GTRP
Int. J. Biological Technology

Virulence markers of Uropathogenic *Escherichia coli* for Trojan- horse drug delivery

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Published: 15, December, 2012; Vol. No. 3(3): 1-5; www.gbtrp.com;

Abstract

A study was carried out in a rural teaching hospital in 2011 to determine the virulence factors such as fimbriae and siderophore production in uropathogenic *Escherichia coli* (UPEC) and to differentiate them from faecal *E. coli*. About 125 isolates of *E. coli* from urine samples of patients with urinary tract infection and 50 faecal *E. coli* isolates from healthy persons were studied. The presence of fimbriae was observed through heamagglutination assay and siderophore production was detected by chrome azurolsulphonate (CAS) qualitative and quantitative assays. Fimbriae were observed in all *E. coli* isolates from urine samples (100%). Siderophore production was observed in 123 urine isolates (98.4%) and the production percentage was in the range of 35% - 90%. Faecal isolates neither produced siderophores nor had the evidence of p- fimbriae. The results of our study suggest that, siderophores can also be used for selective drug delivery using Trojan horse strategy.

Key words: Uropathogenic *E.coli,* Siderophore, Haemagglutination, Trojan- horse strategy.

Introduction

Urinary tract infection (UTI) is one of the most common infections occurring worldwide and throughout the year, affecting people of all ages and both sexes. UTI can be defined as bacterial infections that may involve the lower or upper urinary tract and the inflammatory response thereof. European Association of Urology recommended the colony count of $>10^3$cfu / ml of urine for the diagnosis of acute uncomplicated UTI on urine culture (Grabe *et al*., 2010).Gram-negative microorganisms such as *E.coli*, *Proteus*, *Klebsiella*, *Citrobacter*, *Enterobacter*, and *Pseudomonas* sp and gram-positive organisms such as *Enterococcus faecalis* can cause urinary tract infection.

About 80-90% of urinary tract infections are caused by *E. coli.* Most of the *E.coli* strains act as commensals, whereas some of them cause infection. It is now recognized that there are subsets of feacal *E.coli*, which can colonize periurethral area, enter urinary tract and cause symptomatic diseases, and these are defined as Uropathogenic *E.coli* (UPEC) (Raksha *et al*., 2003). Uropathogenic *E.coli* differs from faecal coliform by possessing several virulence markers. Virulence factors such as adhesins

(fimbriae, certain other mannose-resistant adhesins, and type 1 fimbriae), the aerobactin system (siderophore), are of recognized importance in the pathogenesis of urinary tract infection.

The pathophysiology of bacterial adhesion in the urinary tract is complex. Uropathogenic *Enterobacteriaceae* is electronegative and too small to overcome repulsion by the net negative charge of epithelial cells. As a result, bacterial adhesion cannot occur in the absence of fimbriae or other (non-fimbrial) surface adhesion systems. The fimbriae systems have favorable electrical charge and also promote adhesion via hydrophobicity (Oelschlaeger *et al*., 2002; Mulvey *et al*., 2002).

These fimbriae can agglutinate human erythrocytes. Most organisms require iron as an essential element in a variety of metabolic and informational cellular pathways and the iron acquisition is the key step in the infection process. More than 100 enzymes acting in primary and secondary metabolism possess iron containing cofactors. Uropathogenic *E .coli* produces siderophores (iron chelating agent) for their iron requirement and also the unique outer membrane proteins (OMPs) (Neilands, 1981; Neilands, 1982; Lankford,1973) which serve to recognize process and transport the siderophore-iron complex into the cell.

Siderophore production is found to be more frequent in E. coli from patients with UTI, than in fecal isolates (Bnyan *et al*., 2010). Due to increased drug resistance, there arises the need to develop antimicrobial agents that target the resistance organisms and limit the natural selection of immune variant. Iron uptake systems of bacteria and fungi seem to be potential candidates for such a strategy. The application of siderophore-drug conjugates as "Trojan Horse" drug delivery has garnered due attention, in particular (Wencewicz *et al*., 2009).

Application of virulence factors in diagnostic sector as well as in therapeutic field was less studied. Previous studies show many discrepancies in confirming the siderophore production and the presence of fimbriae as virulence markers for uropathogenic *E. coli*. Hence, the present study was designed to determine the fimbriae and siderophore production of *E. coli* and their influence to differentiate uropathogenic *E. coli* from faecal *E.coli* and the scope to utilize these virulence markers for targeted drug delivery.

