Prevalence of Different Virulence Factors and Biofilm Production in Enteroaggregative
Escherichia coli Isolates Causing Diarrhea in Children in Ifakara (Tanzania)

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Abstract. This study investigated the prevalence of 19 virulence factors and biofilm production in 86 EAEC isolates
causing diarrhea in children less than 5 years of age from Ifakara, Tanzania. Virulence factors were detected by PCR,
whereas biofilm production was determined using a microtiter plate assay. No virulence factor, with the exception of the
aat gene used to identify EAEC, was detected in 11/86 isolates (12.8%). The most frequently detected virulence factor
was the aggR gene in 53 (61.6%) EAEC, followed by antigen 43 in 33.7%, dispersin in 26.7%, yersiniabactin in 22.1%;
autotransporter Sat in 20.9%; Shigella enterotoxin-1 in 16.3%, and heat-stable toxin-1 in 15.1%. Biofilm was produced
in 66/86 (76%) isolates. AggR was the most prevalent virulence factor in the biofilm-forming group (65% versus 38%,
P = 0.032). These results again show the high heterogeneity of virulence factors among EAEC isolates causing diarrhea
in children, and that biofilm may be an important virulence factor, strongly associated with the presence of AggR.

INTRODUCTION

Diarrheal diseases are a leading cause of morbidity and mortality among children in low-income countries. Although
oral rehydration has been shown to reduce early childhood mortality, diarrhea-specific mortality in children less than 5
years of age in Africa has been estimated at about 10.6 per 1,000.1

Many reports have demonstrated the association of entero-
aggregative E. coli (EAEC) with diarrhea in children in de-
veloping countries.2–7 However, in some studies EAEC was
not related to diarrhea.8,9 Overall, even in studies where
EAEC isolates were statistically associated with diarrhea, the
percentage of controls with this enteropathogen was high.
Some early epidemiologic reports implicated EAEC as a
cause of persistent (≥14 days) diarrhea and it is currently
recognized as an important cause of both acute and persistent
diarrhea.10,11 EAEC have been reported to produce bio-
film12,13 and this can explain, at least in part, the high number
of EAEC isolates in controls and the persistence of EAEC.
Antigen 43 is a surface protein involved in bacterial aggrega-
tion and it has been found that expression of Ag43 dramati-
cally enhances biofilm formation in bacteria.14,15

In addition to biofilm formation, a large number of putative
virulence factors have been identified in EAEC strains. How-
ever, no single factor appears to be consistently present in all
pathogenic strains. Most of the genes encoding these poten-
tial virulence factors are located in the 60–65 MDa pAA plas-
mid, from which a probe and also primers used for detection
of the EAEC by PCR have been designed.16,17 In this plas-
mid, genes encoding adherence fimbria (AAFI and AAFII)
as well as the heat-stable enterotoxin-1 (EASt-1, encoded in
the astA gene) have been detected.18–21 Nataro and col-
leagues22 described a transcriptional factor encoded by the
aggR gene, which controls not only the expression of the
adherence factors (AAFI and AAFII), but also chromosomal
genes.23

The pAA plasmid also carries the aap gene, previously
called aspU, which encodes the dispersin. Dispersin is a se-
creted low-molecular weight protein that promotes dispersal
of EAEC on the intestinal mucosa to establish new foci of
infection and facilitates efficient colonization.24

Other factors, such as a pAA plasmid-encoded toxin (Pet),
which is a serine protease autotransporter protein able to
cleave spectrin, a component of the membrane cytoskele-
ton,25–27 or ShET-1, an enterotoxin first identified in
Shigella flexneri 2a,28 have been referred as virulence factors present
in EAEC isolates.

The aim of this study was to investigate the prevalence of 19 virulence factors, some of which have been previously in-
vestigated in EAEC and some not, and biofilm production in
EAEC isolates causing diarrhea in children less than 5 years
of age with acute diarrhea from Ifakara, Tanzania.

