

1 **Abstract**

2 Enterotoxigenic *Escherichia coli* (ETEC) expressing the colonization factor CS6 is widespread
3 in many developing countries including India. The different allelic variants of CS6, caused by
4 point mutations in its structural genes *cssA* and *cssB*, are designated as AIBI, AIIBII, AIIIBI,
5 AIBII, and AIIIBII. A simple, reliable and specific mismatch amplification mutation assay based
6 on real-time polymerase chain reaction (MAMA-qPCR) was developed for the first time for
7 detection of CS6-expressing ETEC along with specification of allelic variations. This assay was
8 based on mismatch nucleotide incorporation at the penultimate base at the 3'-end of the reverse
9 primers specific for *cssA* and *cssB* and was validated using 38 CS6-expressing ETEC isolates.
10 This strategy was effective in detecting all the alleles containing single nucleotide
11 polymorphism. Using MAMA-qPCR we have also tested CS6 allelic variants in 145 ETEC
12 isolated from children with acute diarrhea and asymptomatic infections, the latter serving as
13 controls. We observed that AIBI and AIIIBI allelic variants were mostly associated with cases
14 rather than controls, whereas the AIIBII variants were detected mostly in controls. In addition,
15 the AIBI and AIIIBI alleles were frequently associated with ETEC harboring *est* alone or with *elt*
16 genes, whereas AIIBII allele was predominant in ETEC isolates harboring *elt* gene. This study
17 may help in understanding the association of allelic variants in CS6-expressing ETEC with the
18 clinical features of diarrhea as well as in ETEC vaccine studies.

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1 **Introduction**

2 Enterotoxigenic *Escherichia coli* (ETEC), *Vibrio cholerae* O1 and rotavirus, account for a
3 majority of the most severe form of acute diarrhea that can turn fatal in the absence of timely
4 intervention. The virulence factors of ETEC are one or both of the two enterotoxins (6, 14),
5 commonly known as heat-labile (LT) and heat-stable (ST) depending on their temperature
6 susceptibility, and a group of cell surface proteins, collectively called colonization factor
7 antigens (CFAs). The CFAs enable the organisms to adhere to host intestinal cells effectively to
8 initiate pathogenesis (15, 18) by overcoming intestinal peristalsis and mucous barrier. Several
9 studies indicate that 20-40% of the travelers from developed countries experience ETEC-
10 mediated diarrhea in developing countries that are endemic for diarrheal pathogens (4).
11 According to the World Health Organization (WHO), ETEC is the second most common cause
12 of diarrhea after rotavirus in children less than 5 years of age and this age group is the main
13 target for vaccine implementation (8).

14 In the majority of developing countries, CS6 is one of the major CFAs among ETEC. Till date,
15 more than 20 CFAs are known, of which CS6 is the most prevalent globally (6, 5, 19). Recent
16 studies indicate that infection caused by CS6-expressing ETEC alone account for up to 20% of
17 the total diarrheal cases in south-east Asia (1). Due to the increase in the incidence of ETEC-
18 mediated diarrheal disease, considerable attention has been paid to the development of simple,
19 sensitive molecular methods for rapid identification of this pathogen. Currently, CFA's are
20 detected by normal PCR assay, substituting the conventional immunological tests (7, 12, 13, 16,
21 17).

1 The CS6 operon is composed of four genes, namely *cssA*, *cssB*, *cssC* and *cssD*. The *cssA* and
2 *cssB* genes are the structural genes of CS6 that exhibit variation in different CS6-expressing
3 ETEC isolates (16, 20). Recently, we have documented that *cssA* gene of CS6 has three alleles
4 (AI, AII, and AIII), and *cssB* gene has two alleles (BI, BII) that show differential binding with
5 human intestinal epithelial cells (Caco-2) (16). Following identification of CS6-expressing
6 ETEC by PCR or antibody based detection methods, molecular techniques such as allele-specific
7 PCR and DNA sequencing are helpful in the detection of CS6 variants. However, these methods
8 are laborious and time-consuming for diagnostic purposes. Till date, there is no simple, specific
9 and rapid detection method for ETEC expressing CS6 alleles.

