Contents lists available at www.innovativejournal.in
INNOVATIVE JOURNAL OF MEDICAL AND HEALTH SCIENCE
Available online at
http://www.innovativejournal.in/index.php/ijmhs

ONLINE ISSN:2277-4939
PRINT ISSN:2589-9341

Molecular description of alpha-hemolysin producing Escherichia coli isolates from extraintestinal source.

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Abstract:- Context: *Alpha-hemolysin (Hly) is a common exotoxin produced by Escherichia coli, which plays an important role as a virulence factor in a number of clinical infections. There exist other virulence properties exhibited by such strains whose role in pathogenesis have not yet been clearly elucidated.*

Aims: *Aim of present study was to characterize those alpha-hemolysin producing isolates on the basis of their virulence factors and phylogenetic background.*

Settings and Design: *This descriptive study was carried out in a multi-specialty tertiary care hospital.*

Methods and Material: *Three hundred non-repeat E. coli isolates from in-patients were studied. Isolates were differentiated as haemolytic and non-haemolytic on the basis of both phenotypic methods such as haemolysis on sheep blood agar and genotypic detection of the hly gene. Possession of virulence and drug resistance genes was determined by multiplex polymerase chain reaction (PCR). Phylogenetic analysis was performed by triplex PCR methods. Antibiotic sensitivity testing was performed by disk diffusion method.*

Results: *Of the 300 isolates, it was observed that by phenotypic (haemolysis on blood agar) method, 65(22%) were hemolytic whereas the genotypic method detected 70 (23.5%) isolates possessing alpha haemolysin (hlyA+) marker. B2 phylogroup isolates were found to harbour the hlyA+ marker at a significantly higher ($P < 0.05$) rate. The possession of papC and cnf1 genes was significantly higher in hlyA+ isolates.*

Conclusions: *Alpha-haemolysin producing isolates demonstrated higher virulence properties. The microbiology laboratory may report haemolytic characteristics of the isolates as it may alert the physician of the higher pathogenic potential of the strain and to initiate appropriate treatment.*

Key-words: *E. coli; Alpha haemolysin; phylogroup; virulence factor*

- *Key Messages: Alpha hemolysin producing E. coli isolates were showing higher pathogenic potential in comparison to non- hemolysin producing isolates.*
- *Alpha haemolysin producing isolates mainly belonged to phylogroup B2.*

Introduction:

Extraintestinal infections (EIs) due to *E. coli* are common in all age groups and can involve almost any organ or anatomical site. [1] Pathogenic *E. coli* frequently express combinations of several virulence factors that presumably provide a competitive advantage over other bacteria or host defence mechanisms. [2-4] One well known virulence factor is the potent exotoxin alpha-hemolysin, which enhances virulence in a number of clinical settings. The production of alpha-hemolysin can be easily demonstrated in sheep blood agar where the isolates shown beta-haemolytic colonies. The production of alpha-hemolysin in *E. coli* is due to the expression of *hly* gene. [5,6]

Although several studies have characterized extraintestinal pathogenic *E. coli* (ExPEC) isolates based on their phenotypic, genotypic and clinical properties, little attention has been given to the beta-haemolytic extraintestinal *E. coli* isolates. The aim of the present study was to examine the specific

contribution of *hly* gene to enhance the virulence property in the ExPEC isolates.

Subjects and Methods:

Participants and clinical isolates:

The study was conducted during the period from August 2010 to August 2014, from patients of a tertiary care hospital in South India, after obtaining permission from the institutional ethical committee. Three hundred non-repeat strains of *E. coli* were isolated from specimen such as urine, blood, wound swab, pus, CSF, ascites fluid and intravascular devices from the study population. Samples were processed immediately using standard procedures. Isolates were identified based on gram staining and by standard biochemical tests. [7]

Haemolysin production:

Production of haemolysin was detected by growing the different strains in LB medium overnight (37º C) and dropping 50 µL of this culture on a Petri dish containing sheep blood agar. The cultures were incubated at 37ºC overnight and haemolysin production was verified by the presence of a clear hemolytic halo around the colony. [8]

Genetical studies:

Preparation of Template DNA:

To 500µl of sterile distilled water taken in a micro centrifuge tube was added 4-5 freshly sub-cultured identical colonies of the isolate. This suspension was heated in a water bath at 95^0C for 10 minutes and then centrifuged at 10,000 rpm for 10 minutes. [9] The supernatant containing bacterial DNA was used as template for PCR. *E. coli* strains used as positive controls in the PCR assay were kindly provided by Ms. Lotte Jakobsen (Statens Serum Institut Microbiology & Infection Control 5 Artillerivej, build 46/202 DK-2300 Copenhagen).