MATERIALS AND METHODS

Subjects and strains. The study was carried out in the town
of Ifakara, in the Kilombero district, in South-western Tan-
zania. This town has 40,000 inhabitants, most of whom are
small-scale farmers. All children less than 5 years of age who
were admitted to St. Francis Hospital in Ifakara, Tanzania
because of diarrhea were recruited during the period from
July to September (dry season). Informed consent was ob-
tained from parents and/or close relatives. The study was re-
viewed and approved by the Ethical Committees of the Hos-
pital Clinic (Barcelona, Spain) in a shared authorization with
the St. Francis Hospital.

E. coli strains were isolated from stool samples of 348 chil-
dren using conventional methods.1,8 Diarrhea was defined as
three or more watery or loose stools over a 24-hour period
prior to admission to the hospital.

Characterization of EAEC isolates and detection of viru-
ulence genes. The EAEC strains were identified by PCR am-
plication of the att gene (previously referred to as CVD432
or AA probe), following primers and conditions previously
described29,30 and have been validated by comparison with
the adherence assay.31 The presence of the aggR gene was
detected by PCR using primers designed in this study. The
primers were evaluated using the E. coli 042 strain as a con-
trol. All the primers used are shown in Table 1. In both cases, the PCR conditions were identical to those described below for the amplification of different virulence factors.

The genes encoding the following virulence factors were detected by PCR: heat-stable toxin (ST), heat-labile toxin (LT), enterogaggregative heat-stable enterotoxin-1 (EAST), Shigella enterotoxins 1 and 2 (ShET1 and ShET2), adherence factors AAF/I and AAF/II, the plasmid encoded toxin (Pet), plasmid-encoded cryptic secreted protein (Aap), autotransported toxin Sat, cytolethal-distending toxin (Cdt), yersiniobactin (Fyu), Antigen 43 (Ag43), enteropathogen adherence factor (EAF), intimin (eae), the bundle-forming pili (bfp), bactin (Fyu), Antigen 43 (Ag43), enteropathogen adherence factor (EAF), intimin (eae), the bundle-forming pili (bfp), and the verotoxins VT1 and VT2.

The _agn43_ gene, encoding antigen 43 was amplified using the primers Ag43-F 5' -ACGCACAACCATCAATAAAA-3' and Ag43-R 5' -CCGCCTCCGATCTGAGTCG-3'. Briefly, one colony of each isolate was suspended in 25 μL of sterile water and boiled for 10 minutes. Twenty-five μL of reaction mixture containing 20 mM Tris-HCL (pH 8.8), 100 μL of sterilized water and boiled for 10 minutes. Twenty-five μL of reaction mixture containing 20 mM Tris-HCL (pH 8.8), 100 mM KCL, 3.0 mM MgCl2, 0.1% gelatin, 400 μM dNTPs, and 1 μM of each primer were added, together with 2.5 units of _Taq_ polymerase. The reaction mixture was overlaid with a drop of mineral oil and subjected to the following program: 30 cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, except for amplification of _cdtB_, which was amplified with an annealing temperature of 59°C. The PCR products were detected by electrophoresis on 2% agarose gel and stained with ethidium bromide (0.5 mg/L). At least one amplified product of each different PCR performed was sequenced, to verify the correctness of the amplified products. Controls carrying the specific genes were included in each PCR.

