Characterization of adhesin variants in Indian isolates of enteroaggregative *Escherichia coli*

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Abstract

Enteroaggregative *Escherichia coli* (EAEC) are causative agents of diarrhea, being characterized by aggregative adherence to cultured epithelial cells. In this study, phenotypic properties of EAEC were analyzed with respect to AA, hemagglutination, clump and biofilm formation, all of which are mediated by aggregative adherence fimbriae (AAF). The strains were also screened for AAF types, AAF adhesin variants and Dr adhesin by PCR. Of the three known AAF types, AAF/I and AAF/II adhesin variants were identified. An association between the AAF/ adhesin genotypes and the subtypes/scores of phenotypic properties was sought and it was observed that strains harboring same adhesins displayed different subtypes/scores and vice versa.

Introduction

Enteroaggregative *Escherichia coli* (EAEC) are increasingly recognized as an emerging pathotype responsible for persistent and acute diarrhea in both developing and developed countries (Bhan *et al*., 1989; Paul *et al*., 1994; Nataro *et al*., 1998). EAEC are defined by characteristic aggregative adherence (AA) to HEp-2 cells (Nataro *et al*., 1987). However, this definition seems to cover both pathogenic and non-pathogenic EAEC (Kaper *et al*., 2004) as shown in volunteer studies (Nataro *et al*., 1995) and natural cases of the disease (Bhan *et al*., 1989; Fang *et al*., 1995).

Adherence is an early step in diarrheagenic *E. coli* infections that is mediated by fimbrial/afimbrial adhesins (Torres *et al*., 2005). Most of the EAEC strains harbor a 60–65 MDa plasmid (pAA), which has an operon encoding aggregative adherence fimbriae (AAF) that are responsible for the AA phenotype, hemagglutination (HA) of human erythrocytes, clump and biofilm formation (Nataro *et al*., 1992). So far, three types of AAF – AAF/I/II/III (Nataro *et al*., 1992; Czeczulin *et al*., 1997; Bernier *et al*., 2002) have been reported. AAF are distantly related members of the Afa/Dr family of adhesins but its not known whether they recognize the Afa/Dr receptors which are the decay accelerating factor (CD55) and type IV collagen (Nowicki *et al*., 2001; Servin, 2005). The genes encoding AAF/I are separated into two regions (Nataro *et al*., 1992). Region 1 contains a cluster of four genes, *aggDCBA*, encoding for the chaperone (AggD), usher (AggC), invasin (AggB) and the adhesin/fimbrial subunit (AggA). Region 2 encodes an AraC-like regulator, AggR that is required for AAF/I expression (Nataro *et al*., 1994). AAF/II is genetically, phenotypically and morphologically distinct from AAF/I (Czeczulin *et al*., 1997; Elias *et al*., 1999a). Here, region 1 consists of three genes, *aafADR* encoding for the adhesin (AafA), chaperone (AafD) and the transcriptional activator (AggR) whereas region 2 consists of genes encoding for silent chaperone (AafD′), usher (AafC) and invasin (AafB). The organization of AAF/III operon is similar to that of AAF/I (Bernier *et al*., 2002). Presence of AAF adhesin variants has been indicated in a few studies (Rich *et al*., 1999; Gioppo *et al*., 2000; Bernier *et al*., 2002).

Recently, AA subtypes have also been reported (Gioppo *et al*., 2000; Sarantuya *et al*., 2004) but the role of AA subtypes, AAF types or AAF adhesin variants in EAEC

pathogenesis is not known. Also, no information is available about the type of fimbriae/adhesin variant that is responsible for a given AA subtype. As the results obtained from adherence assays vary from one laboratory setting to another, this information on association of adhesin types with AA subtypes will be useful in identification of subset of pathogenic EAEC. Keeping this in view, the present work was designed to examine the type of the adhesins/adhesin variants expressed by Indian isolates of EAEC, and to look for an association if any, with AA subtypes and other phenotypic characteristics of EAEC.

Materials and methods

Bacterial isolates

Escherichia coli strains were isolated from stools of 170 children (age < 5 years) suffering from diarrhea attending PGIMER, Chandigarh, India. All samples were processed by routine microbiological and biochemical tests. Twenty-seven EAEC strains were obtained from NICED, Kolkata, India. EAEC strains 17-2 (Vial *et al.*, 1988), 042 (Nataro *et al.*, 1985) and 55989 (Bernier *et al.*, 2002) were used as positive controls. *Escherichia coli* strain (166) isolated in this study was used as Afa/Dr adhesin control. Four AAF/I strains were provided by C. Le Bouguenec (Bernier *et al.*, 2002). *Escherichia coli* strain DH5α was used as a recipient strain for the recombinant plasmids and pBluescript was used in cloning experiments.

