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Real Time PCR-based Mismatch Amplification Mutation Assay for the Specific Detection of CS6-expressing Allelic Variants of Enterotoxigenic *Escherichia coli* and its Application in Assessing Diarrheal Cases and Asymptomatic Controls

Subrata Sabui[1], Sanjucta Dutta[1], Anusuya Debnath[1], Avishek Ghosh[1], T. Hamabata[2], K. Rajendran[1], T. Ramamurthy[1], James P. Nataro[3], Dipika Sur[1], Myron M. Levine[4], and Nabendu Sekhar Chatterjee[1]*

[1]National Institute of Cholera & Enteric Diseases, Kolkata, India.

[2]National Center for Global Health and Medicine, Tokyo, Japan.

[3]Department of Pediatrics, University of Virginia School of Medicine, Charlottesville, VA

[4]Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland

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*Corresponding author

Division of Biochemistry, National Institute of Cholera & Enteric Diseases

P33 C.I.T. Road, Scheme XM, Beliaghata, Kolkata - 700 010, India

Phone: +91(33) 2363-3372; Fax: +91(33) 2370-5066

e-mail: nschatterjee@rediffmail.com; chatterjeens@icmr.org.in

Abstract

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

Introduction

Enterotoxigenic *Escherichia coli* (ETEC), *Vibrio cholerae* O1and rotavirus, account for a majority of the most severe form of acute diarrhea that can turn fatal in the absence of timely intervention. The virulence factors of ETEC are one or both of the two enterotoxins (6, 14), commonly known as heat-labile (LT) and heat-stable (ST) depending on their temperature susceptibility, and a group of cell surface proteins, collectively called colonization factor antigens (CFAs). The CFAs enable the organisms to adhere to host intestinal cells effectively to initiate pathogenesis (15, 18) by overcoming intestinal peristalsis and mucous barrier. Several studies indicate that 20-40% of the travelers from developed countries experience ETEC-mediated diarrhea in developing countries that are endemic for diarrheal pathogens (4). According to the World Health Organization (WHO), ETEC is the second most common cause of diarrhea after rotavirus in children less than 5 years of age and this age group is the main target for vaccine implementation (8).

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

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

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

Materials and Methods

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

cultured on normal Luria-Bertani agar (Difco, Sparks, MD) plate and screened for the diarrheagenic *E. coli* (DEC) as described previously (11, 15). After confirmation, all the ETEC isolates were serotyped using commercially available O and H antisera (Denka Seiken, Tokyo, Japan). All the ETEC isolates were stored at -80°C in Luria-Bertani broth (Difco) with 15% glycerol until further use. A well characterized ETEC CS6 strain 4266 (7) and a K12 *E. coli* strain DH5α were used as PCR positive and negative control strains, respectively.

Genomic DNA isolation: ETEC isolates were sub-cultured from -80°C stock on CFA medium (1% Casamino acid, 0.15% yeast extract, 0.005% $MgSO_4$, 0.005% $MnCl_2$, pH 7.4). After 24 hrs incubation at 37°C, total genomic DNA was isolated by NucliSENS EasyMag (biomérieux, Marcy l'Etoile, France) machine according to manufacturer's protocol. One ETEC isolate per patient was used in this study and presence of CS6 was confirmed by PCR as described before (16).

Primer design for mismatch qPCR: The MAMA primer to detect the sequence polymorphism among *cssAI, cssAII, cssAIII, cssBI* and *cssBII* subunit genes was focused on nucleotide position 169 and 474 for *cssA* and *cssB* subunit genes of CS6, respectively (Fig.1 A, B). Two conserved forward primers for *cssA* and *cssB* genes and five allele-specific polymorphism detection primers, RV-cssAI, RVcssAII, RV-cssAIII, RV-cssBI and RV-cssBII were designed (Table 1). At 3′ end, these allele-specific primers contained G, C and A for *cssAI, cssAII* and *cssAIII* and C and A for *cssBI, cssBII*, respectively. To increase the specificity of the 3′mismatch activity, nucleotide C was incorporated (rather than T and/or A) at the penultimate nucleotide of 5′- 3′ reverse primers of all CS6 subunit alleles. A conserved forward primer (FO-parC) and a reverse primer (RV-parC) of the housekeeping gene *parC* were used as an internal control.