Materials and Methods

The study was conducted in the Department of Microbiology of a Tertiary Care Medical College hospital from September 2011 to December 2011. During the study period, *E.coli* isolates from UTI (n=125) and stool samples from healthy persons (n=50) were isolated and identified as described by Bailey & Scott. The isolates were maintained by inoculating nutrient agar butts and stored at room temperature for the detection of fimbriae and siderophore production.

Haemagglutination assay for fimbriae detection

All the *E. coli* isolates were inoculated onto the nutrient broth and incubated at 37ºc for 48 hrs for full fimbriation. The slide haemagglutination test was carried out on a multiple concavity slide. One drop of the broth culture was added to a drop of the 3% suspension of human RBC. The slide was rocked back and forth at room temperature for 5 minutes to observe the presence of clumping. Data were analysed by simple descriptive statistics.

Detection of microbial siderophore production

Siderophore production was detected by inoculating all the *E. coli* isolates on to the CAS agar and incubated at 37ºC for 24 - 48 hrs. Siderophore production was recognized qualitatively by the formation of orange halo around the colonies after 48 hours of incubation. In quantitative detection of siderophores (liquid assay), the isolates were grown in a nutrient broth at 37ºC for 24 hrs. The cells were removed by centrifugation at 3000 rpm for 15 minutes. The culture supernatant (0.5 ml) was then mixed with 0.5 ml of CAS solution and 10µl of shuttling solution (Sulphosalicilic acid). The change in color of the blue dye (Meazurolsulphonate) assay solution to purple orange indicates the presence of siderophore. The optical density of the resultant solution was read using the spectrophotometer at 630 nm after 20 minutes of incubation with appropriate blank (nutrient broth) and reference (Nutrient broth + CAS dye + shuttle solution). The OD values of reference solution and sample were marked as A_r and A_s respectively. The percentage of

siderophore produced was detected using the following formula:
% siderophore production = ($A_r - A_s$ / A_r) x 100

The data were analysed using SPSS software

Results and Discussion

All the 125 (100%) urinary *E.coli* isolates showed the presence of P-fimbriae (Fig. I) and in controls (faecal *E.coli*) (Fig. II), it was not observed in any of the isolates. Siderophore productions were observed qualitatively in 123 (98.4%) urinary *E.coli* isolates and not in any of the faecal isolates (Fig. III and IV). The percentage of siderophore production was in the range of 90% to 35% (Table - 1).

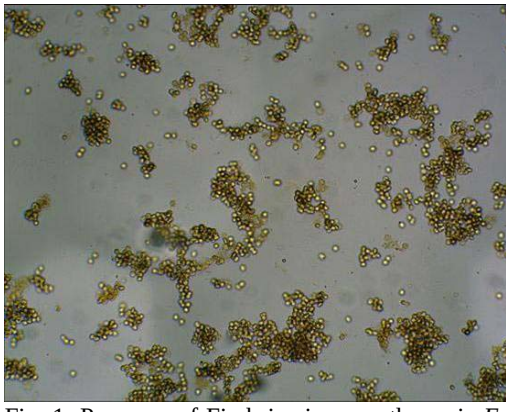

Fig. 1: Presence of Fimbriae in uropathogenic *E. coli*

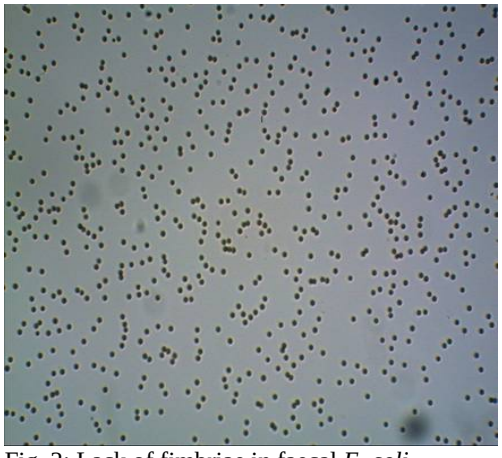

Fig. 2: Lack of fimbriae in faecal *E. coli*

Fig. 3: Qualitative CAS assay for siderophore production by uropathogenic *E. coli*