Clump formation and hemagglutination

Clump formation was checked by the method of Albert *et al.* (1993). The strains were inoculated in trypticase soy broth (TSB) and incubated for 16–20 h at 37 °C under shaking conditions. Hemagglutination was carried out as described by Yamamoto *et al.* (1991) with minor modifications. *Escherichia coli* grown overnight in TSB under static conditions were suspended in phosphate-buffered saline (PBS, pH 7.4). In a 96 well round bottom plate (Greiner, Frickhausen, Germany) 25 μL of bacterial suspension was mixed with an equal volume of 3% (v/v) erythrocytes in PBS containing 1% D-mannose. Hemagglutination was scored as 3+, 2+, 1+ and − (negative) by formation of a mat, a large ring with a center mat, a small ring with a center mat and a solid button, respectively.

Adherence assay and α-hemolysin production

Adherence to HeLa cells was assessed by the method of Cravioto *et al.* (1979). The AA pattern was further subgrouped as: (i) typical adherence pattern – AAt, (ii) low level of aggregative adherence – AAII, (iii) chain-like adherence – CLA and (iv) mixed phenotype – AA/DA (Gioppo *et al.*, 2000; Bernier *et al.*, 2002). For analyzing α-hemolysin production, strains were cultured on blood agar supplemented with 5% defibrinated sheep erythrocytes washed in PBS with 10 mM $CaCl_2$. Plates were examined for zones of hemolysis after 3 and 24 h of incubation at 37 °C (Beutin, 1991).

Biofilm formation

Light microscopic examination and spectrophotometric quantification of biofilm formation was performed as described by Sheikh *et al.* (2001). It was scored as, 1+: partial honeycomb formation, 2+: completely connected biofilm with rare three-dimensional mounds, $> 2+$: partially connected biofilm with three-dimensional mound formation in all the fields with the substratum visible, 3+: significant three-dimensional mound formation in all the fields with the substratum visible and 4+: substratum completely covered.

Standard molecular techniques

All DNA manipulations were performed by standard techniques (Sambrook *et al.*, 1989). The AAF/I adhesin variant $aggA_I$ was amplified from EAEC strain R2 and was cloned in pBluescript SKII (Stratagene, CA) by blunt end ligation.

Screening for diarrheagenic *E. coli* was performed by multiplex/single gene PCR (Le Bouguenec *et al.*, 1992; Chakraborty *et al.*, 2001). *Escherichia coli* strains positive for EAEC specific/pCVD432 PCR (Schmidt *et al.*, 1995) amplifying a region corresponding to a component of putative ATP-binding cassette transporter apparatus, *aatA* (Nishi *et al.*, 2003) and/or showing AA to HeLa cells were analyzed for genes encoding the transcriptional regulator (*aggR*), adhesins and ushers of AAF operons (Czeczulin *et al.*, 1999; Bernier *et al.*, 2002; Kahali *et al.*, 2004) and Dr adhesin (Le Bouguenec *et al.*, 1992). To amplify *aafA*, primers F-5′CCAACACCATTTTATATAAACTT3′ and R-5′ AACTCATATCAGATATCACAGATA3′ (GenBank Accession no. AF012835) were designed from *aafD* and *aggR* regions that flank *aafA* and the 1.8 kb product thus obtained was designated as *aafDR*.

PCR was carried out with *Taq* polymerase for detection assays. High-fidelity PCR master mix. or Expand high-fidelity PCR system (Roche Molecular Biochemicals, Germany) was used to get amplicons to be cloned and/or sequenced.

DNA sequencing, bioinformatics analysis and nucleotide sequence accession number

The double-stranded DNA was sequenced using ABI PRISM Big dye terminator cycle sequencing ready reaction kit (Perkin-Elmer ABI, Foster City, CA). The sequencing primer used for obtaining *aafA* sequence from *aafDR* amplicon was 5′-TACTGGACCACCGAAATGGCCATTCT-3′ whereas

for sequencing *aggA, agg3A, agg3C*, and *afaBC* amplicons the forward primers from the published PCR primer pairs were used (Le Bouguenec *et al.*, 1992; Czeczulin *et al.*, 1999; Bernier *et al.*, 2002). Sequencing data were analyzed with ABI version 3.0.1b3 software and were compared with previously published sequences using BLASTN and BLASTX computer programs at the National Centre for Biotechnology Information. Multiple sequence alignments were performed by CLUSTAL W program. The GenBank accession number for AAF/I adhesin variant $aggA_I$ reported in this study is AY344586.

Decay-accelerating factor (DAF) clustering assay

Decay-accelerating factor clustering assay was carried out by the method of Goluszko *et al.* (2001) with minor modifications. HeLa cells grown on sterile coverslips were incubated with overnight grown bacteria for 1 h and were then fixed in 3.7% formalin in PBS. Next, cells were incubated with polyclonal DAF antibody at room temperature (RT) for 1 h and subsequently with fluorescein isothiocyanate-conjugated goat antirabbit IgG. The slides were observed under a fluorescent microscope (×100 magnification).

