Putative Virulence Genes and Biofilm Production Among Typical Enteroaggregative *Escherichia coli* Isolates from Diarrhoeic Children in Kashmir and Andhra Pradesh

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Abstract Fifty-eight typical EAEC isolates from children with diarrhoea were examined for HEp-2 cell adherence assay, presence of dispersin (*aap*), yersiniabactin (*irp2*), plasmid encoded toxins (*pet*), *Shigella* enterotoxin1 (*set1A*) and cryptic open reading frame (*shf*) putative virulence genes by polymerase chain reaction as well as for biofilm production. All the isolates showed aggregative adherence pattern on HEp-2 cells. All but five isolates (91.3 %) carried *aap* gene. While *irp2*, *pet*, *set1A* and *shf* genes were detected in 68.9, 5.1, 39.6, and 60.3 % isolates, respectively. Thirty-three (64.7 %) isolates out of 51 tested were found to produce biofilm which was found to be significantly associated only with *set1A* virulence gene ($P = 0.025$). Highest amount of biofilm was produced by a strain that possessed all the genes studied. Out of 14 isolates in which the most frequent gene combination (*aap*,

irp2 and *shf*) was observed, only six produced biofilm. It is concluded that there is significant heterogeneity in putative virulence genes of EAEC isolates from diarrhoeic children and biofilm formation is associated with multiple genes.

Keywords EAEC · *aap* · *irp2* · *shf* · *pet* · *set1A*

Introduction

Enteroaggregative *Escherichia coli* (EAEC) have been implicated as emerging enteric pathogens which cause persistent diarrhoea among infants both in developing and industrialized countries [8, 13]. The EAEC strains are characterized by their unique patterns of aggregative or ‘stacked brick’ adherence to HEp-2 epithelial cells in culture [14]. The exact mechanism by which EAEC cause diarrhoea is poorly understood, however, adhesins, toxins and several other virulence factors have been implicated.

Adherence of EAEC to small and large bowel mucosal surfaces in a thick aggregating biofilm has been quantified by an in vitro assay [21]. In addition to biofilm formation, a large number of putative virulence factors have been identified in EAEC strains. Most EAEC strains harbour a high molecular weight plasmid called pAA from which a fragment is used as a detection probe. The pAA also encodes several virulence factors that include fimbrial adhesions, *astA* (enteroaggregative heat-stable enterotoxin 1) [18], *aap* (dispersin protein) [20] and *aatA* (dispersin transporter/CVD432) [15] genes. The plasmid encoded toxins (*pet*) [6] and a cryptic open reading frame known as *shf* [4] have also been described. The transcriptional activator (*aggR*), present on the plasmid is the best studied virulence gene of EAEC. It controls expression of adherence factors, and a larger cluster of genes encoded on pAA

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Table 1 Details of Primer sequences used in this study

Primer	Sequence (5'–3')	Target	Primer conc. (μM)	Product size (bp)	References
aap-f	ATGAAAAAATTAAGTTTGTTATCTT	<i>aap</i>	0.25	351	[12]
aap-r	TTATTTAACCCATTCGGTTAGAGC				
irp2-f	AAGGATTCGCTGTTACCGGAC	<i>irp2</i>	0.25	264	[19]
irp2-r	TCGTCGGGCAGCGTTTCTCT				
pet-f	ACTGGCGGACTCATGCTGT	<i>pet</i>	0.25	832	[12]
pet-r	GCGTTTTTCCGTTCCCTATT				
set1A-f	TCACGCTACCATCAAAGA	<i>set1A</i>	0.25	309	[12]
set1A-r	TATCCCCCTTTGGTGGTA				
shf-f	ACTTTCTCCCGAGACATTC	<i>shf</i>	0.25	613	[4]
shf-r	CTTTAGCGGGAGCATTCAT				

and chromosome [14]. Detection of *aggR* may identify pathogenic EAEC strains called typical EAEC. In addition, some EAEC strains express putative virulence factors encoded on the chromosome such as *Shigella* enterotoxin1 (*set1A*), [7] and Yersiniabactin biosynthesis gene (*irp2*) [19], which are also thought to be involved in EAEC pathogenicity.

Although, there are reports on isolation of EAEC associated with human diarrhoea in India [1], there seems very limited information available about the virulence genes of EAEC of Indian origin [2, 10]. Thus, the present study was carried out to ascertain the putative virulence gene profile, in vitro adherence pattern to HEp-2 cells and biofilm production of EAEC isolates from diarrhoeic children in India.

