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PREVALENCE AND CHARACTERIZATION OF VIRULENCE GENES OF *ESCHERICHIA COLI* IN DIABETIC AND NON-DIABETIC URINARY TRACT INFECTED PATIENTS USING MULTIPLEX PCR TECHNIQUE

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ABSTRACT
The present study was designed to characterize virulence genes of *Escherichia coli* in diabetic and non-diabetic urinary tract infected (UTI) patients. About 299 urine samples were collected from diabetic and non-diabetic patients from Gopi Hospital, Salem. Among them, 30 isolates were found positive and 10 isolates were subjected to Multiplex PCR technique for the detection of fim H (type 1 fimbriae) gene, pap E/F (minor structural subunits of P fimbriae) gene and cnf-1 (cytotoxic necrotizing factor type-1) gene. The study concludes the presence of *E.coli* in UTI patients and shows antimicrobial susceptible pattern of isolated *E.coli*. The identification of fim H and pap E/F genes and also attempts to generate a vaccine against uropathogenic *E. coli*.

INTRODUCTION

Diabetes mellitus is recognized as an epidemic in the Asian sub-continent affecting nearly 25 millions in India alone. Diabetes is a chronic (life time) disease marked by high levels of sugar in the blood. Diabetes mellitus is a major health problem in India. Metabolic abnormalities are due to deficiency of insulin (Kuzuya, 1997).

Diabetes type 2, also known as non insulin-dependent diabetes (NIDDM), accounts for 90-95% diabetic cases are one of the two major types of diabetes in which the beta cells of the pancreas produce insulin but the body is unable to use it effectively because the cells of the body are resistant to the action of insulin (Harding *et al*., 2003).

E. coli was the most frequent isolate from urinary tract infection in diabetic patients and had poor antibiotic sensitivity to conventional antibiotics. Metabolic abnormalities are due to deficiency of insulin: 1. Hypertension and high urinary albumin excretion show significant association with type 2 diabetes. 2. Bacteriuria is more common in diabetic than in non diabetic women because of a combination of host and local risk factors 3. Resistant bacteria are more frequently isolated from diabetic outpatients with urinary infections (Rakhshanda Baqai *et al*., 2008).

Extended Spectrum Beta Lactamases (ESBL) detection systems appear to be acceptable for clinical use and each was more sensitive and convenient than the alternative disk approximation method. The use of these tests may contribute to a wider recognition and more careful monitoring of this emerging resistant problem among some Enterobacteriaceae (*E.coli* and *Klebsiella* spp.). Furthermore, it is likely that these tests will also prove to be useful for selecting strains for more detailed molecular analysis (Martin *et al*., 1999).

These findings provide novel insights into the Virulence Factors (VFs) of extraintestinal pathogenic *E. coli* and demonstrate the new PCR assay's utility for molecular epidemiological studies. However, the eradication of UTI seemed to be more difficult in diabetics than in non-diabetics (Bonadio *et al*., 1999).

FimH, the adhesion portion of type I fimbriae produced by most Enterobacteriaceae including uropathogenic *E. coli*, is a conserved protein involved in bacterial attempts to generate a vaccine against pathogenic Gram-negative bacteria (Connell *et al*., 1996).

In this background the study was designed to assess the prevalence, antibiotic susceptibility pattern and also to detect the distribution of papE/F, fimH, and cnf-1 virulence genes compared with diabetic and non-diabetic urinary tract infected patients in *E. coli* isolates.

MATERIALS AND METHODS

Sample collection

Two hundred urine samples from diabetic and non-diabetic

patients in the age group 10-80years were selected from Gopi Hospital, Salem.

Culture methods

For the isolation of aerobic bacteria, the media used were 5% sheep blood agar, nutrient agar and Hi-chrome UTI agar, which were incubated at 37ºC for 24h. The organisms isolated were identified using standard methods. All the isolates were subjected to antibiotic sensitivity testing on Muller Hinton agar using Kirby-Bauer disk diffusion method.

ESBL Detection

Testing for ESBL production was carried out using Muller Hinton agar plates that were inoculated with standardized inoculum of the isolates compared to 0.5Mcfarland standards to form a lawn culture. Separate commercial discs containing cefotaxime (30μg) with and ceftazidime (30μg) with and without clavulanic acid (10μg) were placed over the lawn culture. An increase in zone size of more than or equal to 5mm for cefotaxime and ceftazidime with and without clavulanic acid was considered to indicate ESBL producing strain as described by Carter *et al*. (2000).