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

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

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

Results

Specificity of MAMA-qPCR primer pairs

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

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

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

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

Correlation between CS6 allelic variants and toxin genes

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

Discussion

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

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

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

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

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

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Figure legends

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

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

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

Target gene	Location	Oligonucleotide Sequence	Amplicon size (bp)
cssAI	55⟶189[a]	FO-cssA: 5′-AGAACAGAAATAGCGACTA-3′	135
		RV-cssAI: 5′GCTCACACTAAATAATAA**C**C-3′	
cssAII	55⟶189[b]	FO-cssA: 5′-AGAACAGAAATAGCGACTA-3′	135
		RV-cssAII:5′-GCTCACACTAAATAATAA**C**G-3′	
cssAIII	55⟶189[c]	FO-cssA: 5′-AGAACAGAAATAGCGACTA-3′	135
		RV-cssAIII:5′-GCTCACACTAAATAATAA**C**T-3′	
cssBI	328⟶ 494[d]	FO-cssB: 5′-ATCAGAAAGGATTCTGGC-3′	167
		RV-cssBI:5′-GTAAAATGATACACTCAAA**C**G-3′	
cssBII	328⟶494[e]	FO-cssB: 5′-ATCAGAAAGGATTCTGGC-3′	167
		RV-cssBII:5′-GTAAAATGATACACTCAAA**C**T-3′	
parC	63⟶162[f]	FO-parC: 5′-TGGATCGTGCGTTGCCGTTTATTG-3′	100
		RV-parC:5′- AATTTGGCGGTAGCATTCAGACCC-3′	

[a], GeneBank Accession Number GQ241334

[b], GeneBank Accession Number GQ241330

[c], GeneBank Accession Number GQ241332

[d], GeneBank Accession Number GQ241335

[e], GeneBank Accession Number GQ241337

[f], GeneBank Accession Number EU561348

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

CS6 allelic Variant	Number of CS6 positive ETEC			
	Case (n=58)	Control (n=13)	OR (95% CI)	P-value
AIBI	36	4	3.68 (0.88-16.41)	0.039*cs
AIIIBI	16	2	2.10 (0.37-15.40)	0.298
AIIBII	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

