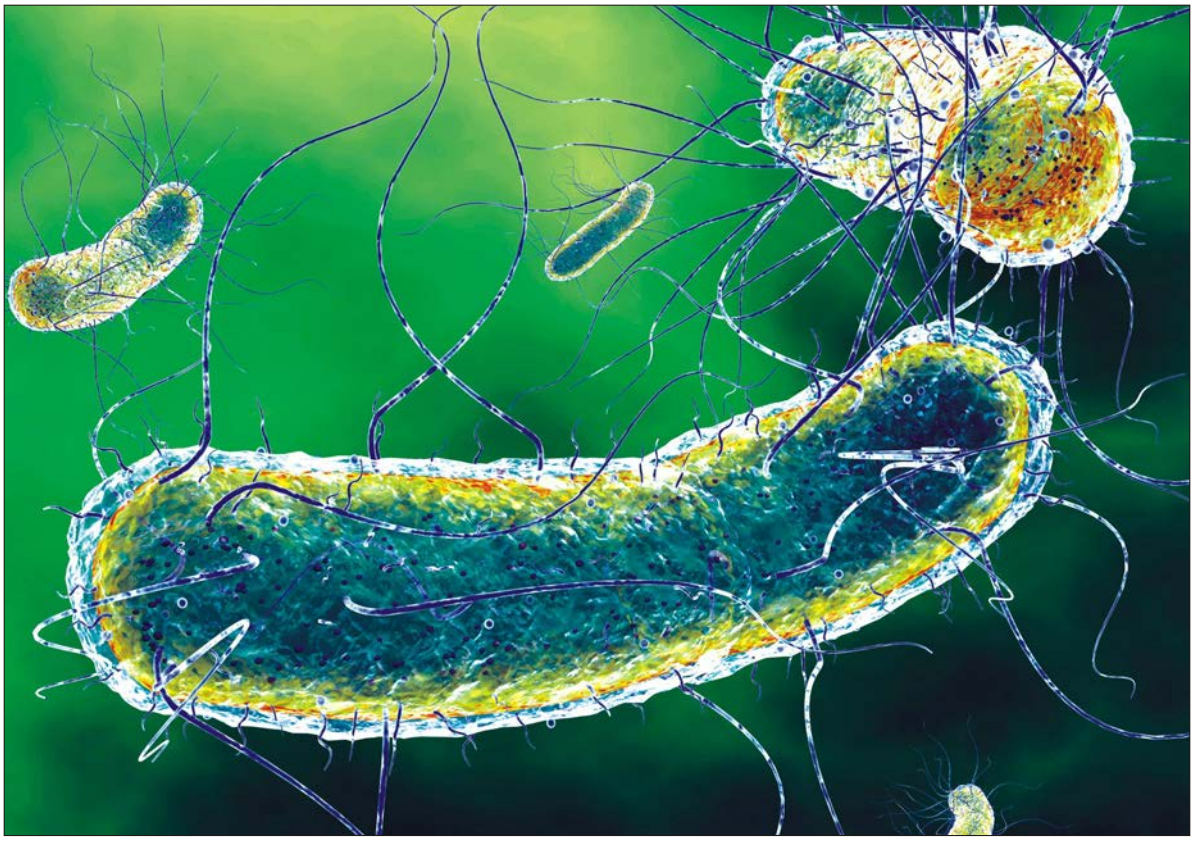

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Antimicrobial Susceptibility Pattern of Biofilm Producing *Escherichia coli* of Urinary Tract Infections