Multiplex PCR for identification of virulence factors

1.5mL of overnight broth culture of *E. coli* was transferred into 2mL micro centrifuge tubes. The tubes were centrifuged at 8000 rpm for 5 minutes. After centrifugation, the supernatant was discarded and the pellet was collected. The Pellet was suspended in 200μL of 1X TE buffer + 100μL of 10% SDS and mixed by vortexing. The tubes were kept in water bath at 60ºC for 20 minutes. Then added with 300μL of Phenol: Chloroform: Isoamyl alcohol mixture (24:25:1) to extract the DNA and mixed completely by vortexing. The tubes were then centrifuged at 10000 rpm for 10 minutes to separate the phases. The aqueous phase containing the DNA was carefully removed and transferred to new tubes. Equal volume of 100% Isopropanol was added to the tubes containing the aqueous phase. It was mixed by inverting the tubes 3 to 4 times. The tubes were then centrifuged at 10000 rpm for 10 minutes to pellet the DNA. The supernatant was discarded and the pellet was collected. To the pellet, 200μL of 70% ethanol was added and centrifuged at 10000rpm for 10 minutes. Then Ethanol was decanted completely and the pellet was air-dried to give purified DNA. Re-suspended the dried DNA pellet in 20μL of TE buffer and dissolved by tapping. DNA solutions were stored at 4ºC for further work.

All the available partial and full-length gene sequences of resistance gene were determined according to protocol with some modification. The Primer was obtained from Sigma, India and used in the PCR comprised Primer CNF-1 F 5′ GAA CTT ATT AAG GAT AGT 3′, and CNF-1 R 5′ CAT TAT TTA TAA CGC TG 3′, PAP E/F F 5′ GCA ACA GCA ACG CTG GTT GCA TCA T 3′ and PAP E/F R 5′ AGA GAG AGC CAC TCT TAT ACG GAC A 3′, FIM H F 5′ TGC AGA ACG GAT AAG CCG TGG 3′ and FIM H R 5′ GCA GTC ACC TGC CCT CCG GTA 3′. The PCR mixture was prepared in thin walled PCR tube in a sterile laminar flow hood. There agent was added as follows. Each PCR reaction mixture (20μL) contained 2μL of template DNA (plasmid DNA), 2μL of 10 X PCR buffer, 0.5μL of (0.5μM) each of the primers, 1μL of 0.2 mM of each deoxynucleotide triphosphate (dNTP'S) and 1μL of Taq DNA polymerase (Con. 5U/μL) and 8.0μL of molecular grade water.

A brief spin was given to settle down the materials than tubes were kept in Theromocycler (Genei). After initial denaturation at 94ºC for 2min, the samples were subjected to 30 cycles of denaturation at 94ºC for 1min, annealing at 62.5ºC for 1min and extension at 72ºC for 1min. A final extension was performed at 72ºC for 5min. Following PCR, aliquots (20μL) of the reaction mixtures were analyzed by Electrophoresis on a 1.5% Agarose gel, containing ethidium bromide (0.2mg/mL), in the presence of an appropriate DNA molecular weight marker. The amplification bands were observed under UV Transilluminater.

Confirmation of DNA by agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal submarine Electrophoresis unit. 30mL of 1 % Agarose gel was prepared with 1X TBE buffer (do not mix) and heated the content to get up to clear solution for casting Agarose gel. After cooling the solution, 7μL of staining dye solution was added into the casting system. The gel was allowed to solidify and then carefully disassembled from the casting system without disturbing the wells and placed in 1X TBE buffer filled electrophoresis tank (the buffer level should be above gel). 5μL of genomic sample DNA mixed with 2μL of gel loading dye and then loaded to gel and simultaneously loaded 3μL of DNA marker provided in the nearby well. The power card terminals was connected at respective positions, run the gel at 50 V, till the gel loading dye migrate more than half the length of gel. The power was switched off and visualized the isolated DNA under UV Transilluminator.

RESULTS AND DISCUSSION

E. coli are most frequent isolated Gram-negative pathogen and increasingly implicated as a cause of diabetic urinary tract infections in world wide. The main reason is increase of Multi drug resistance strains at large hospitals has started to pose great difficulty in selecting antimicrobial agent for management of infection they cause.

From the present study, when screened 200 samples from urine specimen for the presence of *E. coli*. 30 samples were found to be positive (Table 1).

