isolates were isolated, identified and analysed for their antibiotic sensitivity pattern. Resistant isolates were identified and studied for Methicillin Resistant *Staphylococcus aureus* (MRSA) and Extended Spectrum Beta Lactamases (ESBL) by various methods.

The observations were recorded and analysed. The results were as follows:

Out of the 1000 urine samples, 118 (11.8%) were culture positive.(Fig-1). Among 118 isolates, total number of Gram negative bacilli were 78(66.10%)and total number of Gram positive cocci were 40(33.90%) (Fig-2)

Mid stream clean catch urine specimens were collected from one thousand antenatal women without any symptoms of urinary tract infection who attended Obstetrics and Gynaecology outpatient department at Government Kilpauk Medical College Hospital, Chennai from January 2014 to June 2015. Urine samples were collected from pregnant women of different age groups, gravida and trimesters.

The culture positives with asymptomatic bacteriuria in pregnancy were 11.8%. The culture positives were more in the 21-30 years age group, primigravida and in the first trimester. Gram negative bacilli were 78(66.10%), the predominant bacteria isolated in antenatal women with asymptomatic bacteriuria.

Escherichia coli was the major isolate constituting 54(45.76%) followed by *Staphylococcus aureus* 21(17.8%).In the study, antibiotic sensitivity of all Enterobacteriaceae and *Pseudomonas aeruginosa* showed 100% sensitivity to Imipenem and piperacillin/tazobactam. Amoxycillin showed less than 45% sensitivity to all the Gram negative isolates.

Staphylococcus aureus, *Enterococcus faecalis* and *Staphylococcus saprophyticus* showed 100% sensitivity to vancomycin. Among 21 *Staphylococcus aureus*, 17(80.95%) were sensitive to amoxicillin-clavulanic acid, 16(76.19%) to nitrofurantoin and 9(42.85%) to cephalexin.

Out of 75 Gram negative bacilli, 6(8%) were ESBL producers by both screening andphenotypic confirmatory test. Minimum inhibitory concentration (MIC) was done with E-strip containing ceftazidime and ceftazidime with clavulanic acid for the ESBL producers.

Of the 4 ESBL positive *Escherichia coli*, 3(75%) were positive and of the 2 ESBL positive Klebsiellapneumonia, 1(50%) was positive showing a ceftazidime and ceftazidime/clavulanate MIC ratio of ≥ 8.

Table.1 Distribution of organisms (n=118)

Organisms	Percentage
Escherichia coli	54(45.76%)
Staphylococcus aureus	21(17.80%)
Klebsiella pneumoniae	19(16.10%)
Staphylococcus saprophyticus	10(8.45%)
Enterococcus faecalis	9(7.63%)
Pseudomonas aeruginosa	3(2.54%)
Proteus mirabilis	2(1.69%)

Out of the 118 isolates, *Escherichia coli* 54(45.76%) was the predominant isolate followed by *Staphylococcus aureus*. *Proteus mirabilis* was the least common organism isolated 2(1.69%).

Table.2 Antibiotic sensitivity pattern of Gram negative isolates (n=78)

Name of the Drug	*Escherichia coli* (n=54)	*Klebsiella pneumoniae* (n=19)	*Proteus mirabilis* (n=2)	*Pseudomonas aeruginosa* (n=3)
Amoxycillin	24(44.4)%	7(36.8%)	0	Not tested
Amoxycillin/ Clavulanic acid	46(85.2%)	9(47.4%)	1(50%)	2(66.7%)
Cotrimoxazole	39(72.2%)	11(57.9%)	1(50%)	2(66.7%)
Cephalexin	32(59.3%)	7(36.8%)	0	Not tested
Cefotaxime	50(92.6%)	17(89.5%)	2(100%)	Not tested
Ceftazidime	50(92.6%)	17(89.5%)	2(100%)	3(100%)
Gentamicin	35(64.8%)	10(52.6%)	1(50%)	1(33.3%)
Amikacin	42(77.8%)	15(79%)	1(50%)	1(33.3%)
Norfloxacin	30(55.6%)	10(52.6%)	1(50%)	1(33.3%)
Ofloxacin	45(83.3%)	13(68.4%)	1(50%)	2(66.7%)
Nitrofurantoin	48(88.9%)	15(79%)	0	3(100%)
Piperacillin/ Tazobactum	54(100%)	19(100%)	2(100%)	3(100%)
Imipenem	54(100%)	19(100%)	2(100%)	3(100%)

All the Gram negative bacilli were 100% sensitive to Piperacillin/Tazobactum and Imipenem. Nitrofurantoin showed 48(88.9%) and 15(79%) sensitivity to *Escherichia coli* and Klebsiellapneumoniae. Amoxycillin showed 24(44.4%) sensitivity to *Escherichia coli* and 7(36.8%) to *Klebsiella pneumoniae*.