Results and discussion

Enteroaggregative *Escherichia coli* is a diverse pathotype that encode various factors mediating AA to epithelial cells (Nataro *et al.*, 1987). The adherence assay, though it is cumbersome and results vary from one laboratory to another, remains the gold standard method for identification. This can be overcome by identification of genetic factors responsible for AA. AA is mediated by AAF, which are of three main types AAF/I/II/III (Nataro *et al.*, 1992; Czeczulin *et al.*, 1997; Bernier *et al.*, 2002). A number of groups (Rich *et al.*, 1999; Gioppo *et al.*, 2000; Bernier *et al.*, 2002) have indicated the occurrence of adhesin variants among AAF operons and recently, subtypes of AA have also been described (Gioppo *et al.*, 2000; Bernier *et al.*, 2002; Sarantuya *et al.*, 2004). However, the role of AAF/adhesin variants or AA subtypes in pathogenesis and an association between them is yet unknown. This study was designed to identify the AAF/AAF adhesin variants found in Indian isolates of EAEC and to analyze association between the AAF adhesin genotypes and phenotypes encoded by them.

Identification of EAEC strains

Escherichia coli isolated from stool samples were analyzed for adherence patterns. Seven percent (12) of strains isolated from PGIMER, Chandigarh showed AA pattern, 2.9% (5) strains displayed diffuse adherence and only 0.6% (1) strains showed localized adherence. All aggregative strains obtained from PGIMER (12) and NICED, Kolkata (27) were subgrouped and AAt, AAII, CLA and AA/DA patterns were displayed by 41% (16), 28% (11), 7.7% (3) and 5.1% (2) strains, respectively. 7.7% (3) strains were nonadherent and 7.7% (3) strains adhered in an unidentifiable manner (UDA) (Table 1). The percentage of various AA subtypes in different studies is difficult to correlate as AA has been subgrouped in diverse manner, however, as observed in these studies (Gioppo *et al.*, 2000; Bernier *et al.*, 2002; Kahali *et al.*, 2004; Sarantuya *et al.*, 2004) we also found a higher percentage of AAt strains (Table 1). Cell-detaching property and α-hemolysin production was observed in only 1 strain (Table 1). α-hemolysin producing strains were not found in a recent study on Indian EAEC isolates (Kahali *et al.*, 2004) and in another study (Gomes & Marques, 1995) only 13% strains were found to produce α-hemolysin which shows that this factor probably does not have a major role in EAEC pathogenesis.

Ninety-seven percent (38) of the strains that displayed any one of the four AA patterns showed positive results with pCVD32/*aatA* PCR (Table 1) revealing concordance in PCR and adherence assay. Few strains though PCR positive were found to be nonadherent which could be owing to lack of AAF expression and this needs to be analyzed further. The correlation of pCVD32 probe/PCR positivity with AA phenotype varies geographically (Fang *et al.*, 1995; Bernier *et al.*, 2002) but it has been found highly sensitive and specific in strains from Chile and India (Paul *et al.*, 1994; Dutta *et al.*, 1999).

Analysis of AAF-encoded phenotypes

Clump formation ability was scored as 1+, 2+ and 3+ on the basis of thickness of the scum (Fig. 1) (Table 1). 84.6% (33) strains showed clump formation. 17.9% (7) strains gave 3+ score, 41% (16) showed 2+ score, and 28.2% (11) displayed 1+ score. Clump formation correlated with AA to some extent but not with AA subgroups or EAEC-specific

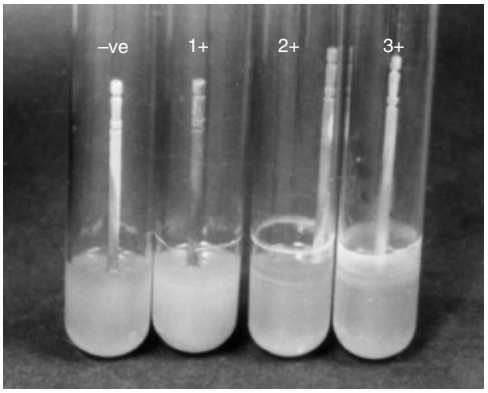

Fig. 1. Scoring of clump formation ability in enteroaggregative *Escherichia coli* strains by means of the thickness of the scum formed at the side of the test tube.

