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Rapid Detection Method for Enteroaggregative *Escherichia coli* Using Simple Clump Formation and Aggregative Assay

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ABSTRACT

Enteroaggregative *Escherichia coli* (EAggEC) strains cause the persistent diarrhea in infants and compromised hosts in developing countries. These strains are currently defined as *E. coli* that adheres to HEp-2 cells in an aggregative adherence (AA) pattern. In this study, we compared 4 different rapid methods for the detection of EAggEC using a PCR assay, clump formation test, glass slide adherence assay, and the HEp-2 cell adherence assay. Out of 683 *E. coli* strains isolated from diarrheal stool samples, we detected 17 *aggR* and/or clump-positive strains, and identified 2 *aggR*-positive, clump-negative strains and 2 *aggR*-negative, clump-positive strains. All the *aggR* positive and clump positive strains also showed positive results in glass slide adherence and HEp-2 cell adherence assays. From all these results, we suggest the following procedure for the rapid identification of EAggEC strains: first, screen *E. coli* strains with the clump formation test and subsequently perform the glass slide adherence assay to observe AA for confirmation.

Keywords: *Escherichia coli*; Aggregative Adherence; Clump Formation; Rapid Detection

1. Introduction

Enteroaggregative *Escherichia coli* (EAggEC) strains cause persistent diarrhea among infants in developing and industrialized countries [1]. EAggEC strains are characterized by aggregative adherence (AA) to HEp-2 cells and expression of aggregative adherence fimbriae (AAF) [2,3]. The AA phenotype of EAggEC strains are associated with the presence of a 60-MDa plasmid (the pAA plasmid) [4,5], which encodes many virulence genes, including an anti-aggregation protein transporter (*pCVD*432, the AA probe) [6,7], enteroaggregative heatstable toxin (EAST; *astA*) [8,9], aggregative adherence fimbriae I (AAF/I; *aggA*), aggregative adherence fimbriae II (AAF/II; *aggA*), dispersin secretory protein (*aap*), and the gene encoding the transcriptional activator AggR (*aggR*), which is needed for the expression of fimbriae.

The gold standard for EAggEC identification remains the HEp-2 cell adherence assay [10]. However, this assay is expensive and cumbersome, and it is thus impracticable in the majority of clinical laboratories. As a rapid test to identify EAggEC, Albert *et al.* [11] reported bacterial clump formation at the surface of liquid culture. On the other hand, it was reported previously that PCR assay is a convenient and sensitive molecular test to detect EAggEC [6,12]. However, both these methods may require the use of the HEp-2 cell adherence assay for the final diagnosis of EAggEC. In this study, we compared 4 different rapid methods for the detection of EAggEC using a PCR assay, clump formation test, glass slide adherence assay, and the HEp-2 cell adherence assay. We suggest the following procedure for the rapid identification of EAggEC strains: first, screen *E. coli* strains with the clump formation test and subsequently perform the glass slide adherence assay to observe AA for confirmation.

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2. Materials and Methods

2.1. PCR Amplification

A total of 683 *E. coli* strains isolated from diarrheal stool samples were examined for the detection of the *aggR* and *pCVD*432 genes by PCR. Pure colonies of each strain was suspended in 3 mL of Luria-Bertani (LB) broth (Sigma-Aldrich Japan, Tokyo, Japan) and incubated overnight at 37˚C with shaking at 115 rpm. One hundred

μL aliquot of each bacterial culture was suspended in 900 μL of Tris-EDTA (TE) buffer, boiled for 5 min, and centrifuged at 10,000 × *g* for 5 min [13]. Supernatant was separated and used as the template for PCR. The primers for the *aggR* gene were as follows: forward, 5'–GTATACACAAAAGAAGGAAGC–3' and reverse 5'–ACAGAATCGTCAGCATCAGC–3' [14], and for the *pCVD*432 gene as follows: forward, 5'–CTGGCGAAAGACTGTATCAT–3' and reverse, 5'–TAATGTATAGAAATCCGCTCTT–3' [15]. The PCR assay was performed as follows: each 25 μL reaction mixture contained 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 1.5 - 2.0 mM $MgCl_2$, 2.5 mM of each deoxynucleoside triphosphate, 1.25 U of Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan), 25 μM of each primer, and 2.5 μL of the DNA template. The mixture was preheated at 94˚C for 1 min, and then amplified for 25 cycles using a thermal cycler (BioRad i-cycler; BIO-RAD, Hercules, CA., USA). Each cycle consisted of denaturation at 94˚C for 1 min, annealing at 55˚C for 1 min, and extension at 72˚C for 1 min. A final extension step was performed at 72˚C for 10 min. The PCR products were subjected to electrophoresis (Mupid-21; Cosmo Bio Ltd., Tokyo, Japan) in agarose gels (Nippon Gene Co., Ltd., Tokyo, Japan) containing ethidium bromide.