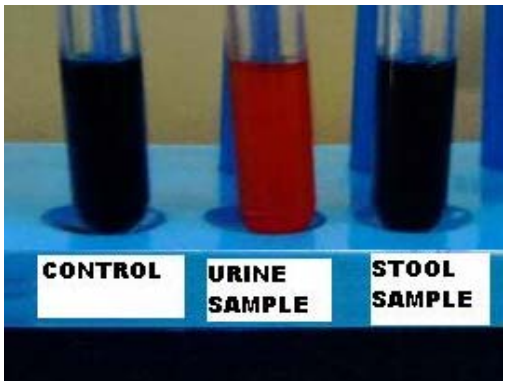

Fig. 4: Quantitative CAS assay for siderophore production by uropathogenic *E. coli*

Table - 1: Quantitative liquid assay for siderophore production

Range of production	% production
Above 90%	44
60% - 89%	15
41% -59%	28
35% -40%	36
Below 35%	Nil

Note: The percentage of siderophore production was in the range of 35% to 90%

Urinary tract infection is one of the most frequently encountered problems in ambulatory medicine. In the present study, 98.4% of urinary *E. coli* isolates produced siderophore and all had fimbriae, but the faecal isolates neither produced siderophore nor had the evidence of fimbriae. These results suggest that siderophore production and the presence of fimbriae can be used as

determinants of virulence which is supported by Vagrali *et al*.,2008). On the other hand, the results are contrary to that of Pal and Gokarn (2010). They have reported that, there was no significant difference in siderophore production in the commensals as well as in the clinical isolates of *E.coli.* However, their research comprised of only a few number of samples.

Molecular epidemiologic evidence from several studies has demonstrated an increased prevalence of Iron among UTI isolates, relative to the fecal isolates (controls). This suggests that iron functions as a siderophore receptor and is an urovirulence factor for UTI, as described in a recent study by Russo *et al.,* (2002).Antibiotics are the only choice of treatment for UTI and the development of antimicrobial resistance is a common problem in antimicrobial chemotherapy. Along with drug inactivators, altered targets and efflux, outer membrane permeability barrier also mediates the resistance to antibiotics. Misuses of bacterial transport systems, especially, the iron transport systems provide the promise to circumvent membrane-mediated resistance (Mollmann *et al*., 2009).

Antibiotics act like bomb, killing both the commensals and pathogens necessitating specific targeting. Besides contributing virulence, siderophores can also be used for selective delivery of antibiotics as enumerated by Henderson *et al*., (2009) using "Trojan horse" strategy. In this technique, antibiotics can be designed like a siderophore which results in the formation of siderophore -antibiotic conjugate known as sideromycin (Nagoba *et al*., 2011). Sideromycin employs siderophores as mediators to facilitate the cellular uptake of antibiotics. The siderophore part of the conjugate is able to scavenge iron and is recognized by cellular Fe-Siderophore uptake systems, while the other part of the conjugate bears an antibiotic activity that uses the siderophore as a Trojan horse and carries out iron transport mediated drug delivery.

Further, siderophores can be used for the treatment of acute iron intoxication and chronic iron overload diseases like hemochromatosis, sickle cell diseases, malaria, and cancer and for removing transuranic elements. Owing to the desirable features of siderophores, researchers can try to block or disrupt the activity of the protein that makes the siderophore, which will serve to recognize the process and transport the siderophore- iron complex into the cell. By such innovative designs, newer types of antibiotics may be introduced so as reduce the antibiotic resistance. This study was limited to single organism in single centre and the type of siderophore produced by *E.coli* was not determined.

Conclusion

Fimbriae was observed in all *E.coli* isolates from urine samples (100%). 123 urine isolates (98.4%) also produced siderophore and the production percentage was in the range of 35%-90%. Faecal isolates neither produced siderophores nor had the evidence of p- fimbriae. This study concludes that the production of siderophores and the presence of fimbriae can be used as virulence markers for UPEC and siderophores can very well be used for targeted antibiotic delivery using "Trojan horse strategy".

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Manuscript Progress Date
Received : 26.09.2012
Revised : 06.11.2012
Accepted : 07.11.2012