**Quantitative biofilm assay.** Biofilm assay was carried out using minimal glucose medium (M63). The strains were grown overnight in LB medium at 37°C without shaking. An aliquot (1.25 μL) of the overnight culture was subcultured in 125 μL of M63 medium with 1% of LB in each well of a polystyrene microtiter plate and incubated at 30°C overnight without shaking. A 1.25 μL of each culture was subcultured again in 125 μL of M63 medium in a new polystyrene microtiter plate, and incubated as cited above. After 24 hours, the culture was removed turning the plate upside down and the biofilm was stained with ethidium bromide (0.5 mg/L). At least one ampiclon was observed.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Virulence factor</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>aafA</em></td>
<td>Fimbria AAF/II</td>
<td>5'-GGGATTGCTACTACCATTA-3'</td>
<td>457</td>
<td>29</td>
</tr>
<tr>
<td><em>astA</em></td>
<td>Heat stable toxin-1</td>
<td>5'-ATGACTCTCTAATCATC-3'</td>
<td>110</td>
<td>30</td>
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<tr>
<td><em>fuuA</em></td>
<td>Yersiniabactin</td>
<td>5'-AGTTAGATGCCGACGGGAA-3'</td>
<td>880</td>
<td>40</td>
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<tr>
<td><em>raf</em></td>
<td>Enteropathogen adherence factor</td>
<td>5'-CAAGGTAAAGAAGAGATGATAA-3'</td>
<td>397</td>
<td>29</td>
</tr>
<tr>
<td><em>cdtB</em></td>
<td>Cytolethal distending toxin</td>
<td>5'-GCAAATGAGCAGATGACGA-3'</td>
<td>430</td>
<td>49</td>
</tr>
<tr>
<td><em>Bfp</em></td>
<td>Bundle-forming pili</td>
<td>5'-TGGACGAGGAGGAAAGCGAGC-3'</td>
<td>260</td>
<td>29</td>
</tr>
<tr>
<td><em>eae</em></td>
<td>Intimin</td>
<td>5'-CGGAGCGAGCAGGAAAGCGAG-3'</td>
<td>881</td>
<td>29</td>
</tr>
<tr>
<td><em>lt</em></td>
<td>Heat labile toxin</td>
<td>5'-GAAAATCTGACGATCAGGAC-3'</td>
<td>322</td>
<td>29</td>
</tr>
<tr>
<td><em>st</em></td>
<td>Heat stable toxin</td>
<td>5'-GAAATGACGATCAGGAC-3'</td>
<td>147</td>
<td>29</td>
</tr>
<tr>
<td><em>pet</em></td>
<td>Plasmid encoded toxin</td>
<td>5'-ACCTGGCGACCTTCTTCGTTG-3'</td>
<td>832</td>
<td>30</td>
</tr>
<tr>
<td><em>set1A</em></td>
<td>Shigella enterotoxin-1</td>
<td>5'-TCAGCTAATCAAGAAGACGATG-3'</td>
<td>309</td>
<td>29</td>
</tr>
<tr>
<td><em>sen</em></td>
<td>Shigella enterotoxin-2</td>
<td>5'-TTACCCATCCCTACGTTGTCT-3'</td>
<td>979</td>
<td>30</td>
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<tr>
<td><em>sxt-1</em></td>
<td>Verotoxin-1</td>
<td>5'-GAAAGAGCTGGCTGGGT-3'</td>
<td>130</td>
<td>29</td>
</tr>
<tr>
<td><em>sxt-2</em></td>
<td>Verotoxin-2</td>
<td>5'-TTCACACACACGCCGATG-3'</td>
<td>346</td>
<td>29</td>
</tr>
<tr>
<td><em>sat</em></td>
<td>Autotransporter Sat</td>
<td>5'-AATGGCGGACATCTGAGT-3'</td>
<td>387</td>
<td>30</td>
</tr>
</tbody>
</table>
biofilm was stained with 175 µL of violet crystal for one minute, washed with 1 × PBS, and air dried for about 1 hour. The colorant was solubilized in dimethyl sulfoxide (DMSO) to measure the absorbance at λ of 550 nm in an automatic spectrophotometer (Anthos Reader 2001, Innogenetics, Spain). The result was considered positive when the absorbance was > 4-fold the value obtained in the well containing bacteria-free medium. All the assays were carried out in duplicate using positive and negative controls. The controls were uropathogenic E. coli strains that had been investigated in depth regarding the production of biofilm.32

Statistical analysis. To analyze the data the Fisher’s exact and the χ² tests were used.

