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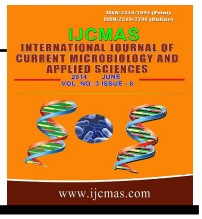

Original Research Article

Multidrug Resistance and Virulence Phenotypes Among Uropathogenic *Escherichia coli*

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A B S T R A C T

Escherichia coli, the most prevalent facultative gram negative bacillus in the human fecal flora, usually inhabits the colon as an innocuous commensal. Urinary tract infection (UTI) is the most common form of extraintestinal *E. coli* infection, and *E. coli* is the most common cause of UTI. Presence of virulence characters strengthens the uropathogenic *E. coli* and provides ways to escape from normal human defenses. Knowledge of the virulence features in addition to the antibiotic sensitivity profiles can greatly influence the treatment strategy adopted and enhance recovery. Most of UTI isolates analyzed showed Multi drug resistance (MAR) index between 0.3 to 0.9 indicating high prevalence of multiple antibiotic resistance. These isolates with resistance to at least 3 or more antibiotics were analysed for expression of virulence characters. All the 15 strains of *E.coli* isolated from suspected UTI were positive for at least 2 or more features among five virulence features tested out of which biofilm formation with cell surface hydrophobicity were highly prevalent indicating these characters play a crucial role in UTI infections.

Introduction

Urinary tract infections are the second most common type of infection in the body, accounting for about 8.1 million visits to health care providers each year (Schappert, 2008). Women are especially prone to UTIs for anatomical reasons with the life time risk of having a UTI is greater than 50 percent (Griebling, 2008).

UTIs in men are not as common as in women but can be serious when they occur. The main causal agent in upper and lower urinary tract infection is *E.coli*, followed by the other Gram negative enteric members viz, *Klebsiella sp.* and *Proteus sp.*

Escherichia coli is one of the common commensal in human intestinal tract. As a normal flora it reinforces the barrier function of the intestinal mucosa, helping it to prevent attachment of pathogenic

microorganisms. However, when *E.coli* enters into other sites, it can cause variety of infectious diseases such as urinary tract infections, wound infections, bacteraemia, meningitis and soft tissue infections. *E.coli* present in the gastrointestinal tract are considered to provide a pool for initiation of UTI (Mucheya Gizachw,2013).

The virulence of uropathogenic *E.coli* (UPEC) is enhanced by production of virulence factors including hemolysins, hemaglutinins, colicins, siderophores and cytotoxic necrotizing factor. Pathogenicity of UPEC also depends on their resistance to phagocytosis and bactericidal action of human serum as well. Ability to adhere to uroepithelial cells is mediated by fimbrial and nonfimbrial adhesions. Further, ability of these microbial strains to produce biofilms and resist the antibacterial agents enhances their pathogenicity (Stewart, 2001). The present study reports the drug resistance pattern and the associated virulence factors observed in *E.coli* isolated from UTI.

Materials and Methods

Clinical isolates

A total of 50 Gram negative isolates from suspected urinary tract infected patients attending Central Hospital, Mettuguda , Hyderabad, during July to September 2013 were included in the present study. All the Gram negative bacilli isolates were identified based on colony morphology on Blood agar, MacConkey's agar, Gram staining and by standard biochemical techniques (Cappunccino 2001,Collee 1989). Sensitivity was determined by disc diffusion technique.Results were interpreted according to CLSI guidelines (Wayne Pa , 2005).

Antibiotic susceptibility testing

Susceptibility of isolates to antibiotics were tested, against the following commonly used for UTI. The spectrum of antibiotics tested include β lactam group- Cefoperazone (CFP:75µg),cefotaxime (CF:30µg),Cefadroxil(CD:30µg), Ceftriaxone (CTX:30µg), Ceptazidime (CPZ:30µg); Aminoglycoside group like Amikacin (AMK:30µg), Netilmicin (NET:30µg), Gentamicin (GEN:10µg) and Sparfloxacin (SF:5µg), Ciprofloxacin (CIP:5µg), Lomefloxacin (LM:10µg) that belong to Quinolone group and a β-lactamase inhibitor- Sublactum (SLB:20µg) obtained from Hi-media, Mumbai. Antibiotic susceptibility testing was done on all isolates using the disk diffusion as per Bauer-Kirby method according to the criteria of clinical and laboratory standards institute (CLSI) (Wayne Pa, 2005).