Table 1. Virulence profiles of EAEC strains

Strain	pCVD32 PCR	HEp-2 assay	Clump formation	Biofilm formation	HA	*aggA*	*aggC*	*aafC*	*aafA/aafDR*	*agg3C*	*agg3A*	*aggR*
AAFI												
17-2	+	CDEC	+	2+	3+	+	+					+
R2	+	AAII	+	+	2+	+	+					+
120	+	AAII	3+	3+	2+	+	+					+
A44*	+	AAII	3+	3+	2+	+	+					+
6602*	+	AAII	2+	2+	2+	+	+					+
SDC21*	+	AAt	2+	3+	3+	−	+					+
BCH152*	+	AAt	+	3+	2+	−	+					+
11006†	+	AAt	+	2+	2+	+	+					+
384P†	+	AAt	2+	3+	2+	−	+					+
56390†	+	AAt	+	3+	+	+	+					+
645125†	+	AAt	2+	2+	2+	+	+					+
AAFII												
042	+	AAt	2+	3+	3+			+	+/+	+		+
T7*	+	AAt	+	+	3+			+	+/+	+		+
T8*	+	AAt	3+	3+	+			+	+/+	+		+
T53*	+	CLA	3+	3+	2+			+	+/+	+		+
T320*	+	AAt	3+	3+	3+			+	−/+	+		+
6894*	+	CLA	3+	> 2+	2+			+	−/+	+		+
179	+	AAII	2+	2+	2+			+	+/+	+		+
590	+	UDA	2+	2+	2+			+	+/+	+		+
AAFIII												
55989	+	AAt	3+	3+	3+					+	+	+
502(3)	+	AAt	2+	3+	3+					+	+	+
444	+	UDA	+	> 2+	2+					+	−	+
497	+	AAII	2+	2+	2+					+	−	+
245	+	UDA	2+	2+	2+					+	−	+
7383*	+	AAt	2+	3+	2+					+	−	+
SDC8*	+	AAII	2+	−	−					+	−	+
BCH157*	+	AAt	2+	+	+					+	−	+
BCH267*	+	NA	−	2+	−					+	−	+
BCH248*	+	CLA	3+	3+	2+					+	−	+
125(1)	+	CDEC	2+	+	2+				−/+	+	−	−
OTHERS												
A43*	−	AAt	+	> 2+	+							+
A103*	+	AAt	+	2+	3+							+
A104*	+	AAII	+	2+	2+							+
A105*	+	AAt	+	3+	3+							+
A106*	+	AAII	+	−	3+							+
7062*	+	AAII	+	> 2+	−							+
503(2)	+	AA/DA	2+	3+	2+							+
125(2)	+	AA/DA	−	2+	−							+
129	+	AAt	2+	3+	2+							+
Com57*	+	NA	−	2+	+							+
Com58*	+	AAt	−	−	2+							+
SDC16*	+	NA	−	−	2+							−
SDC24*	+	AAII	2+	+	2+							−
SDC25*	+	AAt	2+	−	−							+
SDC46*	+	AAt	2+	2+	+							+
SDC55*	+	AAt	+	−	−							+

*Strains obtained from NICED, Kolkata, India.

†Strains obtained from Institut Pasteur, France.

EAEC, enteroaggregative *Escherichia coli*; HA, hemagglutination; AAt, typical adherence pattern; AAII, low level of aggregative adherence; CLA, chain-like adherence; AA/DA, mixed phenotype; UDA, strains adhered in an unidentifiable manner.

PCR (Table 1). Hemagglutination was evident in 85% (33) of the EAEC strains. This could be further divided into a gradation of hemagglutination i.e. 3+, 2+ and 1+ hemagglutination score. On this basis, 17.9% were characterized as being 3+ whereas 53.8% and 15.4% of the strains were 2+ and 1+, respectively. Most of the strains with a 3+ score expressed the AAt phenotype.

Biofilm formation was observed in 84.6% (33) of the EAEC strains (Table 1). A 3+, > 2+, 2+ and 1+ score was again devised. The majority of the strains (33.3%) had a score of 3+ in the biofilm assay. The percentage of the strains scoring > 2+, 2+ or 1+ was 28.2%, 10.3% and 12.8%, respectively. A previous study also defined different biofilm scores among EAEC strains (Sheikh *et al.*, 2001). The ability to grow as biofilms did not correlate with AA patterns or EAEC-specific PCR (Table 1). A possible explanation could be the preferential AA of the strains to culture cells or coverslips (Gomes *et al.*, 1998; Gioppo *et al.*, 2000). The former will show at least one of the AA phenotypes whereas latter would give good biofilm and clump formation scores. But again the biofilm and clump formation could be related partially thus, and an increased number of strains need to be screened to explain this.

Analysis of AAF operons, AAF adhesin variants and Dr adhesin

On analyzing the EAEC strains for fimbrial operons, the transcriptional regulator (*aggR*) was detected in 92.3% (36) strains (Table 1), which is similar frequency to earlier reports (Czeczulin *et al.*, 1999; Kahali *et al.*, 2004; Zamboni *et al.*, 2004). The ushers of AAF operons *aggC, aafC* and *agg3C* were found in 15.4% (6), 18.0% (7) and 43.6% (17) isolates, respectively. But the adhesin genes *aggA, aafA* and *agg3A* could only be detected in 10.3% (4), 12.8% (5) and 2.6% (1) strains, respectively (Table 1). Thus, the accessory genes of the operons were found to be conserved whereas the adhesin gene displayed heterogeneity. These results are similar to those shown in a few previous studies (Rich *et al.*, 1999; Gioppo *et al.*, 2000; Bernier *et al.*, 2002; Kahali *et al.*, 2004). A large percentage (61%) of the strains did not possess show any of the AAF operons and perhaps encode a yet unidentified AAF. Future investigations should therefore target the identification of other fimbrial types.