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ABSTRACT

Urinary tract infections is a serious health threat with respect to antibiotic resistance and biofilms formation being the prime cause for the antibiotic resistance, the present study aimed at performing *in vitro* detection of biofilm formation in *E. coli* strains isolated from urine cultures and to determine their susceptibility pattern using 14 commonly used antibiotics. The present study comprised of 210 urine samples collected from tertiary care hospitals in Palakkad, South India. All the samples were subjected to gram staining, bacterial culture and the *E. coli* isolates were screened for biofilm formation using Tube Method (TM) and Congo Red Agar (CRA) method. Subsequently, the antibiotic sensitivity test was performed by Kirby Bauer-disk diffusion method for the biofilm producing *E. coli* isolates. *E. coli* ATCC 25922 was used as the control strain. Of the 96 (71.0%) *E. coli* isolates, 81 (84.37%) displayed a biofilm positive phenotype under the optimized conditions in the Tube Method and the strains were further classified as strong positive 9 (9.4%), positive 33 (34.4%), weakly positive 39 (40.6%) while in 15 (15.6%) isolates, no biofilm was detected. Screening on CRA does not correlate well with the tube method for detecting biofilm formation in *E. coli*. The rates of antibiotic resistance were 90.6% for erythromycin, 71.9% for amikacin, 65.6% for cotrimoxazole, 59.3% for ampicillin, 56.3% for meropenem and chloramphenicol, 53.1 and 50.0% for tobromycin and gentamicin respectively. Biofilm production in *E. coli* may promote the colonization and lead to increased rate of UTI's and such infections may be difficult to tres as they exhibit multi drug resistance.

Key words: Urinary tract infections, *E. coli,* biofilm, multi drug resistant, antibiotic sensitivity test

INTRODUCTION

Urinary Tract Infections (UTI's) pose a serious health threat with respect to antibiotic resistance and high recurrence rates. Generally there is an agreement among the authors in the literature that the predominant uropathogens acquired from any source are gram negative bacteria with *Escherichia coli* accounting for the highest prevalence in most instances (Moges *et al*., 2002). Community and hospital acquired UTI's are among the frequently encountered infectious diseases (Sobel and Kaye, 2000). Chronic Kidney Disease which is the cause of morbidity and mortality worldwide is also highly prevalent among children, where the main cause is identified due to the poor healthcare system (Mortazavi and Rafiee, 2010). Uropathogenic *E. coli* form intracellular bacterial communities with biofilm like properties within the bladder epithelium (Anderson *et al*., 2004). A biofilm is a population of cells growing on a surface and enclosed within an exopolymer

matrix that can restrict the diffusion of substances and bind antimicrobials. This will provide effective resistance for biofilm cells against large molecules such as antimicrobial proteins lysozyme and complement (Ishida *et al*., 1998). According to a recent public announcement from National Institutes of Health, "more than 60% of all microbial infections are caused by biofilms" (Lewis, 2001). The armament of therapeutic agents available to treat bacterial infections today is restricted to antibiotics developed specifically to kill or stop the growth of individual bacteria (Sritharan and Sritharan, 2004). Antibiotic resistance is the most problematic and costly characteristics of biofilm. Biofilm formation occurs when microorganism attach to a surface and through growth and continuing colonization, spread over the surface (McLean *et al*., 1999). Biofilms can vary in thickness to from a mono cell layer of 6 to 8 cm thick but mostly on an average of about 100 μm thickness (Kumar and Prasad, 2006). Antibiotic resistance of urinary tract pathogens has been known to increase worldwide, especially against commonly used antimicrobials (Kahlmeter, 2003). The antibiotic sensitivity patterns of either one or more of the organisms have been determined to one or more of the commonly used antimicrobial drugs in UTI cases (Gordon *et al*., 2003). Bacteria embedded within biofilms present a challenge to surface decontamination by conventional means (Salamitou *et al*., 2009). In modern clinical microbiology, establishment of bacterial biofilms is considered a pathogenicity trait during chronic infections (Sritharan and Sritharan, 2004). The difficulty in eradicating a chronic infection associated with biofilm formation lies in the fact that biofilm bacteria are able to resist higher antibiotic concentration than bacteria in suspension (Gristina *et al*., 1987). The intracellular biofilm like properties allows bacteria to outlast a strong immune response to establish a dormant reservoir of pathogens inside the bladder cells (Anderson *et al*., 2003). In this context the study was aimed to perform *in vitro* detection of biofilm formation of *E. coli* strains isolated from urine cultures and to determine their susceptibility pattern to 14 commonly used antibiotics.