Materials and Methods

Bacterial Strains

Fifty-eight typical EAEC strains isolated from children with diarrhoea and maintained by the Division of Veterinary Microbiology and Immunology were used in the present study [9]. The isolates were identified as typical EAEC on the basis of possessing the pCVD32 probe and *aggR* gene. Ten of these isolates also possessed heat-stable enterotoxin1 gene (*astA*).

Extraction of Bacterial DNA

Bacterial strains were grown overnight in Luria–Bertani (LB) broth at 37 °C. One ml of this broth culture was pelleted by centrifugation at 5,000×g for 10 min. The bacterial pellet was resuspended in 200 μl of lysis buffer (20 mmol Tris–HCl, 2 mmol EDTA, 1 % Triton-X). The bacterial cells were lysed by boiling for 10 min in a water

bath. The lysate was centrifuged again as before and the supernatant was used directly as template for PCR.

Screening for Putative Virulence Genes

All the EAEC isolates were screened for the presence of *aap*, *irp2*, *pet*, *set1A* and *shf* genes using specific PCR protocols described earlier. [4, 12, 19]. Details of the primers used are given in Table 1.

The PCR assays were performed in Gene Amp PCR System 2400 thermal cycler (Applied Biosystem, USA) and Flexigene thermal cycler (Techne Inc., UK) using 25 μl reaction volume which contained 1U of Taq DNA Polymerase, 200 μM of each dNTP, 2.5 μl of 10× PCR buffer and 1.5 mM of MgCl₂. The DNA from EAEC O42 strain and distilled water served as positive and negative controls, respectively, for each reaction.

HEp-2 Cell Adherence Assay

The HEp-2 cell adherence assay was carried out with 51 EAEC isolates, while other seven isolates could not be revived. The assay was performed over 6 h period according to Nishikawa et al. [16] with slight modifications. Briefly, monolayer of HEp-2 cells on cover slips in 12 well plates (Nunc A/S, Denmark) was grown in the absence of antibiotics. Bacterial strains were grown overnight statically in LB broth at 37 °C. Before the test, monolayers were washed once with Dulbecco's phosphate buffered saline (DPBS) and two ml of basal Eagle's medium containing D-mannose (1 % w/v) without antibiotics was added to each well. Forty microliters of overnight bacterial culture was inoculated into each well and the plates were inoculated at 37 °C for 3 h. The monolayers were washed three times with DPBS and two ml of the growth medium was added to each well. Again after 3 h of incubation, the monolayer was washed thoroughly again

with DPBS and fixed with absolute methanol. The monolayer was then stained with 10 % (v/v) Giemsa and examined under microscope.

Biofilm Assay

Biofilm formation was measured by using a microtitre plate assay with the crystal violet staining as described by Mohamed et al. [12] with little modification. Briefly, all the 51 viable isolates were grown in LB broth containing 0.25 % glucose overnight at 37 °C in a shaking incubator. The culture was then diluted 1:100 in Dulbecco's Modified Eagles Medium (DMEM) containing 0.45 % glucose. Two hundred microlitre of the bacterial suspension was inoculated into individual wells of a sterile 96 well polystyrene plate (Nunc, Denmark) and incubated for 18 h at 37 °C. After incubation the biofilm was fixed with 200 µl Bouin fixative for 15 min and rinsed once with phosphate buffered saline (PBS). The fixed bacterial cells were then stained with 0.5 % crystal violet for 15 min and rinsed thoroughly with distilled water. After air drying, crystal violet was solubilized in 200 µl of ethanol–acetone (80:20, v/v) for 30 min. The optical density was measured at 550 nm (OD₅₅₀) by using an automatic spectrophotometer (Biophotometer, Eppendorf, Germany). Each assay was performed in duplicate and repeated at three different occasions. EAEC strain O42 served as positive control for the strong biofilm production, and the non-pathogenic *E. coli* strain DH5α (non-biofilm producer) served as negative control. The isolates were considered biofilm producer if, OD₅₅₀ reading exceeded the mean plus two standard deviations of the negative control strain which was recorded as 0.232.