Interestingly, a 485 bp product could be amplified from all AAF/II and diffusely adherent *E. coli* (DAEC) strains (data not shown) with the *agg3C* primers (Table 1). Digestion of this amplicon with *PstI* enzyme resulted in fragments of same size in AAF/II and DAEC strains that were different from those shown by rest of the strains including AAF/III prototype strain 55989 (Fig. 2a). Thus, AAF/II and AAF/III strains could be detected with *agg3C* primers and then differentiated by digestion with *Pst*I. On the basis of the digestion pattern only 10 strains were actually found to harbor AAF/III operon.

Using the *afaBC* primers a ~710 bp amplicon could be amplified in all AAF/II isolates whereas a 750 bp amplicon was obtained in case of DAEC strain. In a previous study, the DAEC probe generated similar results (Elias *et al.*, 1999b). The BLASTN search of this ~710 amplicon showed a

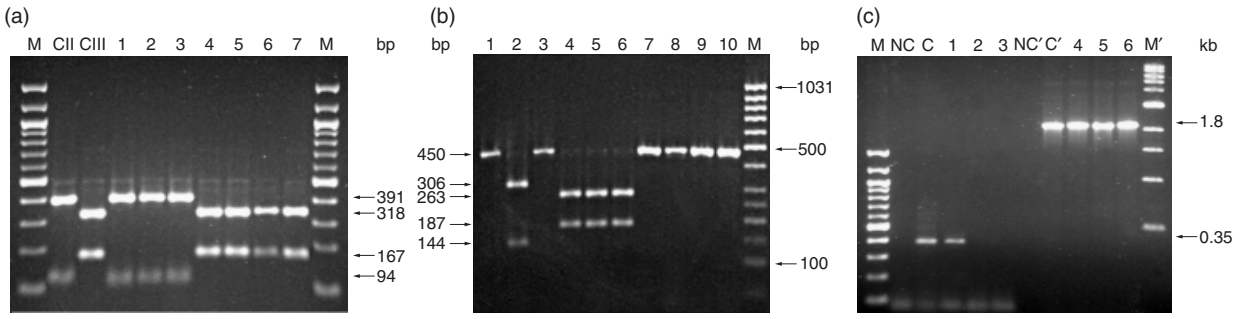

Fig. 2. (a) Gel electrophoresis showing *Pst*I restriction fragment length polymorphism (RFLP) analysis in *agg3C* gene fragments from representative enteroaggregative *Escherichia coli* (EAEC) strains harboring AAF/II or AAF/III operons and diffusely adherent *E. coli* (DAEC) strain. Lane M: 100 bp ladder [New England Biolabs (NEB), MA]; Lane CII: Prototype AAF/II strain (042) showed fragments of 391 and 94 bp; Lane CIII: Prototype AAF/III strain (55989) showed fragments of 318 and 167 bp; Lane 1: DAEC strain (166) also showed 391and 94 bp fragments as obtained in case of AAF/II strains; Lanes 2–3: Representative AAF/II strains (T7 and 590, Table 1); Lanes 4–7: Representative AAF/III strains (502(3), 444, BCH 248 and 7383, Table 1). (b) Gel electrophoresis showing results for *Hinf*I RFLP analysis in *aggA* amplicons (UD, undigested; D, digested). Lane 1: UD Prototype AAF/I strain (17-2) showed a 450 bp *aggA* amplicon; Lane 2: D 17-2 *aggA* showed 306 and 144 bp fragments; Lanes 3–6: Indian EAEC isolates; Lane 3: UD representative Indian isolate R2 showed a 450 bp *aggA* amplicon; Lane 4: D R2; Lane 5: D 120; Lane 6: D 44 all three of these showed 263 and 187 bp fragments; Lanes 7–10: Isolates from Institute Pasteur; Lanes 7 and 9: UD 11006 and 56390 showed a 450 bp *aggA* amplicon; Lanes 8 and 10: D 11006 and 56390 showed a 450 bp product that could not be digested by the enzyme; Lane M: 50 bp ladder (SM0373, MBI Fermentas, Canada). (c) PCR amplification with *aafA* (Lanes NC, C, 1–3) and *aafDR* (Lanes NC′, C′, 4–6) primers in representative AAF/II harboring EAEC isolates. Lane M: 100 bp ladder (NEB); Lanes NC and NC′: Negative control; Lane C & C′: Prototype AAF/II starin (042); Lanes 1 and 4: T7, Lanes 2 and 5: 6894, Lanes 3 and 6: T320 (Table 1); Lane M′: 1 kb ladder (NEB).

sequence corresponding to *aafC* region (2697–3401) of AAF/II operon. However, AAF/II strains did not display the clustering of bacteria in the DAF clustering assay, which is a characteristic feature of DAEC strains that express Dr adhesin (Goluszko *et al.*, 2001). Although AAF/II+EAEC strains carry sequences homologous to Dr operon but they do not recognize DAF as the receptor.