10 In this study, we have developed a rapid qPCR-based mismatch amplification mutation assay
11 (MAMA) (2) for the detection of CS6 allelic variants that can be completed within 2 hrs after the
12 DNA extraction from the ETEC isolates. Using this new assay, we screened 145 ETEC isolates
13 for the detection of different alleles of both *cssA* and *cssB* genes of CS6 and their association
14 with diarrheal and asymptomatic infections.

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16 **Materials and Methods**

17 **Bacterial strains and culture conditions:** Stool specimens were collected from the acute
18 diarrheal children below five years of age from the Infectious Diseases Hospital and B. C. Roy
19 Memorial Hospital for children, Kolkata, India. Stool specimens were also collected from age
20 and sex matched asymptomatic controls from the community. All the stool specimens were
21 examined for common enteric pathogens using standardized methods (10). Three lactose
22 fermenting pink colonies resembling *Escherichia coli* from MacConkey agar plates were sub-

1 cultured on normal Luria-Bertani agar (Difco, Sparks, MD) plate and screened for the
2 diarrheagenic *E. coli* (DEC) as described previously (11, 15). After confirmation, all the ETEC
3 isolates were serotyped using commercially available O and H antisera (Denka Seiken, Tokyo,
4 Japan). All the ETEC isolates were stored at -80°C in Luria-Bertani broth (Difco) with 15%
5 glycerol until further use. A well characterized ETEC CS6 strain 4266 (7) and a K12 *E. coli*
6 strain DH5 α were used as PCR positive and negative control strains, respectively.

7 **Genomic DNA isolation:** ETEC isolates were sub-cultured from -80°C stock on CFA medium
8 (1% Casamino acid, 0.15% yeast extract, 0.005% MgSO₄, 0.005% MnCl₂, pH 7.4). After 24 hrs
9 incubation at 37°C, total genomic DNA was isolated by NucliSENS EasyMag (biomérieux,
10 Marcy l'Etoile, France) machine according to manufacturer's protocol. One ETEC isolate per
11 patient was used in this study and presence of CS6 was confirmed by PCR as described before
12 (16).

13 **Primer design for mismatch qPCR:** The MAMA primer to detect the sequence polymorphism
14 among *cssAI*, *cssAII*, *cssAIII*, *cssBI* and *cssBII* subunit genes was focused on nucleotide position
15 169 and 474 for *cssA* and *cssB* subunit genes of CS6, respectively (Fig.1 A, B). Two conserved
16 forward primers for *cssA* and *cssB* genes and five allele-specific polymorphism detection
17 primers, RV-*cssAI*, RV-*cssAII*, RV-*cssAIII*, RV-*cssBI* and RV-*cssBII* were designed (Table 1).
18 At 3' end, these allele-specific primers contained G, C and A for *cssAI*, *cssAII* and *cssAIII* and C
19 and A for *cssBI*, *cssBII*, respectively. To increase the specificity of the 3'mismatch activity,
20 nucleotide C was incorporated (rather than T and/or A) at the penultimate nucleotide of 5'- 3'
21 reverse primers of all CS6 subunit alleles. A conserved forward primer (FO-*parC*) and a reverse
22 primer (RV-*parC*) of the housekeeping gene *parC* were used as an internal control.

1 **qPCR reaction condition:** qPCR for the detection of mutations was done in a 20µl reaction
2 volume containing 2µl of total genomic DNA (~60ng of DNA in a sterile Tris-EDTA), 1X PCR
3 SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 0.2µM forward and MAMA
4 reverse primers and Milli-Q water to make up the final volume. Thirty cycles of qPCR were
5 carried out as follows: stage 1, 95°C for 15 min (enzyme activation) followed by stage 2, 30
6 cycles of 95°C for 20s, 49°C for 35s, and 60°C, 45 s and stage 3, for dissociation (of each primer
7 from template) on an Applied Biosystem 7500 thermal cycler. Data for analysis were collected at
8 the last step of stage 2. Melting curve was also analyzed at the dissociation step of stage 3 in
9 each experiment to understand the primer dissociation from the template.