Urinary tract infections are the second most common type of infection in males and females. Urinary tract infections account for approximately 8.3 million doctor visits each year, with approximately 20% of all urinary tract infections occurring in

Table 1: Percentage of *E. coli* from collected sample

Sample	Age	No. of isolates	Total no. of samples	Organism isolated	% of *E. coli*
Urine	10-40 41-60 61-80	6 15 9	200	30	15%

Table 2: Percentage of *Escherichia coli* among males and females

Sample	No. of *E. coli* isolated	Male patients				Female patients			
		Diabetic		Non-diabetic		Diabetic		Non-diabetic	
		No.	%	No.	%	No.	%	No.	%
Urine	30	9	30%	6	20%	6	20%	9	30%

Table 3: Colony morphology of *Escherichia coli*

Name of the organism	Sheep blood agar	Hi-Chrome UTI agar	Nutrient agar
Escherichia coli	Non-Hemolytic small colonies	Pink colour colonies due to lactose fermentation	Small colonies 1-2mm in diameter

Table 4: Biochemical tests of *Escherichia coli*

Name of the organism	Oxidase test	Nitrate reduction	Oxidizes glucose	Oxidizes lactose	Oxidizes mannitol
Escherichia coli	No intense deep blue colour appearing within 5 sec indicates negative result	Positive	Positive	Positive	Positive

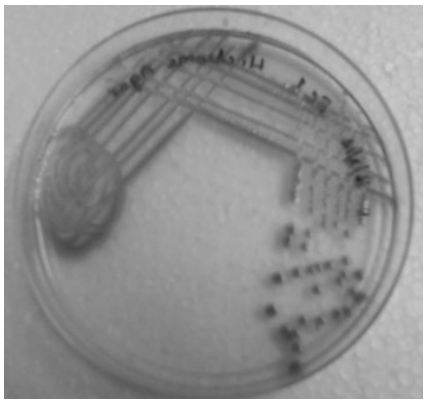

Plate 1: Growth of *E.coli* on hichrome agar

men. The overall lifetime prevalence of urinary tract infections in men between 1988 to 1994 was estimated to be 13,689 cases per 100,000 adult men, based on the National Health and Nutrition Examination Survey (Lan Mo *et al*., 2009).

Present study correlates that the urinary tract infection in diabetic and non-diabetic patients to compare the virulence factors. When screened, frequency of *E. coli* was found to be increased among the male patients showing 30% positively in diabetic patients and 20% in non-diabetic patients and females shows in diabetic is 20% and non-diabetic 30% (Table 2).

E. coli is the most common causative agent of UTIs in both DM and non-DM patients (Bonadio *et al*., 1999). The isolated *E. coli* were subjected for the microscopical examination, its colony morphology, (Table 3, Plate 1) and biochemical characterization (Table 4) was confirmed. Antibiotic sensitivity testing was carried out with different antibiotics against *E. coli* (Table 5, Plate 2) from the results, it was observed that all antibiotics resistant to *E. coli* except co-trimoxazole, cefotaxime and gentamycin. ESBL producing *E. coli* organisms were (Table 6) calculated.

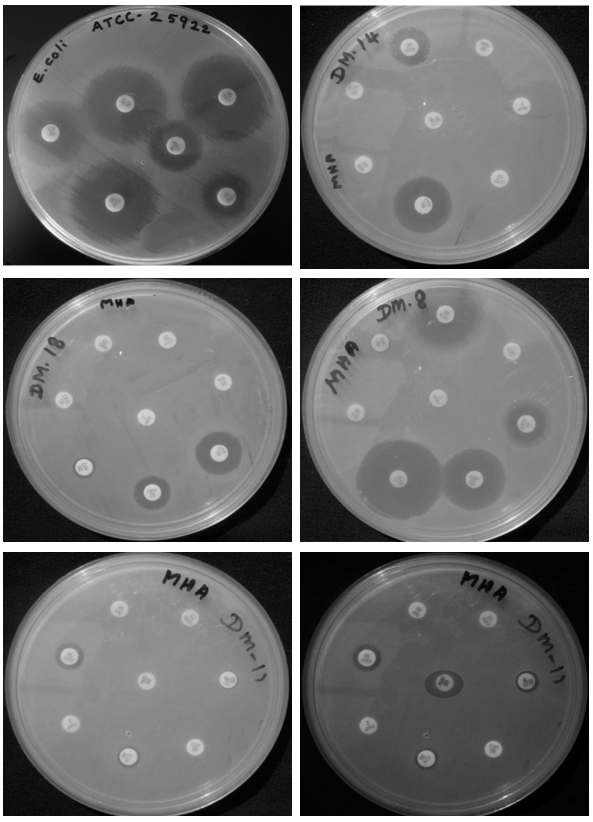

Plate 2: Antibiotic sensitivity plating for *E.coli* with different antibiotic discs

Antibiotic resistance surveillance has a central role among all strategies to manage the problem of antibiotic resistance. The first description was made in the mid 1970s (Sasirekha *et al.,* 2010). In present study, the antibiotic resistance *E. coli* strains are used in the identification of virulence factors.

