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Phylogenetic Grouping and Virulence Genes Distribution in Uropathogenic *Escherichia coli* Isolated from Environmental Samples in Ponnani, South India

Zubair, A.A.[1*] and Sureshkumar, S.[2]

[1] *Post-Graduate Department and Research centre in Aquaculture and Fishery Microbiology, MES Ponnani College, Ponnani South, Malappuram -679586 Kerala, India*
[2] *School of Ocean Science and Technology, Kerala University of Fisheries and Ocean Sciences, Panangad -682506, Kerala, India*
**Email: zubair.mes@gmail.com*

Abstract

Isolation and phylogenetic characterisation of *E.coli* were carried out along with the identification of uropathogenic strains by detection of virulent factors. The samples were collected from Ponnani harbour and the surrounding water bodies. The virulent factor genes were detected with the help of PCR. Isolates were screened for the presence of genes *hly D, afa, sfa* and *pap* and were considered as Uropathogenic *Escherichia coli (UPEC)* if two or more virulent factor genes were present. Phylogroup assignment was carried out by scoring the presence/absence of the genes in the order *arpA*/*chuA*/*yjaA*/. Of the 25 chosen isolates for phylogenetic studies, 8 (32%) belonged to group B_2 and 6(24%) belonged to Group A. The group C, D and E were represented by only one isolate, whereas, 2(8%) isolates represented group B_1. The group F had 4 (16%) isolates.In the current investigation, of the 25 isolates of *E.coli* were selected for the detection of virulence gene by PCR, 17 strains were found to possess one or more virulence factor genes

Keywords: Ponnnai harbour, Phylogenetic studies, UPEC, Virulent factors

1. Introduction

Escherichia coli are by, and large non-pathogenic and are typically found in the alimentary canal of warm-blooded animals. The improper disposal of sewage and municipal waste contribute to the incidence of *E. coli* in water bodies and has become a significant public health problem. The *E. coil* is found to withstand adverse conditions in most aquatic environments, fresh, estuarine and seawater. (Rhodes and Kator, 1988).*E. coli*, as mentioned above, are usually non-pathogenic. However, a few strains may acquire virulent genes by horizontal gene transfer and can emerge as pathogenic, causing various infections.

The pathogenic forms of *E. coli* isolated from infection sites other than intestine are commonly called extraintestinal pathogenic *E. coli* (Russo and Johnson, 2000). ExPEC multiplies in the gut of healthy individual and lives as commensals. When these bacteria reach niches outside the intestine, become pathogenic and cause various types of infection. These bacteria are quite distinct from commensals and intestinal pathogenic forms and possess unique virulent factors. The presence of virulence factors determines the ability of *E. coli* to cause the disease, and these virulence factors help the bacteria to survive and multiply in host cells and withstand adverse conditions (Johnson, 1991; Nataro and Kaper, 1998; Oelschlaeger *et al*., 2002[a]). Based on the site of infection, such forms are further classified into three groups, namely uropathogenic *E .coli* (UPEC), sepsis-associated *E. coli* (SEPEC), and neonatal meningitis associated *E. coli* (NEMEC) (Russo and Johnson, 2003). The predominant form is found to be uropathogenic *E. coli*. An isolate can be considered as ExPEC if it possesses two or more virulent factor genes among the five critical virulent factor genes, as suggested by Johnson and Stell (2000). The immediate reservoir of ExPEC is found to be the intestinal tract of human beings. However, there are reports of community-acquired urinary tract infection caused by ExPEC strains originating from food sources such as chicken meat (Manges *et al*., 2007; Vincent *et al*., 2010). Clermont *et al.* (2000) introduced a teipex PCR method to detect the genes *chu*A, *yja* A, and *tsp* E4C2. The presence or absence of these gnes became the criteria for classifying E.coli isolates into A, B1, B2 or D groups. Clermont *et al.* (2013) revised the method with the introduction of new target gene Arp A and made a quadruplex PCR and classified the *E.coli* into A, B1, B2, C, D, E, F Phylogroups. The phylogroups assignment is carried out by scoring the presence /absence of the genes in the order *arp* A/*chu* A/*yja* A/*tsp* E4C2. Earlier studies in Cochin estuary have revealed that phylogenetic groups B2 and D predominate among the ExPEC isolates, whereas, non ExPEC strains are found to belong to A and B1 group (Divya and Hatha, 2015).