10 **Conventional PCR:** ETEC toxin genes that encode LT and ST were detected by multiplex PCR
11 as described before (11). To check the non-specific amplicons if any, a conventional PCR using
12 the same MAMA primers for all the CS6 alleles were made. PCR was performed in a 20µl
13 volume consisting of 2X PCR Master Mix (QIAGEN Fast Cycling PCR Kit, Qiagen, CA),
14 0.2µM forward and MAMA reverse primers. PCR conditions are as follows: 95°C, 5mins for
15 initial activation of HotStar Taq plus DNA polymerase, 28 cycles of 96°C for 5s; 51°C for 5s
16 and 68°C for 10s, and a final extension step of 1 min at 72°C on an Eppendorf thermal cycler.
17 The amplified products were visualized on a 3% agarose gel after ethidium bromide staining
18 using Geldoc system (BioRad, Hercules, CA).

19 **Statistical Analysis:** CS6 allelic variants in cases and controls were tested by univariate analysis
20 and χ^2 exact tests. Probability level (*P*) value of ≤ 0.05 was considered statistically significant.

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1 **Results**

2 **Specificity of MAMA-qPCR primer pairs**

3 To determine the specificity of MAMA-qPCR, we used the DNA of 38 previously characterized
4 CS6 positive ETEC isolates. CS6 negative ETEC isolates and non-ETEC such as *Vibrio*
5 *cholerae*, enteropathogenic *E. coli*, enteroaggregative *E. coli* and *Campylobacter spp.* were
6 included in the assay. As expected, the assay was negative for CS6 negative ETEC and non-
7 ETEC isolates but was positive for only previously characterized CS6 positive ETEC isolates.
8 Allelic variations were detected in all the CS6 positive ETEC isolates. The internal test control
9 *parC* gave positive C_t values in case of enteropathogenic *E. coli*, enteroaggregative *E. coli*, and
10 all the ETEC isolates (data not shown). A representative gel picture for CS6 allelic variants is
11 shown in Fig. 2. Our results indicate that all the tested MAMA primers were CS6 allele specific
12 and did not cross react with other bacterial genes tested in this study.

13 **MAMA-qPCR based identification of CS6 allelic variants by among ETEC isolates from** 14 **case and controls**

15 Thirty eight previously characterized CS6 positive ETEC isolates were selected to validate the
16 MAMA-qPCR assay. In all these isolates, CS6 alleles were confirmed by DNA sequencing (16).
17 Using MAMA-qPCR, we screened additional 105 ETEC isolates from diarrheal cases and 40
18 from controls. Overall, 49% of the ETEC isolates expressed CS6. In the MAMA-qPCR, AIBI
19 (n=40), AIIIBI (n=18), AIIBII (n=6), AIBII (n=3) and AIIIBII (n=4) were detected in ETEC
20 isolates (Table 2). Most of the ETEC isolates from diarrheal cases harbored AIBI allele (34%) in
21 comparison to the controls (10%). AIBI variant was 9 times significantly higher [OR=3.68 (95%
22 CI=0.88-16.41); $P=0.039$] in cases than in controls. Though it is not significant [OR=2.10 (95%
23 CI=0.37-15.40); $P=0.298$], the AIIIBI variant was 8 times higher in cases than controls.

1 Conversely, the occurrence of AIIBII variant was comparatively more in controls (10%) than
2 cases (2%), which was highly significant ($P<0.001$). In this study, AIBII and AIIIBII variants
3 were detected in very low numbers and the AIBI variant was not found among ETEC isolates.

4 **Correlation between CS6 allelic variants and toxin genes**

5 We have tested the toxin types of ETEC isolates by a multiplex PCR method (11) targeting the
6 *elt* and *est* genes and correlated their occurrence with the specific CS6 allele (Table 3). It was
7 found that 28 (48%) of the CS6-expressing ETEC isolated from diarrheal cases harbored *elt* and
8 *est* genes, of which 22 (76%) were AIBI allelic variant, 5 (17.2%) were AIIIBI and one isolate
9 belonged to AIIBII variant. Out of 13 ETEC isolates from controls, 3 harbored CS6 as well as *elt*
10 and *est* genes. Among 16 *est* positive ETEC isolates from diarrheal cases 6 had AIBI allele, 7
11 with AIIIBI, 2 with AIIIBII and one had AIIIBII. From controls, only 4 ETEC isolates were
12 positive for *est* gene. The *elt* gene was detected in 13 ETEC isolates from cases, in which 8
13 belonged to AIBI allele, 4 isolates to AIIIBI and one to AIBII. Interestingly, most of the AIIBII
14 variant was found to be associated with ETEC isolates from controls that harbored only the *elt*.
15 The most commonly detected toxin encoding genes among CS6 allelic variants in order of
16 frequency were $elt+est > est > elt$. Most of the AIBI allelic variant among cases harbored both
17 *est* and *elt*. The CS6 negative ETEC mostly harbored *est* (data not shown). The serotyping results
18 showed that majority of the ETEC isolates belongs to different serotypes (data not shown).