MATERIALS AND METHODS

Bacterial strains: A total of 210 urine samples were randomly obtained from patients attending various tertiary care hospitals in Palakkad, South India during July 2007-April 2008 were included in the prospective study. Mid stream urine samples collected in a sterile container were inoculated onto 5% Sheep Blood Agar and Eosin Methylene Blue agar by using conventional microbiological procedures.

Detection of biofilm formation

Tube Method (TM): All the 96 *E. coli* isolates were subjected to biofilm production. Number of tests is available to detect biofilm produced by *E. coli;* methods include Tube Method (TM) and Congo Red Agar (CRA) is employed in the present study. A qualitative assessment of biofilm formation was determined as previously described by Christensen *et al*. (1982).

Biofilm formation was considered as positive, when a visible film lined the wall and bottom of the tube (Fig. 1). Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as (-) -negative, (+) -weakly positive, (++) -moderate positive and (+++) -strongly positive.

Congo Red Agar method (CRA): A method of screening biofilm formation described by (Freeman *et al*., 1989) was performed. Positive result was indicated by black colonies with a dry crystalline consistency. Weak biofilm producers usually remained pink, though occasional

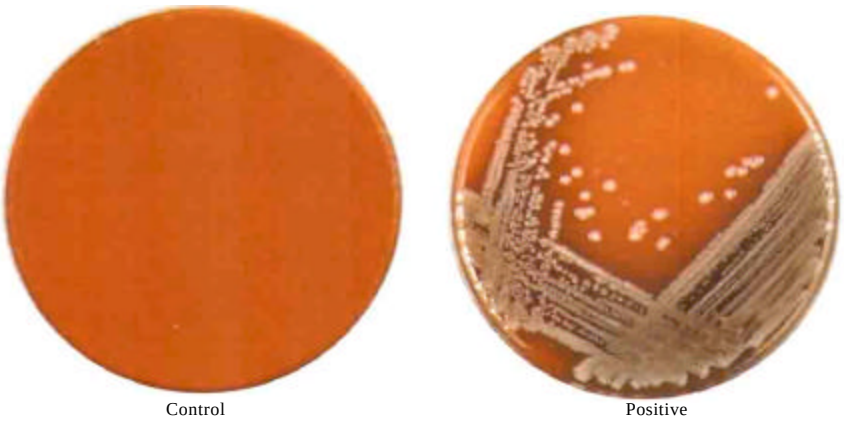

Fig. 1: Biofilm production of *E. coli* by Congo red agar method

darkening at the centers of colonies was observed. A darkening of colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result. The experiments were performed in duplicates and repeated two times.

Antibiotic sensitivity testing-kirby bauer's-disk diffusion method: The interest in antibiotic susceptibility tests for biofilm bacteria has increased in the last few years (Domingue *et al*., 1994). Antibiotic susceptibility to 81 biofilm producing *E. coli* strains to ampicillin (25 μg), imipenem (10 μg), gentamicin (30 μg), amikacin (10 μg), ciprofloxacin (10 μg), tobramycin (30 μg), erythromycin (10 μg), co-trimoxazole (25 μg), tetracycline (30 μg), norfloxacin (10 μg), amoxicillin (10 μg), chloramphenicol (30 μg), meropenem (10 μg) and cephalexin (30 μg) was determined by Kirby Bauer-disk diffusion method with antibiotic containing discs on Mueller-Hinton Agar plate. The results were expressed as susceptible or resistant according to criteria recommended by the Clinical Laboratory Standards Institute (CLSI, 2005).

RESULTS

Of the 210 urine specimens of urinary tract infection processed, 135 (64.3%) specimens showed culture positive and the rest 75 (35.7%) were negative. Among the isolates, the aerobic gram-negative *E. coli* was 96 (71%) and the rest 39 (29%).

Detection of biofilm: Of the 96 (71.0%) *E. coli* isolates, 81 (84.37%) displayed a biofilm positive phenotype under the optimized conditions in the tube method and the strains were further classified as strong positive 9 (9.4%), positive 33 (34.4%), weakly positive 39 (40.6%) while in 15 (15.6%) isolates, no biofilm was detected. However, it was difficult to discriminate between moderate and weakly biofilm producing isolates. The results of Tube Method are shown in Table 1. By CRA method, we obtained very different results, around 33 (34.4%) strains displayed black colonies with a dry crystalline consistency, 39 (40.6%) isolates displayed pink colonies occasionally darkening at the centers on typical biofilm *E. coli* isolates (Fig. 1). With the exception of these 24 (25.0%) isolates showing black colonies without dry crystalline were observed (Table 2).