The *E. coli*, especially UPEC strains, are found to be resistant to different antibiotics since such strains are exposed to different antibiotics administered during treatment of clinical infections. Apart from therapy during infection, bacteria like *E. coli* acquire antibiotic resistance through horizontal gene transfer in water bodies among bacteria originating from different sources.

Ponnani a coastal town in Kerala, South India is densely populated, and there are no sewage treatment plants in Ponnani. Due to the dumping of municipal waste and sewage, water bodies surrounding the Ponnani harbour get polluted with pathogenic bacteria which reach

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nearshore water regularly as there is no natural or anthropogenic mechanism to prevent it. Markets and hospitals in the Ponnani and surrounding areas also frequently discharge the untreated wastewater to Ponnani estuary. Other than hospitals effluents and sewage, waste from poultry farms and retail chicken shops might also contribute to the dissemination of these virulent strains. There are many reports of isolation of UPECstrains from retail chicken meat (Johnson *et al.*, 2005; Vincent *et al.*, 2010; Jakobsen *et al.*, 2010). ExPEC strains of *E. coli* are found to be the leading cause of UTI infection reported worldwide (Russo and Johnson, 2003).

Therefore the main objective of the present study is to detect the presence of uropathogenic strains of *E. coli* in Ponnani harbour and surrounding water bodies along with their phylogenetic classification. The investigation is also aimed to understand the antibiotic sensitivity profile of the isolated UPEC strains.

2. Materials and Methods

2.1 Isolation of *Escherichia coli*

Ponnani fishing harbour is located near the bar mouth, where Ponnani estuary meets the sea, (Fig. 1) and the samples like fish, water, ice and swabs from workers hand, utensils, boat deck and net were collected seasonally for two years (2012-14) From water bodies, fish, water and sediment were collected. The samples were brought to laboratory and enriched in Lauryl Suphate broth(LSB) for 24-48Hrs .From those tubes showing turbidity and gas production, one loopful inoculum was transferred to EC broth for confirmation. For isolation of *E. coli*, inoculum from EC broth was transferred to Eosin Methylene Blue Agar plate and incubated at 37^{0}C for 24 hours. Colonies with blue metallic sheen were presumptively identified as *E. coli* and later confirmed by IMViC tests (Downs and Ito, 2001). Strains positive for phenotypic virulent characteristics (ie Serum resistance, Cell surface hydrophobicity, and hemolytic activity) were selected for genotypic studies Of the 106 isolates obtained in the investigation, 25 isolates were selected for phylogenetic and virulent factor studies employing PCR technique.

2.2 Isolation of DNA from *E. coli*

The pure bacterial colony was inoculated in Luria Bertani broth. 1mL of the culture was transferred to a sterile microcentrifuge tube, and the cells were harvested by centrifugation at 12000 rpm for 10 minutes at room temperature. The supernatant was disposed of and the pellet was resuspended in1mL of 0.85% (w/v) NaCl solution and centrifuged as above. Again the supernatant was discarded and added 600 µL lysis buffers along with 7µL of proteinase-K 5. The mixture was vortexed and incubated at 65°C for 1 hour. An equal volume of chloroform-isoamyl alcohol (24:1) was added with gentle mixing by inverting the tubes for 2-5 minutes. The samples were centrifuged for 15 minutes 12000 rpm at room temperature. The aqueous phase was collected in another microcentrifuge tube without disturbing the interface and lower phase. The steps of chloroform: isoamyl alcohol extraction was repeated. Again the aqueous phase was collected and added the 50µl volume of 3M Sodium