Results

All the EAEC isolates were found to harbour at least one virulence gene studied. The virulence genes under the study were found in 20 different combinations (Table 2). All but seven isolates (87.9 %) were found to harbour the *aap* gene. The *irp2*, *pet*, *set1A* and *shf* genes were detected in 40 (68.9 %), 3 (5.1 %), 23 (39.6 %), and 35 (60.3 %) isolates, respectively. In our study the most frequent gene combination was *aap/irp2/shf* (24.1 %) followed by *aap/irp2/set1A* (12 %), *aap/irp2/shf/set1A* (10.3 %), *aap* (8.6 %), *aap/irp2/astA* (6.8 %), *aap/irp2*, *aap/shf* and *aap/set1A* (5.1 % each) and *aap/shf/set1A* (3.4 %). While *aap/astA*, *aap/irp2/pet*, *aap/irp2/shf/astA*, *aap/irp2/pet/shf/set1A/astA*, *aap/irp2/pet/shf/set1A*, *aap/irp2/shf/set1A/astA*, *aap/shf/astA*, *aap/set1A*, *irp2/shf* and *set1A* combinations were least frequent (1.7 % each).

All the isolates tested exhibited aggregative adherence pattern on HEp-2 epithelial cells in culture. Altogether, 33 (64.7 %) EAEC isolates were found to produce biofilm (Table 2, 3). Of these 33 biofilm-producing isolates, 18 (54.5 %) were from Kashmir while remaining 15 (45.4 %) were from Andhra Pradesh. For the entire group of isolates, the OD₅₅₀ readings ranged from 0.141 to 1.012 U. Higher amount of biofilm (mean OD 0.798 ± 0.013–1.0127 ± 0.005) was produced by isolates that harboured *set1A* along with other genes while highest amount of biofilm (mean OD 1.007 ± 0.005) was recorded in an isolate possessing *aggR*, *aap*, *irp2*, *shf*, *pet*, *set1A* genes. Out of 14 isolates of EAEC possessing *aap*, *irp2* and *shf* virulence genes, six produced biofilm while remaining eight did not.

No correlation was observed between presence of putative virulence genes such as *aggR*, *aap*, *irp2*, *pet*, *shf*, and *astA* and production of biofilm by the EAEC isolates in the present study (Table 3). However, presence of *set1A* was significantly found associated with biofilm production.

Discussion

Though EAEC is an important diarrhoeal pathogen, yet its virulence factors have not completely been characterized. Limited numbers of studies are available that examined the independent roles of many putative EAEC virulence genes in acute diarrhoeal illness. No virulence factor has been identified as common to all EAEC strains. In India, adhesion (fimbrial) variants of EAEC including various putative virulence genes (*aafA*, *aggA*, *agg3A*, *aggR*, *aafC*, *aggC*, *agg3C*, *aap*, *shf*, *astA*, *pet*, *afaBC*, *pic*, *irp2*, and *fliC*) have been reported from Kolkata, only [2, 10].

In the present study, we determined five different virulence genes in addition to *pCVD432*, *aggR* and *astA* in EAEC strains from Kashmir and Andhra Pradesh. Our study showed significant heterogeneity in gene profile among the strains. Besides, *pCVD432* and *aggR*, the most frequent plasmid-borne genes detected among the EAEC isolates were *aap*, *irp2* and *shf*. These findings are in agreement with those of a previous study in Kolkata, India [10] and elsewhere [4, 23]. The *aap* gene is under the control of *aggR*. Earlier finding showed that EAEC isolates carrying *aggR* and *aap* may be phylogenetically or pathogenetically linked [3, 11]. Dispersin is a secreted low-molecular weight protein (10.2 kDa) that coats the bacterial surface and promotes dispersal of EAEC on the intestinal mucosa. Keeping in view the ubiquitous distribution of dispersin protein in EAEC isolates here, it can be exploited as a candidate antigen for vaccine formulation against EAEC induced diarrhoea. However, further studies are needed in this direction for final conclusion. The EAEC strains carrying specific genes under *aggR* control may be