19 **Discussion**

20 Allelic variation in CS6 expressing ETEC is a recent observation that has several implications in
21 the epidemiology of ETEC-mediated diarrhea (16). The screening of these CS6 allelic variants is
22 critical for understanding the correlation of micro-evolutionary changes of this pathogen with

1 clinical severity of the disease and its implication for ETEC vaccine development. Currently,
2 DNA sequencing is the definitive method for identification of CS6 allelic variants. Since
3 sequencing is not practical in studies involving large number of samples and hence it is
4 appropriate to develop a method that is rapid, reliable and cost-effective in clinical and
5 community settings. Considering the existing number of CS6 allelic variants, real-time PCR can
6 effectively be used as an alternative to the DNA sequencing. MAMA-PCR has become the most
7 important tool in identifying single nucleotide variation with epidemiological significance.
8 Previously, this technique was used for the differentiation of cholera toxin B subunit in newly
9 emerged El Tor hybrid strains of *V. cholerae* O1 that has spread in several endemic regions (9).
10 In this study, we have developed a simple, specific, reliable and cost-effective MAMA-qPCR
11 assay for the differentiation of CS6 expressing ETEC variants that can detect nanogram level of
12 DNA and costs about 10 USD per sample, which is much less than DNA sequencing costs.
13 Further to simplify the assay DNA extraction from ETEC isolates by simple heating method may
14 also be adopted.

15 In addition to the formulation of this MAMA-qPCR, we applied the assay to ETEC isolates from
16 children below five years of age with acute diarrhea and age and sex matched controls from the
17 urban community. Many studies with ETEC have shown that CS6 was the most prevalent CF
18 type in developing countries (3, 7, 12, 16). Overall, the AIBI variant was the most prevalent
19 among CS6-expressing ETEC isolates in Kolkata. This inference should be further confirmed
20 with more number of ETEC from diarrheal cases and normal controls. It was also observed that
21 AIBI was the most frequently detected CS6 variant in ETEC isolates from cases, whereas the
22 AIIBII variant was mainly found in the control group. Simultaneous occurrence of LT and ST
23 toxin types in ETEC was significantly higher among AIBI variant whereas AIIBII variant of

1 ETEC were associated with LT alone. Previous epidemiological studies with ETEC have shown
2 that LT toxin-producing ETEC were significantly associated in controls than ST/ST+LT toxin
3 type (12). In our previous study, we have shown that in the presence of BII allele, the CS6
4 expressing ETEC binds to Caco-2 cells with less affinity than the BI allele expressing ETEC
5 (16). Based on our findings, it is possible that the LT-ETEC is associated with less severe
6 clinical cases due to less adherence ability of this allelic type. Further molecular and
7 epidemiological studies are required to prove this presumption.

8 In summary, the MAMA-qPCR method is a reliable, cost-effective and simple method for the
9 detection of CS6 expressing ETEC isolates and its allelic variants. This assay also provides
10 information relevant to severity of ETEC-induced diarrhea and development of vaccines against
11 CS6-expressing ETEC.