RESULTS AND DISCUSSION

Different reports have demonstrated the association of EAEC with diarrhea in children in developing countries, international travelers, or immunocompromised patients.2,4,6,9,33–40 However, the pathogenic mechanisms of EAEC infections are not fully understood. There appears to be a significant heterogeneity of virulence factors among EAEC isolates.30,41 The prevalence of cases of diarrhea with a known etiology in children in Ifakara, Tanzania was 71.8% in the dry season, with the most commonly isolated enteropathogens being diarrheagenic E. coli (37.4%).1 Eighty-six of 348 (24.7%) E. coli isolates recovered from stools of children with diarrhea were EAEC.1 In 21 (24%) of the 86 children included in this study, enteropathogens other than EAEC were found. These enteropathogens were: Rotavirus in 12 (14%) children, Shigella spp. in 5 (6%) children (4 S. flexneri, 1 Shigella dysenteriae), 2 (2.3%) children had Entamoeba histolytica, and 2 children had Salmonella typhimurium and Campylobacter jejuni each. In the present study we investigated the prevalence of different virulence factors and biofilm production in these EAEC isolates.

Of these 86 EAEC isolates, no virulence factor was detected in 11 (12.8%), similar to the figures presented by other reports analyzing the prevalence of different virulence factors in EAEC isolates.15,35,41–43 For instance, Sarantuya and others35 found that 38.8% of the EAEC isolates causing diarrhea did not show any virulence factor. The most frequently detected virulence factor was the transcription factor AggR in 53 (61.6%) of the EAEC, followed by dispersin in 26.7%, yersiniabactin in 22.1%, autotransporter Sat in 20.9%; Shigella enterotoxin-1 in 16.3%, and heat-stable toxin-1 in 15.1%. The remaining virulence factors were detected in less than 10% of the strains, whereas Eaf, AAF/I, VT-1, and VT-2 were not detected (Table 2). The distribution of the different virulence factors was very heterogeneous with only 8 EAEC isolates having greater than or equal to 4 virulence factors (Table 3). A large number of putative virulence factors have been identified in EAEC strains, yet no single factor appears to be consistently present in all pathogenic strains.23 In our study the most frequent gene combinations were aggR/agg43 (18.6%), aggR/aap (17.4%), aggR/sat (14%), and aggR/fyuA (12.8%).

In comparison with other studies performed with EAEC isolated from children in developing countries, our data show slight differences and similarities. Piva and others35 found a high percentage (73% and 82%) of EAEC isolates, causing diarrhea in children from Brazil, positive for EAST and aerobactin, respectively. On the other hand, AAF/I and AAF/II were found in a minority of EAEC isolates. It is important to highlight that in our study AAF/II was detected in only 3 (3.5%) of the EAEC and that these 3 isolates were also AggR+, in agreement with another study that found that many isolates carrying the aggR gene expressed neither AAFI or AAFII adsorbs.44 However, these results are different to data presented in other studies showing a high number of EAEC carrying the aggR and aggI genes.3,11,42 In fact, Okeke and others3 have suggested that the presence of the AAF/II may be a marker for pathogenic EAEC strains. Our study confirms that AggR transcriptional factor, dispersin, heat-stable toxin-1, and Shigella flexneri enterotoxin-1 are among the most prevalent virulence factors found in EAEC.