Fig.1

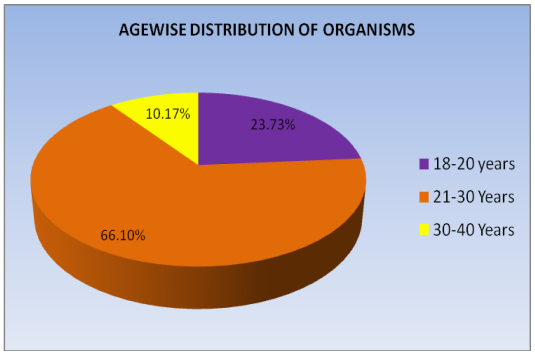


Table.3 Antibiotic sensitivity pattern of Gram positive organisms (n=40)

Name of the Drug	*Staphylococcus aureus* (n=21)	*Staphylococcus saprophyticus* (n=10)	*Enterococcus faecalis* (n=9)
Amoxycillin	8(38.09%)	5(50%)	3(33.30%)
Amoxycillin/clavulanic acid	19(90.47%)	10(100%)	6(66.66%)
Cotrimoxazole	10(47.62%)	7(100%)	5(55.55%)
Cephalexin	9(42.85%)	7(70%)	Not tested
Cefotaxime	15(71.42%)	8(80%)	Not tested
Gentamicin	10(47.62%)	6(60%)	5(55.55%)
Amikacin	15(71.42%)	8(80%)	6(66.66%)
Norfloxacin	9(42.85%)	6(60%)	6(66.66%)
Ofloxacin	17(80.95%)	9(90%)	8(88.88%)
Nitrofurantoin	16(76.19%)	8(80%)	7(77.77%)
Vancomycin	21(100%)	10(100%)	9(100%)

All the Gram positive cocci were 100% sensitive to Vancomycin. *Staphylococcus aureus* and Staphylococcus saprophyticus showed 79.19% and 80% sensitivity to Nitrofurantoin.

Staphylococcus aureus isolates were tested for Methicillin resistant *Staphylococcus aureus* using Cefoxitin disk. None of the *Staphylococcus aureus* isolates were Methicillin resistant *Staphylococcus aureus*.

By the screening test for ESBL and by phenotypic confirmatory test, 4(7.4%) *Escherichia coli* and 2(10.5%) Klebsiellaepneumoniae were identified as ESBL producers. None of the *Proteus mirabilis* isolates were positive for ESBL.

Table.4 Minimum inhibitory concentration of ESBL positive isolates using E-strip containing Ceftazidime and Ceftazidime/Clavulanic acid (n=6)

S.NO	Name of the Organism	Ceftazidime MIC	Ceftazidime + Clavulanic Acid MIC	Ratio	Result
1.	*Escherichia coli*	>32	0.25	128	Positive
2.	*Escherichia coli*	6	0.125	48	Positive
3.	*Escherichia coli*	>32	0.25	128	Positive
4.	*Escherichia coli*	3	0.5	6	Negative
5.	K.pneumoniae	6	0.125	48	Positive
6.	K.pneumoniae	3	0.75	4	Negative

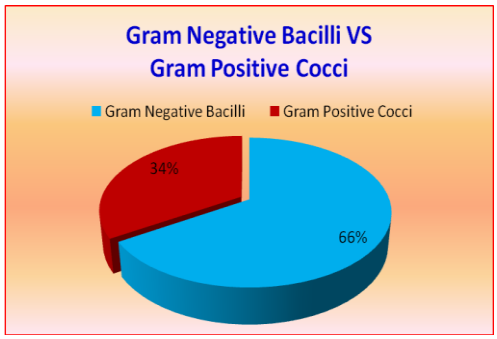
Out of 4 ESBL positive *Escherichia coli*, 3(75%) and of the 2 ESBL positive *Klebsiella pneumoniae*, 1(50%) showed ≥ 8 times reduction in Minimum Inhibitory Concentration.

Table.5 Gene identification in ESBL positive isolates for - TEM, SHV and CTX-M (n=6)

Organism	Total Tested	TEM Positive	SHV Positive	CTX –M Positive
Escherichia coli	4	0	0	2
Klebsiella pneumoniae	2	0	0	1

Among 6 ESBL producers, 3 were positive for CTX-M gene. Of which 2 were *Escherichia coli* and 1 was *Klebsiella pneumoniae*. None of the isolates were positive for the TEM and SHV gene.

Fig.2



6 ESBL producing Enterobacteriaceae which were phenotypically confirmed as ESBL positives were subjected to genotypic test by Polymerase Chain Reaction (PCR). Three genes such as TEM, SHV and CTX-M associated with ESBL production were studied using the relevant primers for the corresponding genes. CTX-M was detected in 3(50%). In the present study, all the 21 *Staphylococcus aureus* isolates were Methicillin sensitive *Staphylococcus aureus* and none were found to be Methicillin resistant *Staphylococcus aureus*.

In conclusion, the present study showed high occurrence of asymptomatic bacteriuria in pregnant women which if not treated, might lead to various maternal and neonatal complications. Urine culture with clean catch mid stream urine is the most sensitive test for its detection preferably in the first trimester. All the sequelae of asymptomatic bacteriuria during pregnancy could be reduced by appropriate antimicrobial treatment early in pregnancy. Hence, screening and management of asymptomatic bacteriuria need to be incorporated as a routine antenatal care for an integrated approach to safe motherhood and newborn health.

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