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1 **References**

- 2 **1. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, Jha P,**
3 **Campbell H, Walker CF, Cibulskis R, Eisele T, Liu L, Mathers C.** 2010. Global,
4 regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet*,
5 **37:1969-1987.**
- 6 **2. Cha R.S., Zarbl H., Keohavong P., ThillyW.G.** 1992. Mismatch amplification mutation
7 assay (MAMA): application to the c-H-*ras* gene. *PCR Methods Appl* **2:14–20.**
- 8 **3. Cohen D, Tobias J, Spungin-Bialik A, Sela T, Kayouf R, Volovik Y, Yavzori M,**
9 **Ephros M.** 2010. Phenotypic characteristics of enterotoxigenic *Escherichia coli*
10 associated with acute diarrhea among Israeli young adults. *Foodborne Pathog Dis.*
11 **7:1159-1164.**
- 12 **4. DuPont, H. L., S. B. Formal, R. B. Hornick, M. J. Snyder, J. P. Libonati, D. G.**
13 **Sheahan, E. H. LaBrec, and J. P. Kalas.** 1971. Pathogenesis of *Escherichia coli*
14 diarrhea. *N. Engl. J. Med.* **285:1–9.**
- 15 **5. Firdausi Qadri, Svennerholm, A. M., Faruque, A. S. G., and Sack, R. B.** 2005.
16 Enterotoxigenic *Escherichia coli* in Developing Countries: Epidemiology, Microbiology,
17 Clinical Features, Treatment, and Prevention. *J Clin Microbiol Rev.* **18:465-483.**
- 18 **6. Gaastra, W. & Svennerholm, A. M.** 1996. Colonization factors of human
19 enterotoxigenic *Escherichia coli* (ETEC). *Trends Microbiol* **4:444-452.**
- 20 **7. Ghosal, A., Bhowmick, R., Nandy, R. K., Ramamurthy, T. & Chatterjee, N. S.** 2007.
21 PCR-Based identification of common colonization factor antigens of enterotoxigenic
22 *Escherichia coli*. *J Clin Microbiol* **45:3068-3071.**

- 1 **8. Lanata, C. F., W. Mendoza, and R. E. Black.** 2002. Improving diarrhoea estimates.
2 WHO, Geneva, Switzerland.
- 3 **9. Masatomo Morita, Makoto Ohnishi, Eiji Arakawa, Bhuiyan, N.A., Suraia Nusrin,**
4 **Munirul Alam, Siddique, A.K., Firdausi Qadri, Hidemasa Izumiya, G. Balakrish**
5 **Nair and Haruo Watanabe.** 2008. Development and validation of a mismatch
6 amplification mutation PCR assay to monitor the dissemination of an emerging variant of
7 *Vibrio cholerae* O1 biotype El Tor. *Microbiol Immunol.***52**:314-317.
- 8 **10. Nair, G. B., Ramamurthy, T., Bhattacharya, M. K., Krishnan, T., Ganguly, S., Saha,**
9 **D. R., Rajendran, K., Manna, B., Ghosh, M., Okamoto, K., Takeda, Y.** 2010.
10 Emerging trends in the etiology of enteric pathogens as evidenced from an active
11 surveillance of hospitalized diarrhoeal patients in Kolkata, India. *Gut Pathog.* **2**(1):4.
- 12 **11. Nguyen, T. V., Le Van, P., Le Huy, C., Gia, K.N. & Weintraub, A.** 2005. Detection
13 and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi,
14 Vietnam. *J Clin Microbiol.* **43**, 755-760.
- 15 **12. Rivera FP, Ochoa TJ, Maves RC, Bernal M, Medina AM, Meza R, Barletta F,**
16 **Mercado E, Ecker L, Gil AI, Hall ER, Huicho L, Lanata CF.** 2010. Genotypic and
17 phenotypic characterization of enterotoxigenic *Escherichia coli* strains isolated from
18 Peruvian children. *J Clin Microbiol.* **48**:3198-203.
- 19 **13. Rodas, C., Iniguez, V., Qadri, F., Wiklund, G., Svennerholm, A. M. & Sjoling, A.**
20 2009. Development of multiplex PCR assays for detection of enterotoxigenic *Escherichia*
21 *coli* colonization factor and toxins. *J Clin Microbiol* **47**:1218-1220.