The ability to form biofilms is a trait that is closely associated with bacterial persistence and virulence, and many persistent and chronic bacterial infections are now believed to be linked to the formation of biofilm.2,23,45 In our study, 66 (76%) of 86 EAEC isolates produced biofilm. When the presence of the different virulence factors was determined in the biofilm-forming EAEC group versus non-biofilm–forming EAEC group, AggR was the most prevalent virulence factor in the first EAEC group. Although ShET-1 was found to be more prevalent in the biofilm-forming group the values did not reach statistical significance. However, the prevalence of CdtB and Pet in the non-biofilm–forming EAEC group was statistically significant (Table 4). On analyzing 57 EAEC isolates, Mohamed and others45 recently found that 53% of the

<table>
<thead>
<tr>
<th>Number of virulence factors</th>
<th>EAEC n (%)</th>
<th>Cumulative %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11</td>
<td>12.8</td>
</tr>
<tr>
<td>1</td>
<td>21</td>
<td>24.4</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>19.8</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>20.9</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>17.4</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* Virulence factors other than the agg gene used to detect EAEC.
EAEC were biofilm producers, which is a slightly lower value than that found in our study. This difference may be explained by the origin of the EAEC isolates. In our study the EAEC isolates were from a limited geographic area in Tanzania whereas the isolates in the Mohamed study were from different countries. On the other hand, among our EAEC isolates there may have been a predominant EAEC clone that produces a bias in our data. However, the heterogeneity in the virulence factors found and the non-genetic relationship observed when the isolates were analyzed by REP-PCR (data not shown) ruled out this possibility. In the same study, the production of biofilm was associated with the virulence genes aggR, setIA, aatA, and irp2. This finding partially agrees with our results because we also found aggR to be associated with biofilm production and a trend to significance for the setIA gene. However, we did not find any difference between the biofilm-forming EAEC group and non-biofilm–forming EAEC group regarding the presence of yersiniabactin.

The Ag43 and the Sat proteins were found in 33.7% and 20.9% of the EAEC isolates, respectively. To our knowledge, the present study is the first report on Sat and Ag 43 in EAEC isolates. Both of these are autotransporter proteins, possessing different functions. Thus, while Sat acts as a cytotoxic toxin, Ag43 acts as an autoaggregation factor. Sat toxin was first described as a virulence factor of uropathogenic E.coli, but further studies have established its presence among diarrhoeagenic pathogens such as Shigella spp., and diffusely adherent E. coli. Similarly, Ag43 has been reported to be found in both uropathogenic and diarrhoeagenic E. coli. Ag43 is a self-recognizing molecule capable of conferring bacterial autoaggregation. Deletion in the agn43 gene generates cells with a very limited ability to form biofilm.

In summary, these results have again shown the high heterogeneity of virulence factors among EAEC isolates causing diarrhea in children, and that the biofilm may be considered as an important virulence factor, strongly associated with the presence of AggR.

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REFERENCES


TABLE 4

Incidence of biofilm (BF+) formation and the presence of several virulence factors among EAEC isolates from children with diarrhea (N = 86)

<table>
<thead>
<tr>
<th>Factor virulence</th>
<th>BF+ (N = 65)</th>
<th>BF– (N = 21)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AggR</td>
<td>42 (64)</td>
<td>8 (38)</td>
<td>0.032*</td>
</tr>
<tr>
<td>Ag43</td>
<td>21 (32)</td>
<td>8 (38)</td>
<td>0.625</td>
</tr>
<tr>
<td>AspU</td>
<td>19 (29)</td>
<td>4 (19)</td>
<td>0.359</td>
</tr>
<tr>
<td>FyuA</td>
<td>13 (20)</td>
<td>6 (29)</td>
<td>0.411</td>
</tr>
<tr>
<td>Sat</td>
<td>13 (20)</td>
<td>5 (24)</td>
<td>0.709</td>
</tr>
<tr>
<td>ShET1</td>
<td>13 (20)</td>
<td>1 (5)</td>
<td>0.100</td>
</tr>
<tr>
<td>EAST</td>
<td>10 (15)</td>
<td>3 (14)</td>
<td>0.903</td>
</tr>
<tr>
<td>CdtB</td>
<td>5 (8)</td>
<td>6 (29)</td>
<td>0.013</td>
</tr>
<tr>
<td>Pet</td>
<td>1 (2)</td>
<td>3 (14)</td>
<td>0.016</td>
</tr>
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</table>

* χ2 test.


