Phenotypic and Molecular detection of Mannose Resistant Haemagglutination in *Escherichia coli* isolates from Antenatal Women with Asymptomatic Bacteriuria

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Abstract

Introduction: Asymptomatic bacteriuria is more common in pregnant females. Early screening and treatment avoids complications like acute pyelonephritis and chronic kidney failure. Escherichia coli is the most commonly encountered organism among them. Certain virulence factors associated with E.coli have a direct link in pathogenic mechanism of organism like adhesins, toxins and hemolysin.

Aim: We aimed at identifying E.coli and demonstrating phenotypic and genotypic presence of mannose resistance by hemagglutination in urinary isolates.

Materials and Method: A cross sectional study during 2014 – 2015 among pregnant females was conducted in a tertiary care hospital. About 1000 urine samples were included in study. Urine culture with antimicrobial susceptibility testing was performed as per standard microbiological conditions. Virulence factor were identified using phenotypic method i.e., haemagglutination test and subsequently confirmed by polymerase chain reaction by looking for Pap A gene expression.

Results: Out of 1000 urine samples, 118 culture positives were seen. Out 118 culture positivity, 54 (45.76%) Escherichia coli isolates causing asymptomatic bacteriuria was identified. Among the 54 isolates, phenotypic method for mannose resistance haemagglutination showed 9 positivity (16.66%), which was confirmed with PCR for Pap A expression.

Conclusion: As asymptomatic bacteriuria is more common in pregnancy, it is a must to screen pregnant females in every trimester even without any specific symptoms. Virulence factors need to be explored in order to understand the pathogenic mechanism and importance of screening the asymptomatic women.

Keywords: Antenatal women, Asymptomatic bacteriuria, Mannose resistant hemagglutination

Introduction

Urinary tract infections (UTI) are the most common infections that are encountered by women with increased prevalence during sexual life and during pregnancy.[1] There is an increased risk of UTI in antenatal women which is due to urinary stasis occurring because of hormone progesterone and also due to various physiological and structural changes that happens during pregnancy.[2] It was estimated that asymptomatic bacteriuria occurs in 2 to 7% of pregnancy women during the first trimester.[3] It is diagnosed by urine culture, which is the gold standard method of diagnosis. Early detection and treatment during pregnancy prevents obstetric complications like acute pyelonephritis and chronic kidney failure for antenatal women and also prevents premature delivery and fetal death.[4] *Escherichia coli* accounts for 50-90% of all urinary tract infections. [5] Strains of *E.coli* causing UTI are different from the fecal isolates and are termed as "Uropathogenic *E.coli*" (UPEC).[6] Certain virulence markers that are chromosomally mediated code these strains.[6] Virulence factors include toxins, fimbriae and adhesins that allow attachment of *E.coli* to uroepithelial cells which inhibit the bacteria from urinary lavage and further allow for tissue invasion, multiplication and pyelonephritis.[7] Major fimbrial antigens associated with pathogenesis of UPEC in urinary tract infections are P fimbriae and Type 1 fimbriae.[8] Based on certain characteristics fimbriae falls under certain categories. The term P fimbriae denote that they are a part of P blood group antigen complex found in human red blood cells. These fimbriae of *E.coli* can recognize α-D-galactopyranosyl-(1-4) β-D-galactopyranoside and then adhere to the epithelial cell receptors that are carrying globoseries glycosphingolipid in their structure. This type of fimbrial antigens are not inhibited by mannose (Mannose Resistant-MR).[8,9] Type 1 fimbriae are mannose sensitive and attach to uroplakins, the major glycoprotein of uroepithelial cells. While P fimbriae are mostly linked with pyelonephritis strains, type 1 fimbriae are associated with cystitis.[8–10] Genes encoding for virulence factors are hlya (hemolysin), cnfl (cytotoxic necrotizing factor 1), iutA (aerobactin receptor), fim H (type 1 fimbriae adhesion), pap (P fimbriae), iroN (catecholate sideophore receptor) and omp T (outer membrane protease T).[11] Mannose resistant P fimbriae are of great concern; hence we aimed at identifying *E.coli* and demonstrating phenotypic and molecular detection of mannose resistance by hemagglutination in urinary isolates.

Materials and Method

This cross sectional study was conducted in Department of Microbiology over a period of one year and six months (January 2014 to June 2015) at a tertiary care teaching hospital. A total of 1000 urine samples were included in study taken from pregnant women with asymptomatic bacteriuria. Women who were having symptoms and signs of UTI, on antimicrobial treatment and structural abnormalities in urinary tract were excluded. Case definition for asymptomatic bacteriuria is actively, persistently growing bacteria in significant counts (10^5 bacteria per ml of urine) without any obvious symptoms. Institutional ethical committee approval was obtained and informed consent obtained for each study subject.