- 1 **14. Sack, R. B.** 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*.
2 *Annual rev of Microbiol* **29**:333-353.
- 3 **15. Sack, R.B.** 1980. *Enterotoxigenic Escherichia coli*: Identification and characterization. *J.*
4 *Infect Dis*, **142**: 279-86.
- 5 **16. Sabui S, Ghosal A, Dutta S, Ghosh A, Ramamurthy T, Nataro J. P., Hamabata T,**
6 **Chatterjee N. S.** 2010. Allelic variation in colonization factor CS6 of enterotoxigenic
7 *Escherichia coli* isolated from patients with acute diarrhoea and controls. *J Med*
8 *Microbiol.* **59**:770-779.
- 9 **17. Vidal, M. R., Valenzuela, P., Baker, K., Lagos, R., Esparza, M., Livio, S., Farfan,**
10 **M., Nataro, J. P., Levine, M. M. & Prado, V.** 2009. Characterization of the most
11 prevalent colonization factor antigens present in Chilean clinical enterotoxigenic
12 *Escherichia coli* strains using a new multiplex polymerase chain reaction. *Diagn*
13 *Microbiol And Infect Dis* **65**:217-223.
- 14 **18. Wolf, M. K.** 1997. Occurrence, distribution, and associations of O and H serogroups,
15 colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli*. *J Clin*
16 *Microbiol Rev* **10**:569-584.
- 17 **19. Wolf, M. K., Andrews, G.P., Tall, B. D., McConnell, M. M., Levine, M. M. &**
18 **Boedeker, E. C.** 1989. Characterization of CS4 and CS6 antigen components of
19 PCF8775, a putative colonization factor complex from enterotoxigenic *Escherichia coli*
20 E8775. *Infect Immun* **57**:164-173.
- 21 **20. Wolf, M. K., Hann, de L. A. M., Cassels, F. J., Willshaw, G. A., Warren, R.,**
22 **Boedeker, E. C. & Gasstra, W.** 1997. The CS6 colonization factor of human

1 enterotoxigenic *Escherichia coli* contains two heterologous major subunits. *FEMS*
2 *Microbiol Lett* **148**:35-42.

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1 **Figure legends**

2 **Fig. 1:** Sequence alignment of *cssA* and *cssB* subunit genes of CS6 expressing ETEC. Three
3 allele of *cssA* and two allele of *cssB* subunit were aligned by using clustalW2 software
4 (<http://www.ebi.ac.uk/Tools/msa/clustalw2>), respectively. Star (*) marks are indicated the
5 conserved nucleotides and gap regions are indicated single nucleotide polymorphisms (SNPs) at
6 different position of *cssA* and *cssB* alleles. The primer regions are indicated by bold and mutated
7 nucleotides of the reverse primer are shown in box. Identical regions between CS6 alleles are
8 indicated in dotted line and the position of the nucleotide shown at right side.

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10 **Fig. 6:** Agarose gel of MAMA PCR product for CS6 allelic variants. A; Lane 1, 2, 3, 4, and 5
11 indicates AI, AII, AIII, BI and BII allele from strain IDH00200, respectively. Lane 6, 7, 8, 9 and
12 10 indicates AI, AII, AIII, BI and BII allele from strain SD173, respectively. Lane 11, 12, 13, 14
13 and 15 indicates AI, AII, AIII, BI and BII allele from strain SD276, respectively. B, Lane 1, 2, 3
14 indicates *parC* gene from strain IDH00200, SD173 and SD276, respectively.

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1 **Table 1.** Oligonucleotide sequences of primers used in MAMA-qPCR assay

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Target gene	Location	Oligonucleotide Sequence	Amplicon size (bp)
<i>cssAI</i>	55—►189 ^a	FO- <i>cssA</i> : 5'-AGAACAGAAATAGCGACTA-3' RV- <i>cssAI</i> : 5'-GCTCACACTAAATAATAA CC -3'	135
<i>cssAII</i>	55—►189 ^b	FO- <i>cssA</i> : 5'-AGAACAGAAATAGCGACTA-3' RV- <i>cssAII</i> : 5'-GCTCACACTAAATAATAA CG -3'	135
<i>cssAIII</i>	55—►189 ^c	FO- <i>cssA</i> : 5'-AGAACAGAAATAGCGACTA-3' RV- <i>cssAIII</i> : 5'-GCTCACACTAAATAATAA CT -3'	135
<i>cssBI</i>	328—►494 ^d	FO- <i>cssB</i> : 5'-ATCAGAAAGGATTCTGGC-3' RV- <i>cssBI</i> : 5'-GTAAAATGATACACTCAA ACG -3'	167
<i>cssBII</i>	328—►494 ^e	FO- <i>cssB</i> : 5'-ATCAGAAAGGATTCTGGC-3' RV- <i>cssBII</i> : 5'-GTAAAATGATACACTCAA ACT -3'	167
<i>parC</i>	63—►162 ^f	FO- <i>parC</i> : 5'-TGGATCGTGCGTTGCCGTTTATTG-3' RV- <i>parC</i> : 5'-AATTTGGCGGTAGCATT CAGACCC -3'	100