Table 1: Biofilm producing *E. coli*-Tube method

S. No	Observation	n (%) of results
1	+++	9 (9.4)
2	++	33 (34.4)
3	+	39 (40.6)
4	-	15 (15.6)

+++: Strong positive, ++: Positive, +: Weakly positive, -: Negative

Table 2: Biofilm production by *E. coli* using Cong Red Agar medium

	Growth on Congo red agar		
Organism	++	+	-
E. coli	33 (34.4%)	24(25.0%)	39 (40.6%)

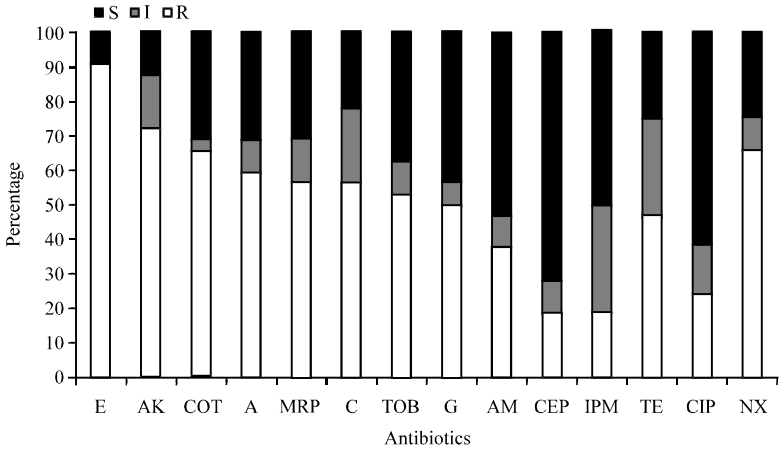

Fig. 2: Antimicrobial susceptibility pattern of biofilm producing *E. coli*

Antibiotic sensitivity testing-Kirby Bauer's-disk diffusion method: All the confirmed biofilm producing *E. coli* strains were subjected to antibiotic susceptibility test. The resistant pattern of erythromycin, amikacin, co-trimoxazole, ampicillin, meropenem, chloramphenicol, tobramycin and gentamicin were found to be in the order of 90.6, 71.9, 65.6, 59.3, 56.3, 56.3, 53.1 and 50.0% respectively (Fig. 2). Resistance was least with amoxicillin (37.5%), cephalexin (18.8%) and imipenem (25%). Intermediate resistance was observed against tetracycline (46.8%). The susceptibility pattern of cephalexin, imipenem, amoxicillin and ciprofloxacin were found to be in the order of 71.8, 65.6, 53.2 and 40.6%. Quinolones are being increasingly used in the therapy of urinary tract infections due to widespread bacterial resistance to commonly used antimicrobials.

Multiple drug resistance patterns of biofilms producing *E. coli*: Out of the total 81 biofilm producing *E. coli* isolated, there was a significant correlation between production and resistance to multiple antibiotics such as ampicillin, cotrimoxazole, norfloxacin, tobramycin, imipenem and meropenem Table 4. These strains were found to show increased biofilm production. Bacterial biofilms are often associated with long-term persistence of organisms in various environments. Bacteria in biofilms display dramatically increased resistance to antibiotics. Biofilms production in

Table 3: Multiple drug resistance pattern of Biofilm producing *E. coli*

Multidrug combination	Resistance (%)
A, Nx, Tb	16 (59.25)
A, Co, Nx	14 (51.85)
A, Co, Nx, Tb	8 (29.62)
A, Co, I, Mr	8 (29.62)

A: Ampicillin, Co: Co-trimoxazole, Nx-Norfloxacin, Tb: Tobramycin, I: Imipenem, Mr: Meropenem

Table 4: Correlation of Biofilm producing Strains and their antibiotic resistance pattern

No. of strains	Resistance (%)
3^{+++}	15 (89)
11^{++}	13 (77)
13^{+}	10 (59)

+++: Strongly positive, ++: Moderate positive, +: Weakly positive

E. coli may promote colonization and lead to increased rate of urinary tract infections. Such infections may be difficult to treat as they exhibit multi- drug resistance. Results are shown in Table 3.