Identification of MDR and determining MAR index

The drug resistance pattern of the isolates was identified by observing the resistance pattern of the isolates to the common antibiotics (Krumperman,1983).A multiple drug resistance (MDR) phenotype is taken as resistance to 3 or more antimicrobial agents. Multiple antibiotic resistance (MAR) index, referred to as the number of antibiotics to which test isolate displayed resistance divided by total number of antibiotic to which the test organism was calculated as it provides a good indicator to evaluate the heath risk of the environments for each test isolates (Krumperman , 1983)

Detection of virulence factors

The virulence features like hemolysin production, cell surface hydrophobicity,

hemagglutination capability, gelatinase, β lactamase production and biofilm production of the MDR isolates were determined.

Haemolysin production

Plate hemolysis test was carried out for detection of hemolysins produced by isolates using modified Blood agar medium with 5% of normal human blood (Collee,1989).

Cell surface hydrophobicity

The cell surface hydrophobicity of isolates was determined by using Salt Aggregation Test(SAT) (Savita, 2011) as briefly described. Ten microliter of isolate suspension made in phosphate buffer was mixed with equal amounts of ammonium sulphate solution of different molar concentrations (0.2, 0.4, 1, 1.4, 2M) on a glass slide. Visible clumping or aggregation of the organism was observed for one minute while rotating . UPEC strains that had SAT value less than or equal to 1.4M were considered hydrophobic. Strains showing aggregation in 0.002M phosphate buffer (pH 6.8) alone were taken as auto agglutination.

Gelatinase test: Gelatinase production was tested using gelatin agar (Collee,1989). The plate is inoculated with test organism and inoculated at 37^{o}C for 24h.The plate is then flooded with mercuric chloride solution . Development of opacity in the medium and zone of clearing around colonies were considered positive for gelatinase.

Detection of β lactamase production by isolates: Production of β lactamases was determined by iodometric method by Sng et al (1980) for all 15 isolates of *E.coli* . The mechanism of the test involves a preliminary positive starch test as evidenced by the filter paper strip becomes purple when iodine solution is added. The test area of the strip turns white if penicilloic acid is produced by the action of penicillinase which converts the iodine to iodide, which is then no longer available to form the purple starch-iodine complex.

Biofilm production: Congo red agar (CRA) medium was prepared with brain heart infusion broth , sucrose, agar and congo red indicator (Freeman *et. al*, 1989). CRA plates were inoculated with test organism and incubated at 37^{o}C for 24 hrs. Black colonies with dry consistency indicate strong biofilm formation. Brownish or reddish growth was considered as negative biofilm formation.

Results and Discussion

Out of 50 Gram negative isolates obtained in UTI infected samples, 15 isolates were identified as *E.coli* and these strains were studied for virulence features. All the *E.coli* strains were multidrug resistant being resistance to 3 or more antibiotics tested. The presence of multi drug resistance may be related to the dissemination of antibiotic resistance among hospital isolates of *E.coli*. As shown in Table 1 maximum resistance (93.33%) was observed with Lomefloxacin, followed by Cefoperazone (80%) and Cefadroxil (80 %). Higher sensitivity was observed with Netilimicin followed by Amikacin and Gentamicin where the resistance percent of isolates was between 6.66% to 20.00%.

As indicated in Fig 1 highest MAR index was 0.91 seen in one isolate, whereas 4 isolates showed a Mar index of 0.75 and majority of the isolates have MAR above

0.2 A MAR index greater that 0.2 implies that the strain of such bacteria originate from an environment where several antibiotics have been used (Ehinmidu,2008). The present results suggest that a very large proportion of the *E.coli* isolates have been exposed to antibiotics resulting in a alarming trend of rise of multiple antibiotic resistance and accumulation of MAR. It also reiterates the role of several external factors, like exposure to antibiotics, overdosing and indiscriminate usage of antibiotics in the hospital and environment which is playing a crucial role in increased MAR index and leading to the spread in both commensal and pathogens.

The studies of expression of virulence phenotypes among the UTI isolates and comparison of antibiotic profile and virulence phenotypes expressed among *E.coli* isolates with MAR index above 0.6 as shown in Table 2 and 3. The results showed that a large proportion of the isolates (73.30 %) had the capacity to form biofilms which is an important factor determining virulence. Bacterial cells in the biofilm often display a variety of phenotypic differences from those in the planktonic culture. These include some phenotypic changes such as in motility, production of extracellular polysaccharide and increased resistance to antibiotic and host defense system. The treatment of bacterial infections becomes hard as 20% of the bacterial genes are expressed differently among biofilm producers leading to better protection against antibiotics compared to free living cells (Stewart et al 2001; Whiteley et al 2001).

