# Development of a novel duplex PCR for detection of virulence-associated genes characteristic of EAHEC O104:H4

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## Introduction

The aggregative pattern of adherence (AA) exhibited by enteroaggregative *E. coli* (EAEC) is a plasmid-associated property which correlates with aggregative adherence fimbria I (AAF/I). The genes *aggA* [a major fimbrial subunit gene], *aggC* [outer membrane usher], and *aggD* [periplasmic fimbrial chaperone] are involved in biogenesis of AAF/I. **The objective of this study was to develop a novel duplex PCR for detection of genes *aggA* and *aggC*.**

## Material and Methods

### Sample collection

Several *E. coli* reference strains were used as positive controls for PCR. These strains were as follows: an EAHEC O104:H4 (strain 11-2027 positive to AAF/I), kindly provided by Dr. A. Fruth (NRC at the Robert Koch Institute, Branch Wernigerode); an EAEC O86:H47 (strain 06-01104 positive to AAF/I), an EAEC O86:H2 (strain 07-000842 positive to AAF/I) and three EAEC O3:H2 (strain 17-2, 05-06913-2, 05-3008 positive to AAF/I), kindly provided by Prof. Dr. U. Dobrindt (Institute of Hygiene and the National Consulting Laboratory on Haemolytic Uraemic Syndrome, University of Münster). Several *E. coli* reference strains were used as negative controls for PCR. These strains were as follows: an EHEC O157:H7 (strain EDL 933) and an *E. coli* O6 (strain ATCC® 25922).
*E. coli*-strains were cultivated at 37 °C for 12 h on nutrient agar (Difco, USA). Isolates were stored at -18°C.

### Design Primers

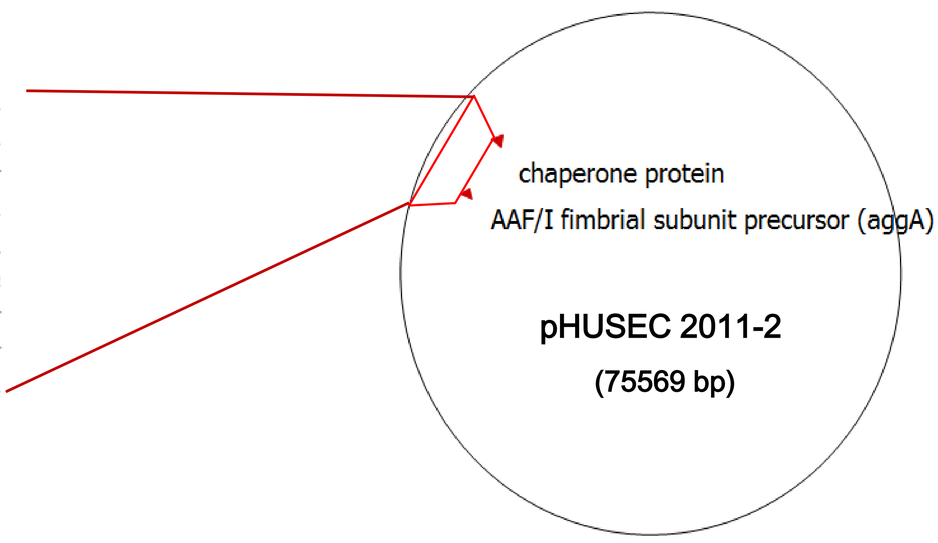
In our work, primer sequences for *aggA* virulence gene were checked against several plasmid genomes in GenBank [e.g. HE610901.2; CP003291.1; s below **Fig. 1, 2**] using Clone Manager Professional 9 Professional Edition (Sci-Ed Software, USA). Their sequences were checked against several known microbial genomes other than *E. coli* using the BLAST N tool (Basic Local Alignment Search Tool; National Center for Biotechnology Information, Bethesda, Maryland, USA) to verify that there were no unspecific matches. The melting temperature for each primer was calculated with Clone Manager Professional (v. 9.2).
For amplification of a 850 bp fragment of *aggC* gene were used primers according to Boisen et al., 2008.

**Fig. 1. Complete sequence of *aggA* gene [503 bp] which encoding structural subunit of aggregative adherence fimbriae I (AAF/I)**

complement (61253..61756) locus_tag="HUS2011 pII0094„

```
61201                                                        ttaaaaat
61261 caactgcagc atggagtatc attgtatatt cgcctggtat taatacttga gttttagggt
61321 tgactgaaac acttgcgttc agaaatttgt tcccgcttgt caatttttta taaatttttg
61381 catcagttcc ttggttcaca aaatctgcac ctccatctat agatggattc aatgtgtatt
61441 tttgttttgc aactttagca tgctgtagga cccacttatt agcgggacct gttccccata
61501 accagacctt tgcaccggct ttaatacatt gatcacttgt cgttactttt gcggaaaatc
61561 ctattggtgt tgttgacgat acaccaacag ttttgtggagg agttgtagtt atagtaacag
61621 gacaatcatt tgtaacggtg aggcggattg tctgagttgt ttgttgtgac gcagcgttag
61681 ccccccccaga tagaagacta actaaaccca aagtaatgta tagattcttt cttctcatat
61741 tttttaatgt tttcat
```