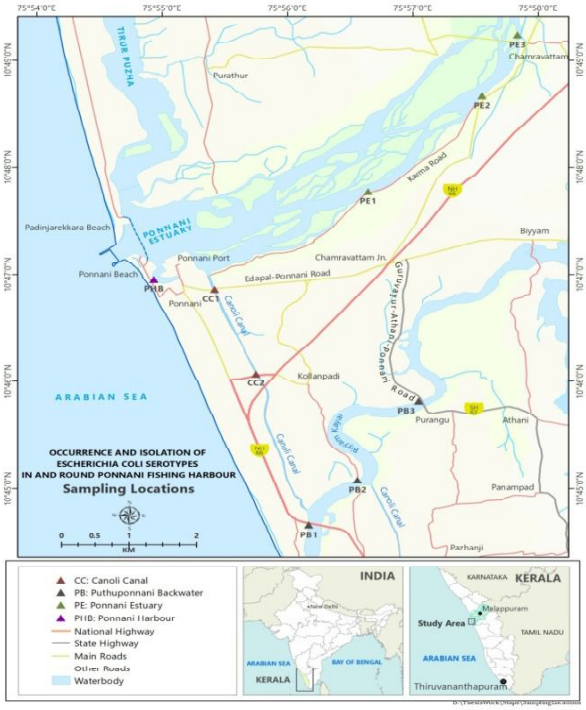

Fig. 1. Study area Ponnani harbour and surrounding water bodies

Acetate (pH 5.2) followed by an equal quantity of cold isopropanol to precipitate the DNA and centrifuged it again at room temperature for 5 minutes at 12000 rpm. The supernatant was discarded and rinsed the pellet twice with 70% ethanol, followed by maintaining the tubes for 1hour in vacuum desiccators. The desiccated DNA samples were resuspended entirely in 50µl of DNA dissolving buffer (TE buffer) and stored at -20° C. Purity of DNA was judged by the optical density ratio at 260:280 nm with the help of UV spectrometer.

2.3 Detection of virulent factor genes

The virulent factor genes were detected with the help of PCR (Table 1). Isolates were screened for the presence of genes *hly D, afa, sfa* and *pap* and were considered as ExPEC if two or more virulent factor genes were present (Johnson and Stell, 2000). PCR was performed in a final reaction volume of 25 µL in 200 µl capacity thin-wall PCR tube. Composition of reaction mixture for PCR was deionized water-18.2µl,Taq buffer without $MgCl_2$-2.5µl, $MgCl_2$ (15 mM)-1.0µL, forward primer (10 pm/µL)-0.5µl, reverse primer (10 pm/µL)-0.5µl Taq DNA polymerase (5U/µL)-0.5µ, template DNA (20 ng/µL)-2.0µL. PCR tubes having the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes consisting of all the components were transferred to the thermal cycle. The PCR protocol designed for 30 cycles for the primers used is given in (Table 2).

2.4 Phylogenetic analysis

Clermont *et al.* (2000) introduced a triplex PCR method to detect the genes *chuA*, *yjaA*, and *tspE4C2*. The presence or absence of these three genes became the criteria for classifying *E.coli* isolates into A, B1, B2 or D groups. Clermont *et al.* (2013) revised the method with the introduction of new target gene *ArpA* and made a quadruplex PCR and classified the *E. coli* into A, B1,B2,

Table 1. Primers used for detection of virulent factor genes of *E. coli* (Zhu *et al*., 2017; Liu *et al*.,2015)

Primer/ Gene Name		Primer sequence	Product Size
papC	**F**	GTGGCAGTATGAGTAATGACCGTTA	205 bp
	R	ATATCCTTTCTGCAGGGATGCAATA	
sfa	**F**	CTCCGGAGAACTGGGTGCATCTTAC	410 bp
	R	CGGAGGAGTAATTACAAACCTGGCA	
Afa	**F**	GGCAGAGGGCCGGCAACAGGC	594 bp
	R	CCCGTAACGCGCCAGCATCTC	
hly D	**F**	CTCCGGTACGTGAAAAGGAC	900bp
	R	GCCCTGATTACTGAAGCCTG	