DISCUSSION

The prevalence of uropathogenic organisms has been remarkably consistent with Gram-negative organisms accounting for most infections (Bukharie and Saeed, 2003). This trend is in agreement with previous studies (Moges *et al*., 2002; Bukharie and Saeed, 2003). According to a study conducted in tamil nadu it was found that among the isolated organisms, *E. coli* (31.5%) was the predominant uropathogen (Manikandan *et al*., 2011). In South Jordon, the prevalent uropathogen was found out to be *E. coli* (53.24%) isolated from 119 patients infected with UTI (Khleifat *et al*., 2006) prevalence rate of *E. coli* was reported to be 21.5% in Nigeria (Umeh *et al*., 2007).

Both the methods described here are based on the enhancement of exopolysaccharide production by using enriched media, TSB in the Christensen method (Christensen *et al*., 1982) while the Congo red agar method also requires the use of a highly nutritious medium-in this case, brain heart infusion broth with 5% sucrose supplementation. Added sucrose has been used for the detection of glucan production by streptococcal isolates (Vera and Power, 1980). Congo red stain was chosen because; it has been used as a stain for showing the presence of the exopolysaccharide of aquatic gram negative bacilli by light microscopical examination (Freeman *et al*., 1989).

In Gram negative organisms, alterations in outer membrane porin proteins due to mutations would lead to decrease in permeability through the outer membrane, so that less drug reaches the target site (Catherine and Gary, 2002). This may be responsible for the higher rate of resistance for the Gram negative organism. Despite the high degree of susceptibility of the bacteria tested to quinolones there should be concern about the low level of resistance. Some workers used molecular methods to study the mechanism of resistance in fluoroquinolones reported that their use might become limited by the emergence of resistance (Chen *et al*., 2001). Fluoroquinolones are indeed very effective in stopping the growth of a biofilm. At the same time, restricted diffusion can protect the biofilm from a degradable antimicrobial activity. Retarded diffusion will decrease the concentration of antibiotic entering the biofilm helping an enzyme like β-lactamse destroy the incoming antibiotic (Giwercmann *et al*., 1991). Biofilm resistance to killing has generally been

assumed to be a feature shared by the bulk of biofilm cells or at least to be present in a sizeable part of the population, such as cells in the deeper layers of a thick biofilm which have less access to nutrients and which will grow more slowly (Costerton *et al*., 1999). Resistance to aminoglycosides which have trouble penetrating the biofilm, is indeed a shared feature of the bulk of biofilm cells (Brooun *et al*., 1999). The penetration of aminoglycosides is strongly restricted by the exopolymer matrix. In *E. coli* the increasing concentrations of ciprofloxacin or imipenem caused an initial decrease in the number of viable cells of a biofilm by 2 to 3 orders of magnitude while the remaining small population was essentially insensitive to a further increase in the drug concentration (Ashby *et al*., 1994). The low level of resistance may become high due to selective pressure of exposure with constant use as a result of drug abuse and arbitrary prescription of these agents.

In the present study about 55.5% of the isolates showed resistance against 8 drugs. Biofilm facilitates the adherence of these microorganisms to biomedical surfaces and protect them from host immune system and antimicrobial therapy (Dunne, 2002). It was found that the resistance pattern varies depending upon the strains, when analysed among the strains exhibiting resistance to various commonly used antibiotics with the strains producing strong positive, weakly positive, moderate positive and negative.

CONCLUSION

The implication of these findings is that antimicrobials that have wide Gram-negative coverage is particularly effective against *E. coli* may be used in the empiric therapy of urinary infections. A greater understanding of the nature of biofilm producing *E. coli* in chronic recurrent urinary tract infections will help in the development of new and more effective treatment for these problematic diseases.

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