To identify adhesin variants in AAF/I strains, *Hinf*I restriction fragment length polymorphism (RFLP) of *aggA* amplicons was carried out. Fragments of same length were obtained in case of all Indian isolates and one of the strains from Institut Pasteur, Paris. However, this pattern was different from that of AAF/I control strain, 17-2. Further, *aggA* amplicons obtained from other two strains from Institut Pasteur, Paris, could not be digested (Fig. 2b). The *aggA* amplicon from one of the Indian isolates (R2) was cloned and sequenced. The BLASTN analysis of this clone showed 89% and 86% identity to 17-2 *aggA* and a previously reported variant $aggA_{457}$ (Rich *et al.*, 1999), respectively. Sequencing and BLASTN analysis of all the *aggA* amplicons

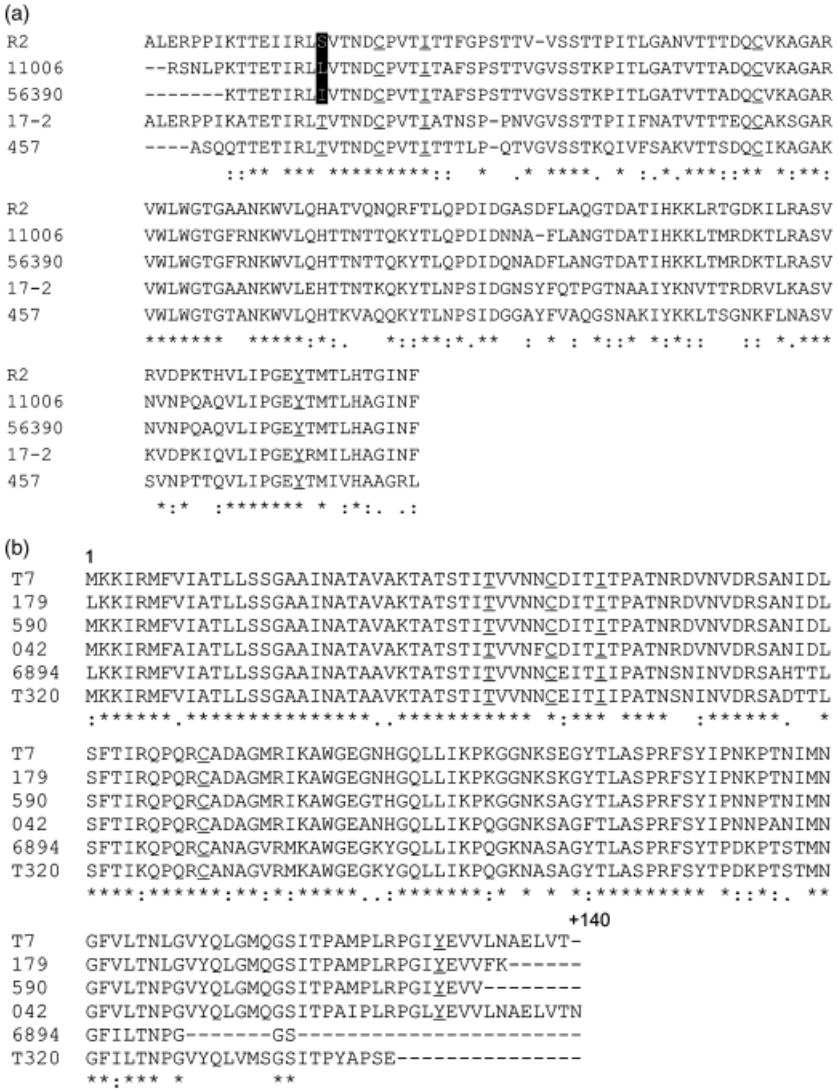

Fig. 3. (a) Sequence alignment of predicted amino-acid sequences derived from the genes encoding AggA from different AAF/I harboring enteroaggregative *Escherichia coli* (EAEC) strains (R2 as the representative $AggA_I$ harboring strain). The threonine residue in the prototype AAF/I strain – 17-2 has been replaced by serine, leucine and isoleucine in R2, 11006 and 56390 strains, respectively. These residues are depicted in white on a black background. '*' and ':', '::' below denote identical and related residues, respectively. (b) Sequence alignment of predicted amino-acid sequences derived from the genes encoding AafA from different AAF/II harboring EAEC strains. The whole AafA sequences along with the signal sequence are shown. The AafA sequences of Indian EAEC isolates are shorter than that of the control strain (042) because of the truncation during sequencing reaction. Strains T7 and T8 showed identical AafA sequence and T7 is shown as the representative strain.'*' and ':', '::' below denote identical and related residues, respectively.