3 ^a, GeneBank Accession Number GQ241334

4 ^b, GeneBank Accession Number GQ241330

5 ^c, GeneBank Accession Number GQ241332

6 ^d, GeneBank Accession Number GQ241335

7 ^e, GeneBank Accession Number GQ241337

8 ^f, GeneBank Accession Number EU561348

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1 **Table 2.** ETEC-CS6 allelic variants detected by MAMA-qPCR from case and control

CS6 allelic Variant	Number of CS6 positive ETEC			P-value
	Case (n=58)	Control (n=13)	OR (95% CI)	
AIBI	36	4	3.68 (0.88-16.41)	0.039 ^{*cs}
AIIIBI	16	2	2.10 (0.37-15.40)	0.298
AIBII	2	4	0.08 (0.01-0.63)	0.009 ^{*ct}
AIBII	1	2	0.10 (0.00-1.55)	0.084
AIIIBII	3	1	0.65 (0.05-17.84)	0.563

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*cs =statistically significant association with cases

*ct =statistically significant association with control

1 **Table 3.** Relationship between toxin genes and CS6 allelic variants of CS6 expressing ETEC

CS6 allelic variants	Case*(n=58)			Control [#] (n=13)		
	<i>elt+est</i>	<i>elt</i>	<i>est</i>	<i>elt+est</i>	<i>elt</i>	<i>est</i>
AIBI	22	8	6	2	2	-
AIIIBI	5	4	7	1	-	1
AIBII	1	-	1	-	4	-
AIBII	-	1	-	-	-	2
AIIIBII	-	-	2	-	-	1

2 Case*, CS6 expressing ETEC was isolated from diarrheal patients as a mixed or sole pathogen
 3 Control[#], CS6 expressing ETEC was isolated from normal children as a mixed or sole pathogen
 4 n, total number of ETEC isolates
 5 -, indicates ETEC were not detected of that toxin type

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cssA

Forward Primer
5' → 3'

cssAI ATGAAGAA.....CCATGCC**AGAACAGAAATAGCGACT**AAAAACTTCCCAGTATC 89
cssAIII ATGAAGAA.....CCATGCC**AGAACAGAAATAGCGACT**AAAAACTTCCCAGTATC 89
CssAII ATGAAGAA.....CCATGCC**AGAACAGAAATAGCGACT**AAAAACTTCCCAGTATC 89

3' ← MAMA Reverse Primer 5'

cssAIACAAATCCAG.....GTAAA.....GAG**GTTTATTATTTAGTGTGAGC**TAAACTG 196
cssAIIIACAAATCCAG.....GTAAA.....GAG**ATTTATTATTTAGTGTGAGC**TAAATTG 196
CssAIIACGAATACAG.....GTGAAA.....GAG**CTTTATTATTTAGTGTGAAC**TAAACTG 196

cssAIGGGTTAGGACGACTCGTA.....ATGATTC.....TGATAAAGGGAGAAAAA.....GTGCA 348
cssAIIIGGATTAGGACGACTCGTA.....ATGATTC.....TGATAAAGGGAAAAAAA.....GTACA 348
CssAIIGGGTTAGGACGACTAGTA.....ATGCTTC.....TGAGAAAGGGAAAAAAA.....GTGCA 348

cssAI GAGGTTACGCCT.....TAGAGC.....GAGATAAAGAAATACCTCCTGGGAT.....AACTAA 465
cssAIII GAGGTTACGCCT.....TAAAGC.....GAGATAAAGAAATACCTCCTGGGAT.....AACTAA 465
CssAII GAGGTTACACCT.....TAAAGC.....GGGAAAAAAAATATCTCCTGGAAT.....AACTAA 465

cssB

cssBI ATGTTGAAAAAATTATTCCGGCTATTGTATTAATTGCAGGAACTTCCGGAGTGGTAAAT 60
cssBII ATGTTGAAAAAATTATTTCCGGCTATTGCATTAATTGCAGGAACTTCCGGAGTGGTAAAT 60

cssBIGATTCGGATCCGAAACTGAATTCACAGTTATA.....GTACCAACAGA 251
cssBIIGATTCGGACCCGAAACTGGATTTCACAGTTATA.....GCACCAACAGA 251

Forward Primer
5' → 3'

cssBI TATTAT**ATCAGAAAGGATTCTGGC**.....AGTTTATGGCAGGGCAAAAAGGCTCCTTTT 382
cssBII TATTAT**ATCAGAAAGGATTCTGGC**.....ACTTTATGGCAGGACAAAAAGGATCCTTTC 382