Phylotyping analysis:

Phylogenetic analysis was performed by triplex PCR based methods as described by Clermont *et al*. [10] Briefly, a combination of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (*TSPE4.C2*) allows the determination of the main phylogenetic groups of *E.coli* (these being A,B1,B2 & D).

Detection of virulence factor (VF) genes by multiplex PCR assay:

Two sets of multiplex PCR were developed to detect following genes:

Set 1: A PCR assay was performed to detect *papC*, *cnf1* & *neuC* genes as per primers and conditions described earlier with minor modification. [11]

Set 2: Another PCR assay was performed to detect *hlyA*, *fimH* & *iutA* genes as per primers and conditions described earlier with minor modification. [11]

Statistical analysis:

The statistical analysis was performed by using SPSS, version 17.0. Correlation of numerical data with drug resistance and virulence was done using Pearson's correlation coefficient and all categorical data were correlated by chi–square test. $P< 0.05$ was considered statistically significant.

Results:

Of the 300 isolates, it was observed that the phenotypic method (haemolysis on blood agar) detected 65 (22%) of isolates as alpha hemolysin positive whereas the genotypic method detected 70 (23.5%) isolates as carrying the *hlyA* gene.

Regarding phylogrouping, sixty-one isolates were found to belong to phylogroup A and 27 strains to group B1, both phylogroups which are known to be commensal groups. Among the virulent groups (phylogroups B2 & D), 104 were from group B2 and 108 were from group D. On analysis of the distribution of phylogroup among the hemolysin positive (hlyA+ve) and hemolysin negative (hlyA-ve) isolates, incidence of B2 group isolates was significantly higher ($P < 0.05$) in hly+ve isolates. (Table 1).

The distribution of the virulence genes among the hlyA+ve and hly-ve strains were as follows: *fimH* (91% vs 90%); *iutA* (73% vs 66%); *papC* (91% vs 31%) *cnf1* (77% vs 7%) and *neuC* (3% vs 5%) respectively. A significant positive correlation was observed with *papC and Cnf1* in between hly+ and hly- strains. (Table 1)

Discussion:

In the present study, nearly 23% of the isolates possessed the *hlyA* gene. A comparison of hly+ve and hly-ve isolates in relation to their phylogroups detected the significant predominance of hly-ve isolates in commensal phylogroups A and B1 and also from virulence group D whereas hly+ve isolates mainly belonged to the virulent phylogroup B2. This indicates that hly+ve isolates were more virulent in comparison to hly-ve isolates.

The most important secreted virulence factor of Extra intestinal pathogenic *E. coli* is α-haemolysin, and in the present study we found a significant correlation between hly+ve isolates and the possession of the virulence genes *papC* and *cnf1*. We also observed higher percentage of *iutA* gene among the hly+ve isolates. Based on these findings we can assume that the haemolytic strains are carrying maximum number of virulence genes. This finding is similar to the study done by Sharma *et al* [12] wherein they also reported the presence of multiple virulence factors among the haemolytic isolates. We also observed that 5 isolates were phenotypically non hemolytic nevertheless they possessed the *hlyA* gene, and this difference may be due to the inability of the isolates to express *hlyB* and *hlyD* genes which regulate the haemolysin secretion by the bacterial cell or maybe there was too less an amount of haemolysin secretion into the medium which was insufficient to cause haemolysis on sheep blood agar [13,14].Our study findings suggest that non-haemolytic isolates were less virulent in comparison to haemolytic isolates. Based on our finding we can suggest that clinical microbiology laboratories may report the haemolytic property of the isolates to the physician as it may help them to better understand the pathogenic potential of the isolate causing the infection. However, further investigations are required to explain the mechanisms at play behind these findings.

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Table 1: Phylogeny and possession of virulence factors by hly positive and hly negative strains of E. coli

	***hlyA* Positive (N=70)**	***hlyA* Negative (N=230)**	**P value**
Phylogenetic distribution			
Group A (n=61)	1(1.5%)	60 (26%)	0.001
Group B1 (n=27)	2(3%)	25(11%)	0.32
Group B2 (n=104)	45(64%)	59(25.5%)	0.001
Group D (n=108)	22(31.5%)	86(37%)	0.21
Virulence factor genes			
papC	64 (91%)	72 (31%)	0.001
cnf1	54(77%)	16(7)	0.001
fimH	64(91%)	207 (90%)	0.42
iutA	51(73%)	152(66%)	0.093
neuC	2(3%)	12 (5%)	0.12