showed that *aggA* from the Indian isolates and one from Institute Pasteur (645125) had the same sequence as seen in case of R2, this *aggA* variant was designated as $aggA_I$. Strains 11006 and 56390 have almost identical *aggA* sequences and their closest homologs were found to be *aggA* of 17-2, $aggA_I$ and $aggA_{457}$ with 84%, 87% and 86% identities, respectively. Multiple sequence alignment data of the predicted AggA amino-acid sequence (Fig. 3a) defined residues characteristic of Dr family of adhesins (Rich *et al*., 1999). In case of 11006, 56390 and all $aggA_I$ strains threonine at position 44 was found to be substituted by, isoleucine, leucine and serine, respectively (Fig. 3a). The significance of these replacements needs to be explored further.

To identify the *aafA*-like allele, amplification was carried out with *aafDR* primer pair (Fig. 2c). Sequencing and BLASTN analysis of these amplicons revealed that five strains (Table 1), which had earlier shown amplification with *aafA* primers, have 96–97% identity to the *aafA* of the prototype strain 042. The other two strains (6894 and T320) from which a PCR product could be amplified with *aafDR* but not with *aafA* primers (Table 1), had 89% identity to *aafA* of the prototype strain 042 and thus harbor an *aafA* variant. Thus, this novel PCR assay can be used to identify *aafA* variants. The multiple sequence alignment data of the predicted amino-acid sequences of AafA adhesins (Fig. 3b) highlighted the variable residues. The residues characteristic of Dr family of adhesins were found to be conserved in AafA adhesins as well.

In case of AAF/III strains the adhesin *agg3A* could be amplified in only one strain. So, either rest of the strains express an *agg3A* variant or it is also possible that as the *agg3C* amplicon represents a conserved region of AAF operon (Bernier *et al*., 2002), these strains might harbor a novel AAF operon. In one of these strains {125(1)} *aafDR* was also detected so either this strain harbors AAF/II and AAF/III operon or it has a AAF operon that has homology to both operons.

As regards the AAF/I and AAF/II strains, AA phenotype, clump and biofilm formation scores could not be correlated to the adhesin genotype (Table 1; Figs 4 and 5). AAF/III harboring strains also displayed all types of AA patterns and biofilm formation scores (Table 1).

The EAEC adhesins were thus found to be allelic in nature and a few of these have been identified in this study. The accessory genes of the AAF operons were found to be conserved but the adhesin showed heterogeneity**.** Also, like AA phenotype, biofilm and clump formation could also be subgrouped/scored and these properties are probably shared by all members of the AAF family. The differences in phenotypes encoded by adhesins of a given genotype in different strains could be due to differential expression of adhesin as seen in preliminary protein expression studies

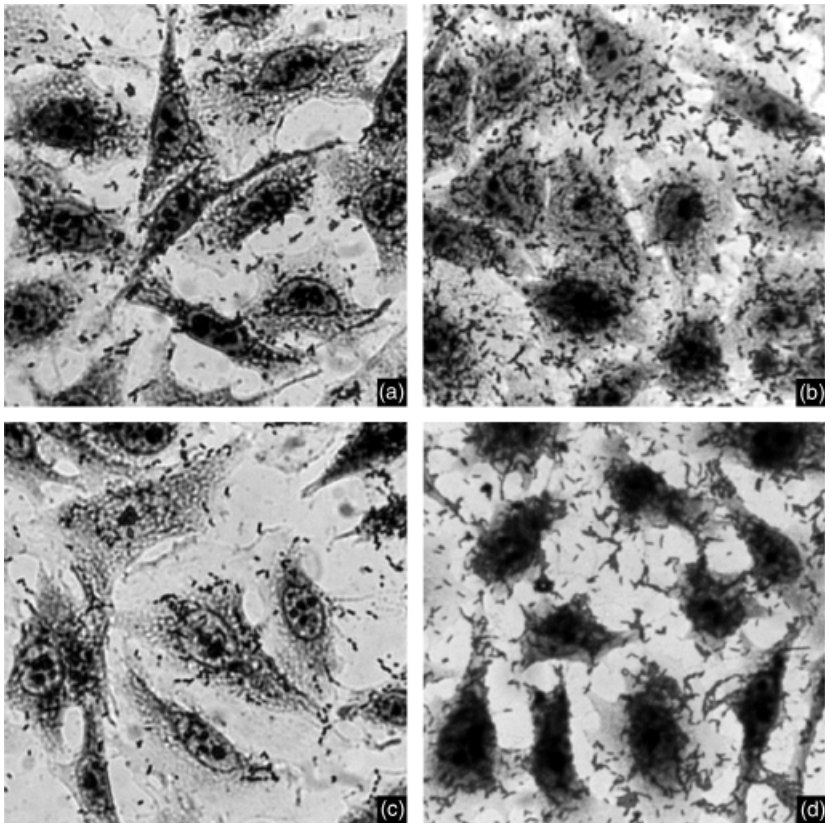

Fig. 4. Enteroaggregative *Escherichia coli* strains harboring identical or almost identical adhesins display different aggregative adherence phenotypes (Table 1, Fig. 3b). (a) T7 and (b) T8 although show the AAt type AA but the patterns are distinct; (c) 6894 and (d) T320 though harbor almost identical *aafA* adhesin they exhibit CLA and AAt, respectively.