*cs =statistically significant association with cases

*ct =statistically significant association with control

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

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

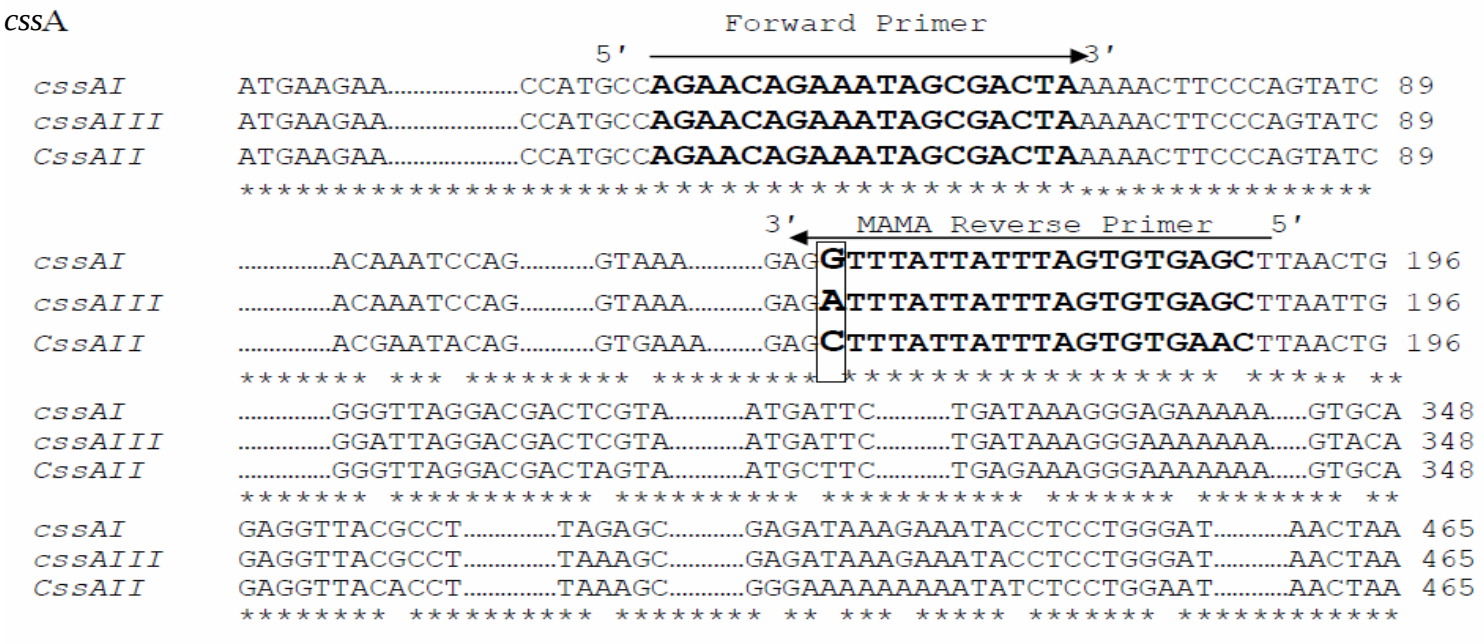
Case*, CS6 expressing ETEC was isolated from diarrheal patients as a mixed or sole pathogen

Control#, CS6 expressing ETEC was isolated from normal children as a mixed or sole pathogen