MAMA Reverse Primer
3' ← 5'

cssBICAGGA**C**ATTTGACTGTATCATT**T**TACAGCAATTAA 504
cssBIICAGGA**A**ATTTGACTGTATCATT**T**TACAGCAATTAA 504

Fig1

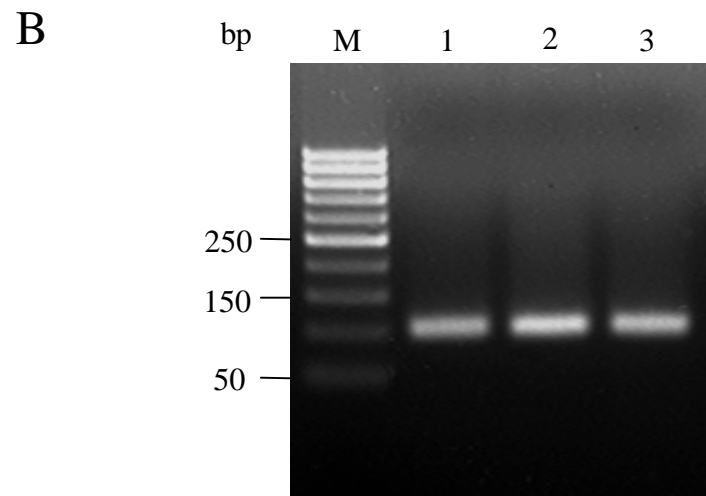
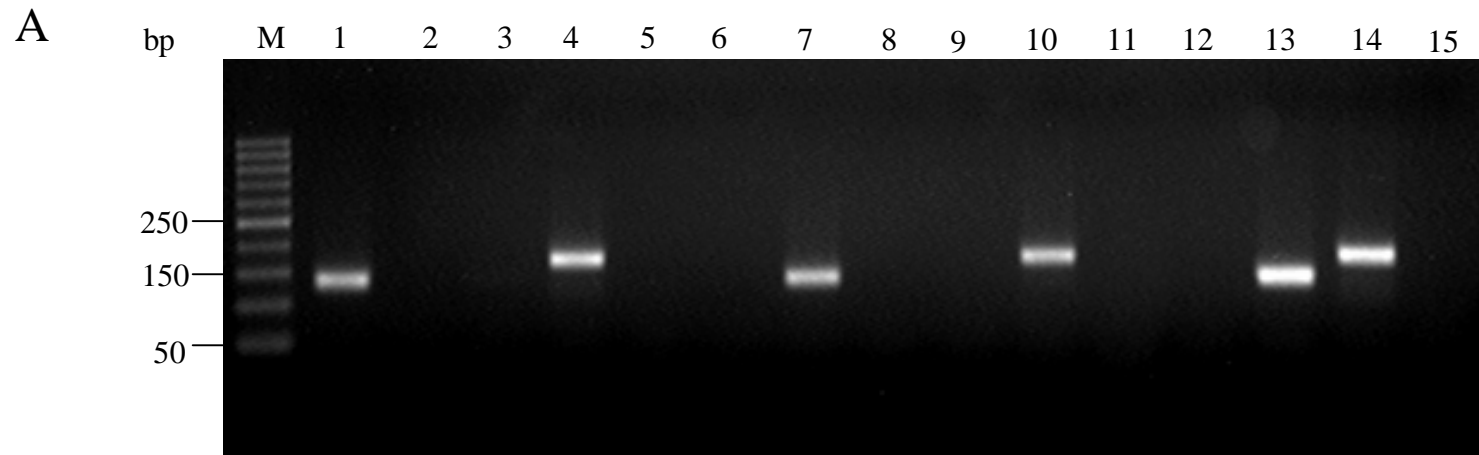


Fig 2

Sabui *et al*, 2011