Table 5: Antibiotic sensitivity pattern for *E.coli*

	Number and Percentage of Sensitivity and Resistant strains of *Escherichia coli*					
S.No	Name of the antibiotic	Disc content	Sensitive		Resistant	
			No.	%	No.	%
1	Ciprofloxacin	30mcg	-	-	30	100%
2	Cefotaxime	30mcg	4	13.30%	26	86.70%
3	Ceftazidime	30mcg	-	-	30	100%
4	Ampicillin	2mcg	-	-	30	100%
5	Amaxyclav	30mcg	-	-	30	100%
6	Cefpodoxime	10mcg	-	-	30	100%
7	Cotrimaxazole	25mcg	4	13.30%	26	86.70%
8	Gentamycin	30mcg	4	13.30%	26	86.70%

Table 6: ESBL producing *Escherichia coli* details

Total no. of specimen	Total no. of *E. coli* isolation	Total no. of ESBL producing *E. coli*	% of ESBL producing *E.coli*
200	30	26	86%

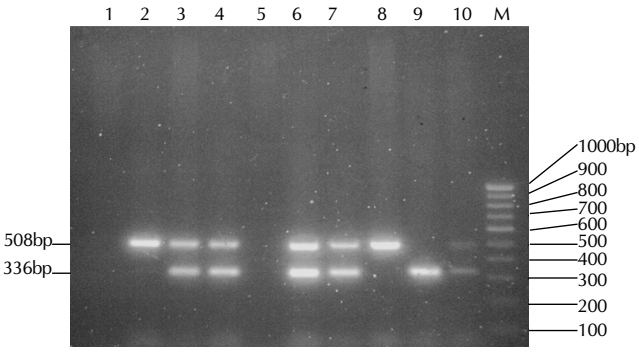

Plate 3: Amplification and identification of virulence genes from *E.coli* by multiplex PCR and Agarose Gel electrophoresis

E. coli was the most frequent isolate from urinary tract infection in diabetic patients and had poor antibiotic sensitivity to conventional antibiotics (Rakhshanda Baqai *et al*., 2008). Present study also indicates *E. coli* strains are more resistant. Interestingly all are found to be ESBL producing and multidrug resistant strains. But all strains showed co-trimoxazole sensitive it is somewhat different from Hagon *et al*. (2010) study. Because Hagon suggest that the antibiotic susceptibility testing indicated that these clinical isolates also showed high frequencies of resistances to common UTI therapies including trimethoprim-sulfamethoxazole (Co-trimoxazole). These were performed to study the antimicrobial susceptibility pattern of *Escherichia coli*. They were subjected to Multiplex PCR technique for the detection of fimH (type 1 fimbriae,) gene, papE/F (minor structural subunits of P fimbriae) gene and cnf-1 (cytotoxic necrotizing factor type-1) gene. FimH (type 1 fimbriae,) gene, papE/F (minor structural subunits of P fimbriae) genes are found in our study. But cnf-1(cytotoxic necrotizing factor type-1) gene was not found in present study. Uropathogenic *E. coli* (UPEC) isolates are a genetically heterogeneous group that exhibit several virulence factors associated with colonization and persistence of the bacteria in the urinary tract. The virulent strains of UPEC that cause cystitis typically produce, at least, one adhesion system. Adhesins can also contribute to virulence, promoting colonization, invasion and replication within uroepithelial cells (Mulvey, 2002). Present study characterizes the gene of *Escherichia coli* by multiplex PCR method to find fimH gene from ten clinical isolates. Out of ten clinical samples, five are diabetic and remaining five are non-diabetic patients. Out of five clinical isolates in diabetic patients we found three fimH genes and out of five clinical isolates in non-diabetic patients we found four fimH genes. All antibiotic resistant strains were done in polymerase chain reaction (PCR) for characterization of virulent gene like papE/F (336bp) and fimH gene (508bp) (Plate 3). The cnf-1 gene not found in all *Escherichia coli* strains.