Biofilm formation in *E.coli* isolates is an important reason as to why the infections cannot be effectively treated and cured. *E.coli* infections are usually associated with the surface of either human tissue or indwelling devices such as catheters, used in urinary tracts and hence regarded as biofilm associated bacterial diseases. Although hemolysin is the main virulence factor by which *E. coli* causes acute prostatitis, the association between hemolysin and biofilm formation can result in increased ability of *E. coli* strains to persist in the prostate also. Organisms like *Citrobacter sp.* are also known to produce virulence factors known to associate with CAUTIs (catheter associated UTI) like fimbria, flagella, capsules, biofilms, siderophores, bacteriocins and LPS(Mark Shirtliff, 2009). Bacteria are lysed due to activity of complement system.

The alternate pathway of complement activation is potentially important than classical pathway in lysis. Bacterial resistance to killing by serum results due to individual to combined effects of capsular polysaccharides, lipopolysaccharides and surface proteins. In the present study 33.50% isolates were observed to be mucoid. The hydrophobic nature of isolate also aids the bacteria to adhere to various surfaces for colonization (Sharma *et al,* 2005). Four out of 15 isolates, *i.e.*26.6% of the isolates from urine were observed to be hydrophobic. This is consistent with the several earlier studies were more than 25-30% hydrophobicity was observed which promoted adherence of bacteria to uroepithelial cells (Sharma et al, 2007; Raksha *et al,*2003).

The most important secreted virulence factor of uropathogenic E. coli is a lipoprotein called α-haemolysin (HlyA), which is associated with upper UTIs such as pyelonephritis.

Table.1 Antibiotic resistance pattern observed among *E.coli*

Antibiotics	Concentration (mcg)	No. of resistant isolates	Percentage observed
Lomefloxacin	10	14	93.33
Sparfloxacin	5	11	73.33
Ciprofloxacin	5	10	66.66
Netilmicin	30	1	6.66
Gentamicin	10	3	20.00
Amikacin	75	2	13.33
Sulbactum	20	10	66.66
Cefoperazone	75	12	80.00
Ceptazidime	30	12	80.00
Cefotaxime	30	10	66.66
Cefadroxil	30	12	80.00
Ceftriazone	30	11	73.33

Figure.1 MAR index among the *E.coli* isolates

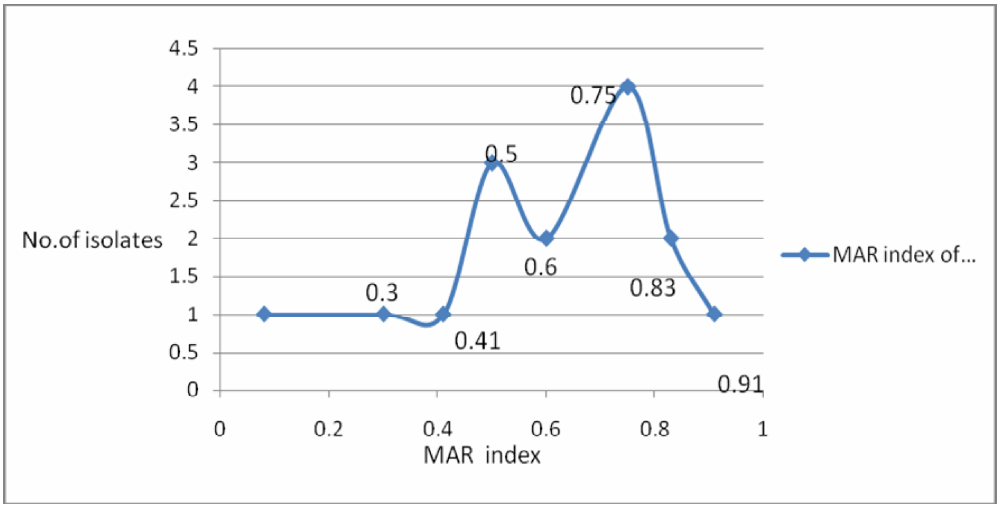

Table.2 Virulence phenotypes tested among *E.coli* UTI isolates

Virulence character	No. of isolates positive	(%) of Positive isolates
Mucoid nature	5	33.30
Hydrophobicity	4	26.66
Hemolysin production	4	26.66
Biofilm production	11	73.30
Penicillinase producers	5	33.30