**Fig. 2. Complete-plasmid sequence [Access No. HE610901.2] carrying *aggA* and *aggC* genes** (Kuenne et al., 2011)




### PCR

For DNA extraction and purification we used the foodproof® StarPrep One Kit (BIOTECON Diagnostics, Germany). PCR was carried out in a final 25 µL volume containing 13.3 µL of nuclease-free-water (Qiagen, Germany), 2.5 µl of 10x Polymerase Buffer [15mM $MgCl_2$] (Thermo Fisher Scientific, Germany), 0.5 µl of $MgCl_2$ [50 mM] (Eurogentec, Germany), 1.0 µl dNTP-Mix [ 10Mm each] (Sigma Aldrich, Germany), 1.0 µl of each primer *aggA* rev / fw (10µM each), 1.5 µl of each primer *aggC* rev / fw (10µM each), 0.2 µl of Polymerase AmpliTaq® [5 U/µl] (Thermo Fisher Scientific, Germany). Finally, 2.5 µL of the different DNA templates were added. PCRs were conducted using a personal cycler (Biometra, Germany). Amplification conditions were: 94°C for 5 min followed by 30 cycles of 30 s at 94°C, 1 min at 57°C, 1 min at 72 °C and a final extension step of 10 min at 72°C. DNA fragments of 330 bp and 850 bp are separated on a 1.5% agarose gel (Biozym, Germany).

## Results

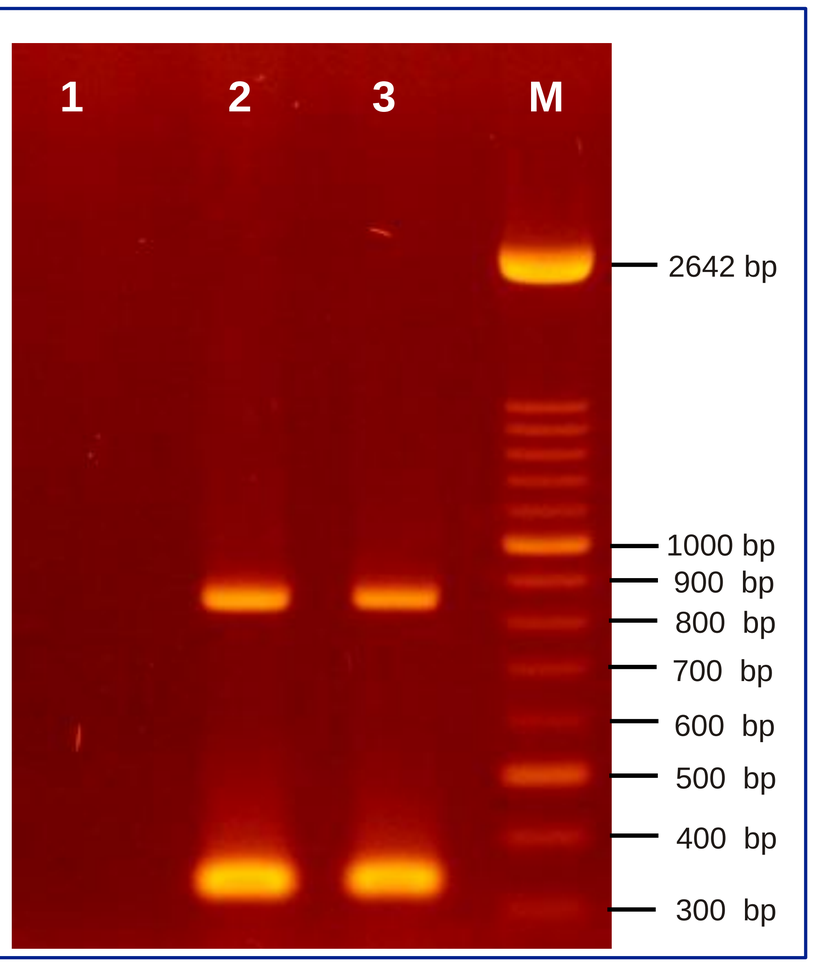



**Fig. 3. Amplification of *aggC* and *aggA* genes coding fimbria AAF/I**

**lane 1:** **Negative Control (*E. coli* O6, ATCC® 25922)**
**lane 2:** **EAHEC O104:H4, strain No.11-2027 (*aggA* / *aggC*)**
**lane 3:** **EAEC O3:H2, strain No. 05-06913-2 (*aggA* / *aggC*)**

**lane M:** **DNA molecular weight marker XIV (Roche Diagnostics)**

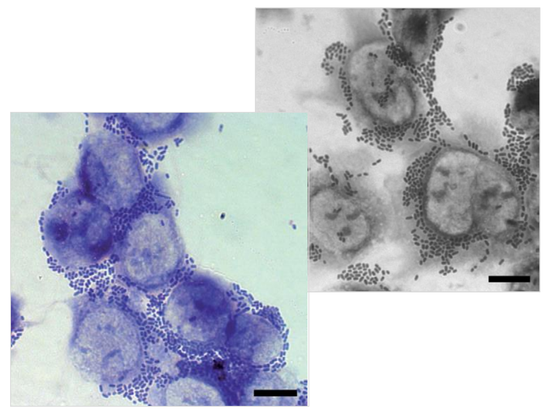


**Fig. 4: Aggregative adherence to cultured intestinal epithelial cells** (Bielazewska et al., 2011)

## Conclusion

**We developed a new duplex PCR as rapid assay that is able to detect two virulence genes (*aggC, aggA*) in EAHEC [EAEC] isolates. We thank Claudia Walter and Karin Simon for excellent assistance.**

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