n, total number of ETEC isolates

-, indicates ETEC were not detected of that toxin type

cssA



cssB

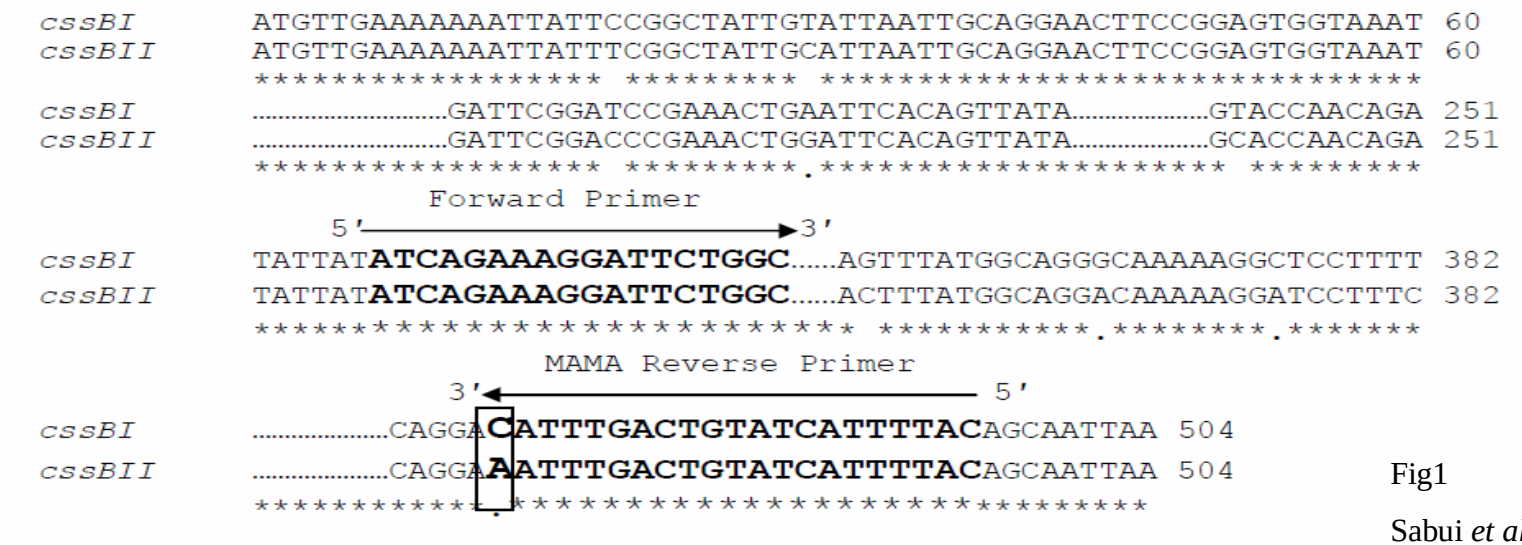
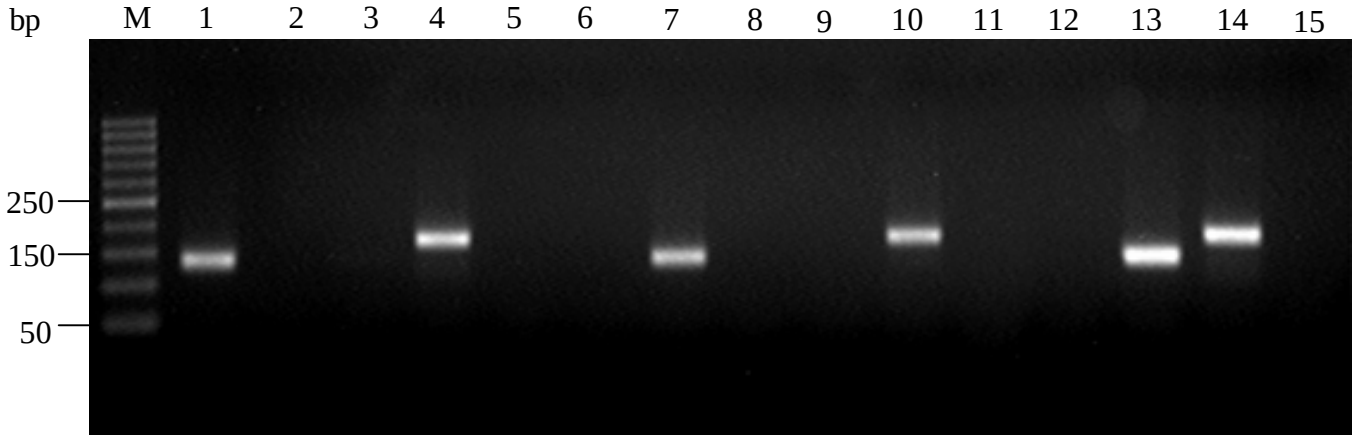


Fig1

Sabui *et al*, 2011

A



B

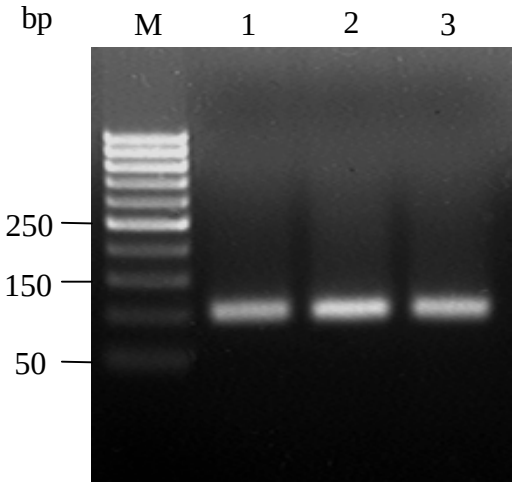


Fig 2

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