Lindberg *et al*. (1985) conclude the amino and carboxyl termini of papE and papF and most of the *E. coli* pilins show striking similarities. Both papE and papF possess the structural and functional characteristics of a pilin protein. The major pilin, papA is rapidly degraded in the absence of papD, a protein required for the assembly of the pilus. Also papE and papF are unstable in papD and thus behave like the papA pilin in this aspect of pap-pilus biogenesis. The present study indicates the papE/F gene in diabetic and non-diabetic urinary tract infected patients out of ten, five are diabetic and remaining five are non diabetic UTI *E. coli* strains. Out of five in diabetic patients the papE/F were found only in 2 patients and out of five non-diabetic the papE/F which is found in four patients. Present study will be important in understanding the role of the virulence factors like papE/F (336bp) P fimbriae tip pilins, fimH (508bp) type 1 fimbriae, cnf-1 (543bp) cytotoxic necrotizing factor type 1 in causing UTIs in both diabetic and non-diabetic patients, we have rigorously established a multiplex PCR assay, capable of detecting virulence factor highly prevalent among UTI isolates. This in turn may lead to the development of universal vaccines to prevent such infections.

REFERENCES

Bonadio, M., Meini, M., Gigli, C., Longo, B. and Vigna, A. 1999. Urinary tract infection in diabetic patients. *Urol. Int.* **63(4):** 215-9.

Carter, M. W., Oakton, K. J., Warner, M. and Livermore, D. M. 2000. Detection of Extended - Spectrum beta - lactamases in Klebsiella with the Ozoid combination disk method. *J. Clin. Microbiol.* **38:** 4228-32.

Connell, I., Agace, W., Klemm, P., Schembri, M. and Marild S. 1996. Type I fimbrial expression enhances *Escherichia coli* virulence for the urinary tract. *Proc. Natl Acad Sci USA.* **93:** 9827-9832.

Harding, G. K., Zhanel, G. G., Nicolle, L. E. and Cheang, M. 2003. Antimicrobial treatment in diabetic women with asymptomatic bacteriuria. *N. Engl. J. Med.* **8(10):** 957-958.

Hagon, C., Erin Amando, L., Lloyd, David, A., Rasko, Gary, J., Faerber, Harry L. T. and Mobley 2010. *Escherichia coli* global gene expression in urine from women with urinary tract infection. *PLOS Pathogens.* **6(11):** e1001187.

Kuzuya, T. 1997. Diagnosis and classification of diabetes mellitus. *Asian Med. J.* **40:** 271-277.

Lan, Mo., Xin-Hua Zhu, Hong-Ying, H., Ellen Shapiro, David, L. H. and Xue-Ru, W., Tamm-Horstall 2009. Ablation of the Tamm-Horsfall protein gene increases susceptibility of mice to bladder colonization by type 1- fimbriated *Escherichia coli*. *Am. J. Physiol. Renal Physiol.* **286(4):** 795-802.

Lindberg Frederick, Bjorn, L. and Staffam, N. 1985. Gene products specifying Adhesion of uro-pathogenic *Escherichia coli* are minor components of pili. *Proc. Natl. Acad. Sci.* **83:** 1891-1895.

Martin, G., Cormican, Steven, A., Marshall and Ronald, N. J. 1999. Detection of Extended-Spectrum Beta - Lactamase (ESBL) Producing strains by the Etest ESBL screen. *J. Clinical Microbiology.* **34(8):** 1880-1884.

Mulvey, M. A. 2002. Adhesion and entry of uropathogenic *Escherichia coli*. *Cell. Microbiol.* **4:** 257-271.

Nicolle, L. E., Wayne, P. 2005. National Committee for Clinical Laboratory Standards, Urinary tract infecion in diabetes. *Curr. Opin. Infect. Dis.* **18(1):** 49-53.

Rakhshanda, B., Mubashir, A. and Ghulam, R. 2008. Urinary tract infection in diabetic patients and Biofilm formation of Uropathogens. *Diabetic Clinic.* **7(17):** 101-105.

Sasirekha, B. R., Manasa, P., Ramya and Sneha, R. 2010. Frequency and antimicrobial sensitivity pattern of Extended spectrum b-lactamases producing *E. coli* and *Klebsiella pneumonia* isolated in a Tertiary care hospital. *Al Ameen J. Med. Sci.* **3(4):** 265-271.