Sample collection and preliminary examination: Urine specimens were collected by proper instructions; mid-stream clean catch sample collected in a wide mouthed container fitted with tight lids, properly labeled and transported to microbiology department without any delay. Colour, turbidity and any deposits in the urine samples were observed macroscopically. Then one drop of uncentrifuged urine was smeared, air dried, heat fixed and Gram stained. Significant bacteriuria correlated with the presence of at least 1 organism/oil immersion field on examining 20 fields.

Urine culture – Semi quantitative method: A semi quantitative calibrated loop (one loop = 0.01ml) was used to inoculate a loopful of urine onto Nutrient agar, 5% sheep blood agar and cysteine lactose electrolyte deficient agar. Culture plates were incubated and standard microbiological identification techniques were used to isolate the organism and for species differentiation. Colonies were counted using colony counter; when at least two consecutive urine samples showed more than or equal to 10^5 colony forming units per ml of urine of a single species with no UTI symptoms, it is taken as a case of asymptomatic bacteriuria. *Escherichia coli* isolates were included for further identification of virulence factors.

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing was performed by Kirby Bauer`s disc diffusion method using Mueller Hinton agar as per CLSI guidelines 2014 (M100-S24).

Virulence factor – Haemagglutination test: *E.coli* strain taken from nutrient agar plate was inoculated in peptone water (pH 7.0) and grown at 37^0C for 24 hours. Human O erythrocytes were taken in Alsever solution and washed twice with phosphate buffered saline (PBS), made into a 3% suspension. A drop of bacterial culture from peptone water was mixed with a drop of 3% erythrocytes suspension in a clean glass slide. Slide was rotated for one minute at room temperature and observed for presence or absence of macroscopic haemagglutination. In order to test the effect of D-mannose on haemagglutination, one drop of mannose (25mg/ml of PBS; pH 6.8) was added prior to addition of RBC suspension and looked for any difference in the degree of haemagglutination subsequently.

Mannose Resistant Haemagglutination gene detection by Polymerase Chain Reaction (PCR): Overnight culture was used for DNA extraction using DNA purification kit (Helini biomolecules, Chennai). Master mix 2X used has 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM Magnesium chloride and 1µl of 10mM dNTPs mix, polymerase chain reaction additives. Primers used for Pap A gene were

Forward primer: 5'-GGCGCTGACAGAAGGTGCCATT- 3';

Reverse primer: 5'-CATGCCCAGTTCCCGGCCTTTT- 3'

Product size: 145 bp

PCR steps used were: Initial Denaturation: 94ºC for 5 minutes; Denaturation: 94ºC for 30seconds in cycles of 35; Annealing: 58ºC for 30 seconds in cycles of 35; Extension: 72ºC for 30 seconds in cycles of 35; Final extension: 72º C for 5 minutes. For visualization of PCR products, agarose gel electrophoresis (Agarose, 50X TAE buffer, 6X gel loading buffer, Ethidium bromide) with UV trans illuminator was used.

Results

Out of 1000 urine samples, 118 culture positives were seen. Out 118 culture positivity, 54 (45.76%) *Escherichia coli* isolates causing asymptomatic bacteriuria was identified.

Among the 54 isolates, phenotypic method for mannose resistance haemagglutination showed 9 positivity (16.66%). Genotypic confirmation of mannose resistant haemagglutination by Pap A gene identification confirmed the presence of gene in all nine isolates. (Fig. 1)

By antimicrobial susceptibility testing, all the *Escherichia coli* isolates were found to be 100% sensitive to Piperacillin/Tazobactum and Imipenem. Cefotaxime and Ceftazidime showed 92.6% sensitivity, Nitrofurantoin 88.9% and Amoxycillin showed 44.4%. (Table 1)

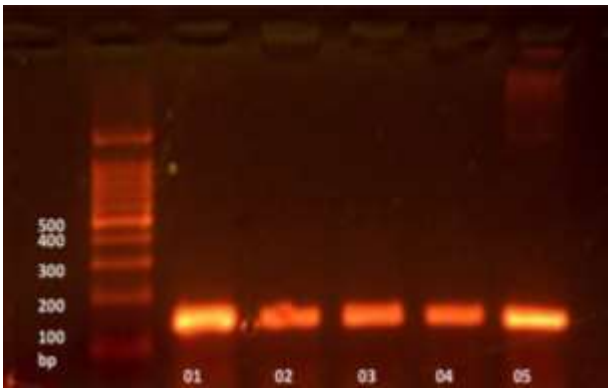

Fig. 1: Gel electrophoresis image showing positivity for Pap A gene

Table 1: Antimicrobial susceptibility pattern of *Escherichia coli* isolates (n=54)

