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 enteroaggregative E.coli (EAEC) with diarrhea in children in 
developing 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 aggregation and it has been found that expression of Ag43 dramatically 
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 
potential virulence factors are located in the 60–65 MDa pAA plasmid, from which a probe and also primers used for detection 
of the EAEC by PCR have been designed.16,17 In this plasmid, genes encoding adherence fimbria (AAFI and AAFII) 
as well as the heat-stable enterotoxin-1 (EAST-1, encoded in the aat 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.3,8 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 aat 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), enterotoxicagregative heat-stable enterotoxin-1 (EAST), Shigella enterotoxins 1 and 2 (ShET1 and ShET2), adherence factors AggF/I and AggF/II, the plasmid encoded cryptic secreted protein (Aap), autotransporter toxin Sat, cytolethal-distending toxin (Cdt), yersiniobactin (Fyu), and Antigen 43 (Ag43). 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

### Table 1

<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>aat</td>
<td>Antiaggregation protein transporter</td>
<td>5′-CTGGCGAAAAGACGTGATCAT-3′</td>
<td>629</td>
<td>29</td>
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<tr>
<td>aap</td>
<td>Dispersin</td>
<td>5′-GTATGATAGAATCCGCTGTT-3′</td>
<td>242</td>
<td>44</td>
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<tr>
<td>aggR</td>
<td>Transcription factor</td>
<td>5′-AGCGGGTGGCTCCCAAGATGATGAC-3′</td>
<td>699</td>
<td>This study</td>
</tr>
<tr>
<td>agg43</td>
<td>Antigen 43</td>
<td>5′-ACGCACAACCTCAATTAAA-3′</td>
<td>600</td>
<td>This study</td>
</tr>
<tr>
<td>aggA</td>
<td>Fimbria AAF/I</td>
<td>5′-TTAGTCTCTCTATCTAGG-3′</td>
<td>457</td>
<td>30</td>
</tr>
<tr>
<td>aafA</td>
<td>Fimbria AAF/II</td>
<td>5′-TGCGATTGCTCTACTTATAT-3′</td>
<td>242</td>
<td>30</td>
</tr>
<tr>
<td>astA</td>
<td>Heat stable toxin-1</td>
<td>5′-ATGGCAATCAAGACATGATGAC-3′</td>
<td>110</td>
<td>30</td>
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<tr>
<td>fyuA</td>
<td>Yersiniobactin</td>
<td>5′-TGATTAACCCCGGGAGCGGAA-3′</td>
<td>880</td>
<td>49</td>
</tr>
<tr>
<td>eaf</td>
<td>Enteropathogen adherence factor</td>
<td>5′-CAGGGTAAAGAGAGAGTLTATATTA-3′</td>
<td>397</td>
<td>29</td>
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<td>cdTB</td>
<td>Cytolethal distending toxin</td>
<td>5′-TTATGACCGTGATTGGCTT-3′</td>
<td>430</td>
<td>40</td>
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<tr>
<td>Bfp</td>
<td>Bundle-forming pili</td>
<td>5′-CTTCACATGATCAGGCGAG-3′</td>
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<td>29</td>
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<tr>
<td>eae</td>
<td>Intimin</td>
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<td>881</td>
<td>29</td>
</tr>
<tr>
<td>lt</td>
<td>Heat labile toxin</td>
<td>5′-CTTCTAGTGATCCAGGAGAC-3′</td>
<td>322</td>
<td>29</td>
</tr>
<tr>
<td>st</td>
<td>Heat stable toxin</td>
<td>5′-CGTACATGCTCTGCAACTTC-3′</td>
<td>147</td>
<td>29</td>
</tr>
<tr>
<td>pet</td>
<td>Plasmid encoded toxin</td>
<td>5′-ACTGCGGAGCTACCTGTCG-3′</td>
<td>832</td>
<td>29</td>
</tr>
<tr>
<td>setlA</td>
<td>Shigella enterotoxin-1</td>
<td>5′-TCAGCTACCATCAAGAAGAC-3′</td>
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<td>29</td>
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<tr>
<td>sen</td>
<td>Shigella enterotoxin-2</td>
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<tr>
<td>sxt-1</td>
<td>Verotoxin-1</td>
<td>5′-CTTCAACGACCCCGCTGAC-3′</td>
<td>130</td>
<td>29</td>
</tr>
<tr>
<td>sxt-2</td>
<td>Verotoxin-2</td>
<td>5′-CTTCAGGATCCATTTATAT-3′</td>
<td>346</td>
<td>29</td>
</tr>
<tr>
<td>sat</td>
<td>Autotransporter Sat</td>
<td>5′-AACCCCTGTAAGAGAGACTGAGC-3′</td>
<td>387</td>
<td>29</td>
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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. 3, 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), and 2 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 presence of different virulence factors in EAEC isolates. 15, 35, 41–43 For instance, Sarantuya and others 6 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, AAFl, 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/aggF (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 others 35 found a high percentage (73% and 82%) of EAEC isolates, causing
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, set1A, 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 set1A 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 Ag43 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 Escherichia coli. But further studies have established its presence among diarrheagenic pathogens such as Shigella spp., and diffusely adherent E. coli. Similarly, Ag43 has been reported in both in uropathogenic and diarrheagenic E. coli. Ag43 is a self-recognizing molecule capable of conferring bacterial autoaggregation. Deletion in the aggR gene generates cells with a very limited ability to form biofilm. In our study we did not find an association between the presence of Ag43 in EAEC isolates and biofilm formation. 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.

### Table 4

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

* χ² test.

REFERENCES