Table.3 Antibiotic profile and Virulence phenotypes expressed among *E.coli* isolates with MAR index above 0.6

Isolate	Resistance to number of antibiotics tested	Antibacterial resistance phenotype	Virulence features expressed
Isolate 1	11	CFP,CF,CD,CTX,CPZ,AMK, NET, SF, CIP,LM,SLB	Biofilm+; Hemolysin+ ; Hydrophobic + ; Penicillinase producers +
Isolate 2	10	CFP,CF,CD,CTX,CPZ,GEN , SF, CIP,LM,SLB	Biofilm+; Hemolysin+ ; Hydrophobic + ; Penicillinase producers +
Isolate 3	10	CFP,CF,CD,CTX,CPZ,GEN , SF, CIP,LM,SLB	Biofilm+ ; Hemolysin+; Hydrophobic +; Penicillinase producers +
Isolate 4	09	CFP,CF,CD,CTX,CPZ, SF, CIP,LM,SLB	Biofilm+ Hemolysin- ; Hydrophobic + ; Penicillinase producers +
Isolate5	09	CFP,CF,CD,CTX,CPZ, SF, CIP,LM,SLB	Biofilm+; Hemolysin-; Hydrophobic +; Penicillinase producers +
Isolate 6	09	CFP,CF,CD,CTX,CPZ, SF, CIP,LM,SLB	Biofilm+; Hemolysin- ; Hydrophobic +; Penicillinase producers +
Isolate7	09	CFP,CF,CD,CTX,CPZ, SF, CIP,LM,SLB	Biofilm+; Hemolysin- ; Hydrophobic +; Penicillinase producers +
Isolate8	08	CFP,CF,CD,CTX,CPZ, CIP,LM,SLB	Biofilm+ ; Hemolysin- ; Hydrophobic -; Penicillinase producers -
Isolate9	08	CFP,CF,CD,CTX,CPZ, CIP,LM,SLB	Biofilm+; Hemolysin-; Hydrophobic -; Penicillinase producers -

Hemolysin production is associated with pathogenicity of *E.coli*, especially in the more severe forms of infection. At high concentrations, HlyA is able to lyse erythrocytes and nucleated host cells, a process that may enable extraintestinal pathogens like UPEC to better cross mucosal barriers, damage effect or immune cells, and gain enhanced access to host nutrients and iron stores (Justyna Bien,2012; Johnson,1991). In the present study 4 isolates (26.66%) of *E.coli* showed

presence of alpha hemolysis around the colonies on Blood agar plates. Hemolysin is known to confer selective advantage to the pathogen by releasing iron from lysed erythrocytes and enhances pathogenicity by destroying phagocytic cells and epithelial cells. Hemolysin production has also been shown previously to influence pathogenicity (Naveen *et al*,2005;Raksha *et al*,2003 and Jhonson *et al*,2002). Hemolysins has been proposed to inflict direct cytotoxic effects on renal epithelium.

Alpha-hemolysin producing organisms are observed more lethal with dermonecrotic and toxic effects on series of host tissues and cells including RBCs, leucocytes, epithelial and endothelial cells. The frequency of isolation of hemolytic *E.coli* significantly associates with the severity of infection. Similar percentage of strains were observed to produce penicillinase with 10 isolates resistant to sulbactam. The mechanism for resistance to Sulbactam is basically due to TEM-1 β-lactamase due to either an alteration in the control of gene expression or simply to an increase in the number of copies of the β-lactamase gene in the plasmids.

Table 3 gives a comparative analysis of Antibiotic profile and Virulence phenotypes expressed among *E.coli* isolates with MAR index above 0.6. All the organisms with MAR index above 0.6 were positive for 2 or more virulence factors and those isolates with MAR index of 10 and above (resistant to 10 and more antibiotics) were positive for all virulent factors testes for which is alarming. The treatment of *E.coli* infections is increasingly becoming difficult because of combination of multidrug resistance and multiple virulence factors exhibited by the organism.

The knowledge of drug resistance pattern in a particular geographical area will very help in control and spread of these infections. Hence the knowledge of trends in virulence features such as hemolysin production, cell surface hydrophobicity, hemagglutination capability and biofilm production are pertinent in evaluating the pathogenicity of isolate. This knowledge, in addition to the antibiotic sensitivity profiles can greatly enhance the treatment strategy adopted for UTI causing organisms and could guide the choice for optimal antibiotic therapy for successful treatment thus improving the outcomes for patients with severe UPEC infections.

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