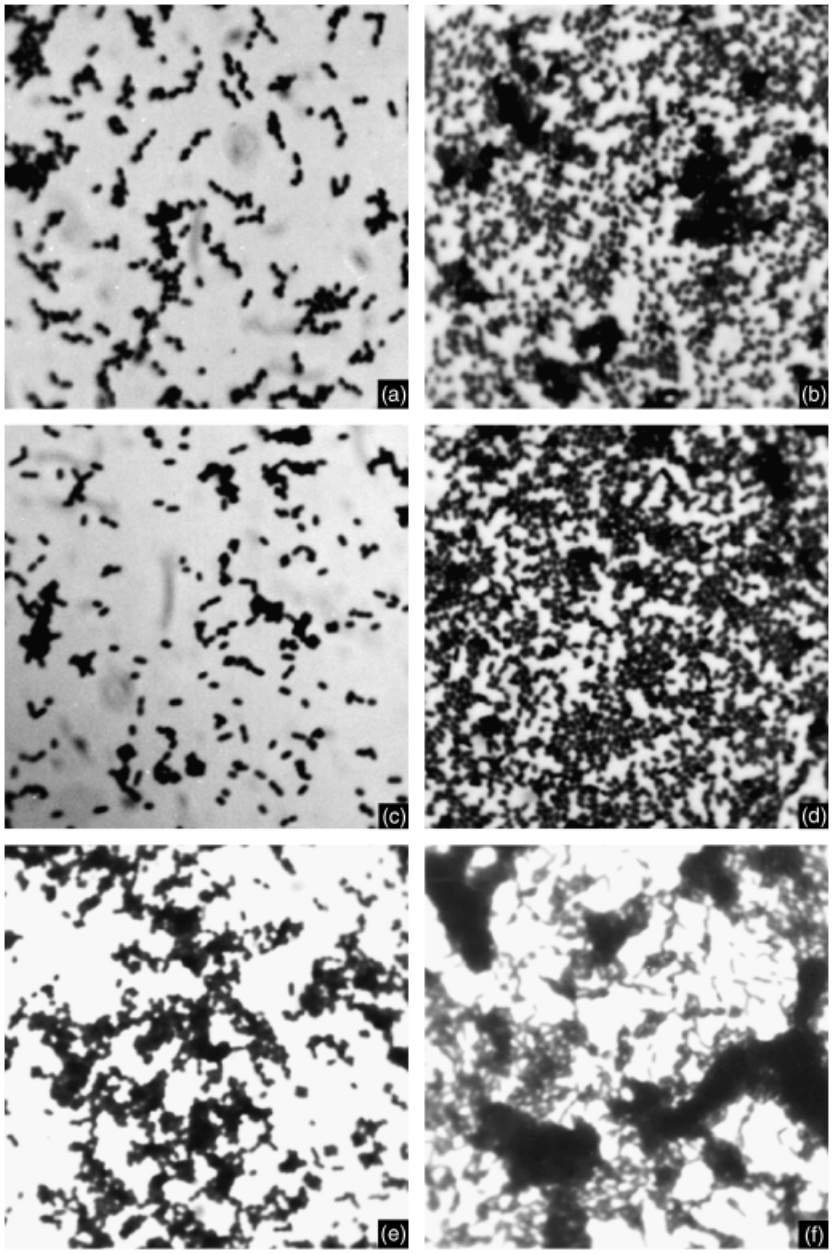

Fig. 5. Enteroaggregative *Escherichia coli* strains with identical adhesin sequences show different biofilm formation scores. Strains (a) R2 and (b) 120 both harbor *aggA*, but show 1+ and 3+ score, respectively (Table 1, Fig. 3a); (c) T7 (1+) and (d) T8 (3+) harbor *aafA* with identical sequence but display a 1+ and 3+ score, respectively (Table 1, Fig. 3b); (e) 6894 and (f) T320 (3+) harbor almost same *aafA* but show > 2+ and 3+ score (Table 1, Fig. 3b).

(data not shown) and this in turn might determine the pathogenic nature of the EAEC strains. Further work in this direction will prove useful not only in identification of pathogenic subset of these strains but also help us to understand their pathogenesis.

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