Table 2. PCR cycles for virulence gene amplification

	Process	Temperature	Time
1	Initial Denaturation	95°C	12 minutes
2	Denaturation	94°C	30 seconds
3	Annealing	63°C	30 seconds
4	Extension	72°C	30 seconds
	Go to step 2 for 29 times		
5	Final elongation	72°C	10 minutes
	An end for 4°C forever		

C, D, E, F, phylogroups. Phylogroup assignment is carried out by scoring the presence/absence of the genes in the order *arpA*/*chuA*/*yjaA*/*tspE4C2*.This method led to the assignment of an *E. coli* isolate to one of the seven phylogenetic groups (A, B1, B2, C, D, E, and F) and *Escherichia coli* cryptic clades I to V. The primers employed are shown in the (Table 3). All PCR reactions were carried out in a final volume of 25 µL containing1 ìL of total DNA, 0.2 µM of each primer, and 12.5 ìL of EmeraldAmp® GT PCR Master Mix (Takara, Japan).PCR reactions were performed as per thefollowing conditions: denaturation was conducted for 4 min at 94 °C, 30 cycles of 5 s at 94 °C and 20 s at 57 °C (group E) or 59 °C (quadruplex and group C), and a final extension step of 5 min at 72 °C, on a ProFlex™ 3x32-Well PCR system (Applied Biosystems, United States). PCR products were resolved by electrophoresis on a 1.5% agarosegel (Sigma-Aldrich, United States) stained with ethidium bromide (Sigma-Aldrich, United States) and visualised by Gel Documentation System (BioRad Gel Doc™ EZ Imager, United States).

2.5 Antibiotic sensitivity studies of uropathogenic *E. coli* (UPEC)

Antibiotic sensitivity studies were carried out by disc diffusion method (Bauer *et al*., 1966) along with ESBL production by following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013).

3. Results

Of the total 106 isolates, 25 isolates were selected for phylogenetic studies and detection of virulence genes.

3.1 Phylogenetic groups

The results of the phylogenetic analysis are shown in (Fig. 2 and Fig. 3). Of the 25 selected isolates for phylogenetic studies, 8 belonged to group B_2(32%) and 6 belonged to Group A(24%) . The incidence of other groups was comparatively less. The group C, D and E were represented by one isolate each, whereas, 2 isolates represented group B_1(8%). The group F was represented by 4 isolates(16%). The remaining two isolates could not be included in any of the groups described above since the distribution of target genes were in an entirely different order from that of the standard pattern accepted for classification and were considered as unknown.

3.2 Prevalence of virulence factor genes

In the current investigation, of the 25 isolates of *E. coli* selected for the detection of virulence gene 17 strains were found to possess one or more virulence factor gene as

Table 3. Primer sequences used in the extended quadruplex phylogenetic typing method (Clermont *et al* ., 2013)

Primer/Gene Name		Primer sequence	Product Size
*chu*A	**F**	ATGGTACCGGACGAACCAAC	288bp
	R	TGCCGCCAGTACCAAAGACA	
*yja*A	**F**	CAAACGTGAAGTGTCAGGAG	
	R	AATGCGTTCCTCAACCTGTG	211bp
*tsp*E4C2	**F**	CACTATTCGTAAGGTCATCC	
	R	AGTTTATCGCTGCGGGTCGC	152
*arp*A	**F**	AACGCTATTCGCCAGCTTGC	400bp
	R	TCTCCCCATACCGTACGCTA	
arpA (ArpAgpE)	**F**	GATTCCATCTTGTCAAAATATGCC	301bp
Group E	**R**	GAAAAGAAAAAGAATTCCCAAGAG	
trpA(trpAgpC)	**F**	AGTTTTATGCCCAGTGCGAG	
GroupC	**R**	TCTGCGCCGGTCACGCCC	219bp
trpA(trpBA)	**F**	CGGCGATAAAGACATCTTCAC	
Inernal control	**R**	GCAACGCGGCCTGGCGGAAG	489