Table 2 Virulence gene profile of EAEC isolates

Serogroup	No. of isolates	<i>aap</i>	<i>irp2</i>	<i>pet</i>	<i>set1A</i>	<i>shf</i>	<i>astA</i>	Biofilm production
O1	1	+	+	–	+	–	–	+
O1	1	–	–	–	+	–	–	ND
O1	1	+	+	–	–	–	+	–
O1	1	+	–	–	–	+	–	–
O9	1	+	+	+	–	–	–	+
O12	1	+	+	–	–	+	–	+
O12	1	+	–	–	+	+	–	+
O27	1	+	+	–	–	+	+	+
O27	1	+	+	–	–	–	–	–
O44	1	+	+	–	+	+	–	+
O44	1	+	+	–	+	–	–	ND
O51	1	+	+	–	+	+	–	+
O73	1	+	+	–	+	+	–	+
O86	1	+	–	–	–	–	–	+
O86	1	+	–	–	–	+	–	+
O86	1	+	+	–	+	–	–	+
O86	1	–	–	–	+	+	–	ND
O97	1	+	+	–	–	–	–	–
O97	1	+	–	–	+	+	–	+
O100	1	+	+	–	–	+	–	–
O100	1	+	+	–	–	–	+	+
O101	1	+	+	–	–	+	–	–
O127	1	+	+	–	+	+	–	ND
O127	1	+	+	–	–	–	+	–
O127	1	+	+	+	+	+	+	+
O127	1	+	+	–	+	+	+	+
O153	1	+	–	–	–	–	–	–
O153	1	+	–	–	–	+	–	–
O153	1	+	+	–	–	+	–	–
O153	2	+	+	–	–	+	–	+
O153	1	+	+	–	–	+	–	–
O164	1	+	+	–	+	–	–	ND
O164	1	+	–	–	–	–	–	+
Rough	1	+	–	–	–	–	–	–
Rough	2	–	–	–	+	+	–	–
Rough	1	+	+	+	+	+	–	+
Rough	1	+	+	–	–	+	–	–
UT	1	+	–	–	+	+	+	+
UT	3	+	+	–	–	+	–	+
UT	1	+	–	–	–	–	+	+
UT	1	+	+	–	–	–	+	+
UT	1	+	–	–	–	–	–	+
UT	1	+	–	–	–	+	+	+
UT	1	+	+	–	–	+	–	–
UT	1	+	+	–	–	–	–	+
UT	2	+	+	–	–	+	–	ND
UT	1	–	+	–	–	+	–	–
UT	2	+	+	–	+	+	–	+

Table 2 continued

Serogroup	No. of isolates	<i>aap</i>	<i>irp2</i>	<i>pet</i>	<i>set1A</i>	<i>shf</i>	<i>astA</i>	Biofilm production
UT	3	+	+	–	+	–	–	+
UT	1	+	–	–	+	–	–	–
Total	58	53	40	03	23	35	10	33

Table 3 Biofilm production and virulence genes of EAEC isolates from diarrhoeic children in India

Gene	No. of isolates		<i>P</i> *
	Biofilm ⁺ (<i>n</i> = 33)	Biofilm [–] (<i>n</i> = 18)	
<i>aggR</i>	33 (100)	18 (100)	
<i>aap</i>	33 (100)	16 (88.8)	0.12
<i>irp2</i>	23 (69.6)	12 (66.6)	1.00
<i>pet</i>	3 (9.0)	0 (0)	0.54
<i>set1A</i>	16 (48.4)	3 (16.6)	0.025
<i>shf</i>	20 (60.6)	12 (66.6)	0.76
<i>astA</i>	8 (24.2)	2 (11.1)	0.46

Figures in parentheses indicate %

* *P* values represent biofilm producers versus biofilm nonproducers (Fisher's exact test)

important alone or in combination with other virulence factors and when present may indicate pathogenic strains of EAEC. It is also evident from the recent data that *aggR* controls both plasmid borne virulence factors and chromosomal genes associated with clinical illness [5]. The chromosomal gene *irp2* was detected in 68.9 % strains. These findings also corroborate with the findings in Kolkata, India [10]. The frequency of other plasmid borne genes (*pet*, and *set1A*) was found to be low. Thus our results indicate that the EAEC isolates were heterogenic in possessing their virulence factors, which has been also observed elsewhere in the world.

The formation of a heavy biofilm may be related to the diarrhoeagenicity of the organisms and perhaps, to their ability to cause persistent colonization and diarrhoea [22]. In the present study 35.2 % isolates did not produce any quantifiable biofilm although all the strains were *aggR* positive. This corroborates with the findings of other workers [17, 20] who also observed no correlation between *aggR* and biofilm formation. However, Mohamed et al. [12] observed that isolates carrying *aggR* alone or in combination with other virulence genes were strongly associated with biofilm formation. Similarly, other virulence genes except *set1A* were also not associated with biofilm formation in the present study.

In summary, there is significant heterogeneity of putative virulence genes among the EAEC isolates of Indian

origin too. A limited number of genes (*pCVD432*, *aggR*, *aap*, *irp2* and *shf*) appeared to be commonly associated with EAEC. No correlation was observed between presence of *aggR*, *aap*, *irp2*, *pet*, *shf* and *astA* genes and biofilm formation by EAEC isolates. However, *set1A* gene was significantly associated with EAEC biofilm formation.

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