Drug	No of Sensitive isolates	%	No of Resistant isolates	%
Amoxycillin	24	44.44	30	55.56
Amoxycillin/ Clavulanic acid	46	85.18	8	14.82
Amikacin	42	77.78	12	22.22
Cephalexin	32	59.26	22	40.74
Cefotaxime	50	92.6	4	7.4
Ceftazidime	50	92.6	4	7.4
Cotrimoxazole	39	72.22	15	27.78
Gentamicin	35	64.81	19	35.19
Imipenam	54	100	0	0
Nitrofurantoin	48	88.89	6	11.11
Norfloxacin	30	55.55	24	44.45
Ofloxacin	45	83.33	9	16.67
Piperacillin-Tazobactum	54	100	0	0

Table 2: Antimicrobial susceptibility pattern among MSHA and MRHA *Escherichia coli* isolates

Antimicrobials	MSHA (n=45)		MRHA (n=9)	
	No. of sensitive isolates	No. of resistant isolates	No. of sensitive isolates	No. of resistant isolates
Amoxycillin	20 (44.44%)	25 (55.55%)	4 (44.44%)	5 (55.55%)
Amoxycillin/ Clavulanic acid	39 (86.67%)	6 (13.33%)	7 (77.77%)	2 (22.22%)
Amikacin	36 (80%)	9 (20%)	6 (66.66%)	3 (33.33%)
Cephalexin	26 (57.78%)	19 (42.22%)	6 (66.66%)	3 (33.33%)
Cefotaxime	43 (95.56%)	2 (4.44%)	7 (77.77%)	2 (22.22%)
Ceftazidime	43 (95.56%)	2 (4.44%)	7 (77.77%)	2 (22.22%)
Cotrimoxazole	32 (95.56%)	13 (28.89%)	7 (77.77%)	2(22.22%)
Gentamicin	28 (62.22%)	17 (37.78%)	7 (77.77%)	2(22.22%)
Imipenam	45 (100%)	0	9 (100%)	0
Nitrofurantoin	39 (86.67%)	6 (13.33%)	9 (100%)	0
Norfloxacin	26 (57.78%)	19 (42.22%)	4 (44.44%)	5(55.55%)
Ofloxacin	37 (82.22%)	8 (17.78%)	8 (88.88%)	1(11.11%)
Piperacillin-Tazobactum	45 100%)	0	9 (100%)	0

Discussion

In our study, out of 118 culture positive urine samples, 45.76% were contributed by *Escherichia coli*. This is in accordance with many studies supporting that *E.coli* is the most common organism isolated in asymptomatic bacteriuria in pregnant females.[12–14] Among many virulence factors associated with urinary infections by *E.coli*, MRHA was analyzed in our study, as it is one of the most common characteristics suggested in few studies.[15,16] Mannose resistant haemagglutination (MRHA) was positive is 9/54 (16.66%) isolates in our study. Various studies have shown different distribution with a range of 15-40% MRHA (P Fimbriae) and it is mainly due to different sample sizes.[15–17] In our study population, we got a significant number of MRHA isolates, as pregnancy is a vulnerable group. Since there is a good proportion seen, it is important to screen to avoid complications like pyelonephritis. Especially, in case of asymptomatic bacteriuria if not properly screened for virulence factors, 30-50% of cases will end up in pyelonephritis.[18] Risk of decline in renal function should be caught up in mind and all pregnant females need to be screened for asymptomatic bacteriuria and if positive, isolates should be tested for expression of virulence factors. Expression of Pap A gene is an additional confirmatory evidence for the phenotypic detection of MRHA strains.[19] In our study all the phenotypically detected MRHA isolates were subjected to detection of Pap A gene expression and 100% positivity was obtained. This confirms the virulence and suggests that Pap A gene is responsible for adhesion mediated through P fimbriae.[20] The importance of P fimbriae is that its host influence which triggers the bacterial population in the urinary tract.[18]

Antimicrobial susceptibility testing of *Escherichia coli* isolates revealed 100% sensitivity to Piperacillin/Tazobactum and Imipenem. This is supported by few studies which also shows no resistance to these group of drugs.[21] Increased resistance was seen for Norfloxacin and Cephalexin, when compared to other drugs. This warrants that oral drugs needs to be started with caution and only after performing suitable susceptibility testing. Details regarding preliminary screening of urine samples have been published already.[22]

Conclusion

Virulence factors expressed by *Escherichia coli* has a direct link in causing complications like pyelonephritis. As asymptomatic bacteriuria is more common during pregnancy, it is a must to screen pregnant females in every trimester even without any specific symptoms. Due to difference in antimicrobial susceptibility patterns in due course, physicians should be informed about the changing trends and importance of antenatal screening even without symptoms.

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