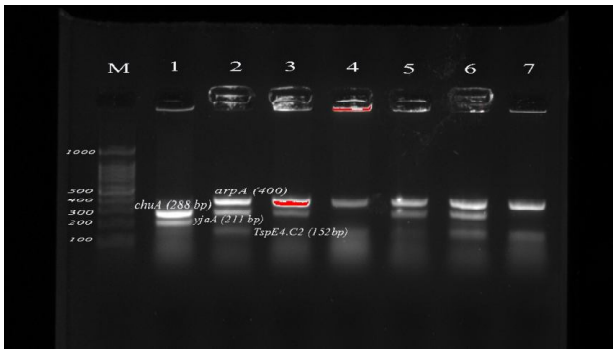

Fig. 2. PCR products of genes *chuA, arp A, yja, tspE4C2*

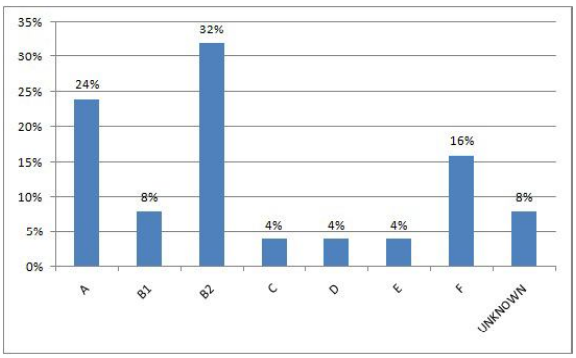

Fig. 3. Phylogenetic groups of *Escherichia coli* isolated from Ponnani

shown in the (Fig. 4). These 17 isolates can be considered as uropathogenic *E. coli* since the protein synthesised by these genes plays a vital role in the survival and proliferation of these bacteria in the human urinary tract. The gene *pap* C was present in 13 isolates (52%) whereas *hly* D was present in 8 isolates (32%). Six isolates (24%) showed the presence of the virulence factor gene *sfa* while five isolates (20%) showed the presence of *afa* gene. Five isolates had three virulent factors genes. Of this five, three isolates were of (*hly*D+*sfa*+*pap*C) virulent genes, and the remaining two isolates were of (*hly*D+*afa*+*pap*C) genes (Table 4).

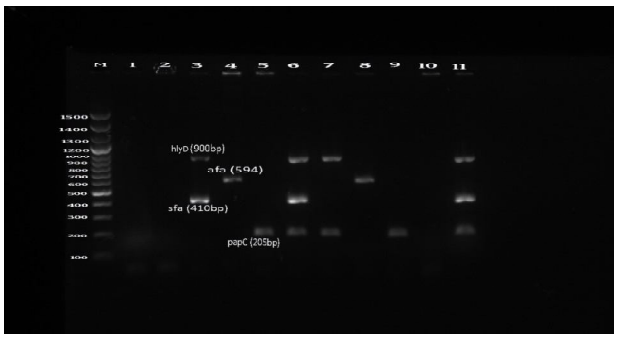

Fig. 4. PCR products of virulent genes *papC, sfa, afa, hlyD*

Table 4. Prevalence of virulent genes among different phylogenetic groups

	Virulent genes	% of incidence in all the isolates n=25	The incidenceof different phylogenetic groups								
			A (n=6)	B_1 (n=2)	B_2 (n=8)	C (n=1)	D (n=1)	E (n=1)	F (n=4)	unknown (n=2)	Total
1	*papC*	-52%	2	-	6	1	1	-	1	2	13
2	*hly* D	-32%	-	-	5	-	1	-	1	1	8
3	*sfa*	-25%		1	3		1	1			6
4	*afa*	-20%	1	-	2	1		1			5

4. Discussion

The phylogenetic studies and virulent factor studies show the incidence of pathogenic forms, especially uropathogenic forms in water bodies in and around Ponnani harbour. As stated before, these forms might have entered the estuaries and harbour premises from municipal sewage or hospital waste of nearby areas.

The results revealed the prevalence of phylogenetic groups B_2 and A among the 25 isolates chosen for studies and are more or less similar to findings of previous investigation in Cochin estuary (Divya and Hatha, 2015). The results also agree with the conclusions of the phylogenetic analysis of ExPEC strains carried out worldwide (Khan *et al.*, 2017). In all these findings, the dominant phylogenetic group was B_2. It has been reported previously that most *E. coli* strains responsible for UTI or other extraintestinal infections belong to group B2 or, less frequently, group D (Picard *et al.*, 1999). In another study in Commensal *E. coli* isolated from piglet in Poland showed predominance of B1 followed by A group (Bok *et al.*, 2020)

However, in contrast to these results, in another study in Iran on the prevalence of phylogenetic groups A and D were observed among the uropathogenic strains (Alizade *et al.*, 2013). In the current study, the incidence of A group was also significant when compared with other groups. These results are in agreement with results of some previous investigations which demonstrated a higher rate of phylogenetic group A among extraintestinal *E. coli* (Dadie *et al.*, 2014: Saeed *et al.*, 2009). Geographical factors influence the distribution of phylogenetic groups and virulent genes, and thus there may be variation in the prevalence of these groups in different areas (Piatti *et al.*, 2008). The groups A and B_1 are not generally considered as pathogenic but can cause opportunistic infection in immuno-compromised individuals (Johnson, 2002).

The incidences of other groups are significantly low, except the F group, which was represented by four isolates. Since these groups are newly introduced by the revised method of Clermont *et al.*, 2013 there is not enough data to compare the present result with other studies. In a similar investigation in Iran phylogenetic group B2 was most predominant (39.3%), followed by unknown (27.1%), E (9.3%), C and clade I (each 6.4%), B1 (5%), F and D (each 2.9%), and A (0.7%) (Iranpour *et al.*, 2015).

The present investigation detected one or more virulent factor genes in 17 isolates. The highest prevalence was in the B_2 group. All the other groups had a meagre percentage of virulent factor genes. However, the D group represented by a single isolate had three virulent factor genes. Whereas, group A represented by six strains, which had only three virulent factor genes. These results are almost consistent with the findings of a similar investigation carried out in Cochin estuary (Divya and Hatha, 2015). The prevalence of VF factor genes among Group B_2 has also been reported by various studies carried out in *E. coli* strains isolated from UTI infections worldwide. (Clermont *et al.* 2000; Mokracka *et al*., 2011: Johnson *et al*., 2005).However, in contrast to the results of the present investigation, in another study in Iran virulent genes were observed predominantly in phylogenetic groups A

In the current investigation, *pap* C, (52%) showed the highest incidence, followed by h*ly* D (32%). The prevalence of *sfa* (25%) and *afa* (20%) were comparatively lower in the present investigation. The virulent genes *pap, sfa* and *afa* encode for the factors involved in the adhesion of bacteria to the mucosal cells of the urinary tract (Mulvey *et al*., 2002; Agarwal *et al*., 2012). These results are in agreement with observations of the similar investigation conducted in Iran where Uropathogenic strains of *E. coli* showed the prevalence of virulent genes like *pap* (20.5%) *saf* (20.5%) and *afa* (8.3%)(Mohajeri *et al.,* 2014).

5. Conclusion

The incidence of phylogenetic Group B_2 in water bodies in Ponnani is an indication of presence of pathogenic *E. coli*, especially uropathogenic strains in the water bodies. The B_2 group demonstrated a comparatively higher incidence of virulent factors such as *pap* C, *hly D*, *afa* and *sfa*. Thus there is a potential risk of spread of infection among the local population in the nearby areas as the water from these water bodies are utilised for various household purposes. Stringent measures should be implemented in the harbour to prevent the utilisation of estuarine and nearshore water for washing the fish, utensils and premises to ensure the quality of the product originating from there.

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