2.2. Clump Formation Test

All the 683 *E. coli* strains were examined for the formation of bacterial clumps visible as a thick scum in a liquid culture. A total of 6 broth media [Müeller-Hinton (MH) broth (Kanto Chemical Co., Tokyo, Japan), tryptic soy broth, nutrient broth, heart infusion broth, brain heart infusion broth (Sigma-Aldrich Japan, Tokyo, Japan), and LB broth] were tested to find out the suitable medium for the clump formation. For this each *E. coli* strain was cultured in 3 mL of the broth media and incubated for 20 h at 37˚C with shaking at 100 rpm.

2.3. Glass Slide Adherence Assay

To assess the AA, 20 mL of MH broth medium was taken in a 50 mL tube containing a glass slide. The broth medium was inoculated with 100 μL of an overnight MH broth culture (prepared earlier) and was incubated for 6 h to 18 h at 37˚C with shaking at 115 rpm[11]. The glass slide dipped in the culture medium was then taken out, fixed in methanol, stained with Giemsa stain and examined under a microscope.

2.4. HEp-2 Cell Adherence Test

For the HEp-2 cell adherence test, 200 μL of a HEp-2 cell suspension containing approximately 1.0×10^5 cells per mL of Dulbecco's modified Eagle medium (DMEM; Life Technologies Co., Tokyo, Japan) supplemented with 10% fetal bovine serum and 0.45% glucose, was added to a 35 mm petri dish (Iwaki, Japan) and incubated for 44 h at 37˚C [16]. The cells were then washed with phosphate-buffered saline (PBS) 3 times and covered with DMEM again. In addition, a dish containing 40 μL of a bacterial culture in MH broth was incubated for 16 - 18 h at 37˚C, adjusted to McFarland standard 1, and further incubated for another 3 h at 37˚C and 5% CO_2. The dishes were then washed with PBS 5 times, and the cells were fixed with methanol, stained with Giemsa stain, and examined under a microscope.

3. Results

Out of the 683 *E. coli* strains examined, a total of 15 *aggR*-, *pCVD*432-gene positive strains were detected by the PCR. However, no *aggR*-positive, *pCVD*432-negative or *aggR*-negative, *pCVD*432-positive strains were detected.In the clump formation test, we compared 6 broth media (MH, tryptic soy, nutrient, heart infusion, brain heart infusion, and LB broth) and all broth media were found to develop same thick scum (**Figure 1**). Therefore, the MH broth medium was used in the subsequent experiments. Thirteen of the 15 *aggR*-positive strains and 2 of the *aggR*-negative strains were found to form scum (*i.e.*, clump-positive) in the clump formation tests. However, 2 of the *aggR*-positive strains and 668 of the *aggR*-negative strains were not found to be clump-positive (**Table 1**).

In glass slide adherence test, AA were observed for different time periods between 6 h and 18 h of incubation. After 6 h, only a few diffused colonies of adherent bacteria were observed. However, between 8 - 18 h of incubation, the bacteria were too overgrown to see distinct colonies. Therefore, in this study, the glass slides incubated for 7 h were taken as optimum and were examined (**Figure 2**). Accordingly, to demonstrate the diagnostic

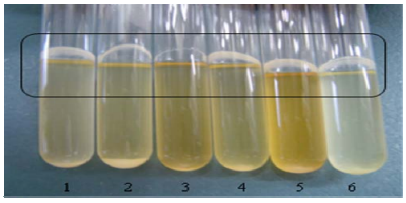

Figure 1. The results of clump formation test as a scum by six broths. 1: Müeller-Hinton broth; 2: Tryptic soy broth, 3: Nutrient broth; 4: Heart infusion broth; 5: Brain heart infusion broth; 6: LB broth.

Table 1. The results of PCR assay (*aggR*, *pCVD*432) and clump formation test in this study (n = 683).

		aggR + *pCVD*432	
		positive	negative
clump formation test	positive	13	2
	negative	2	666

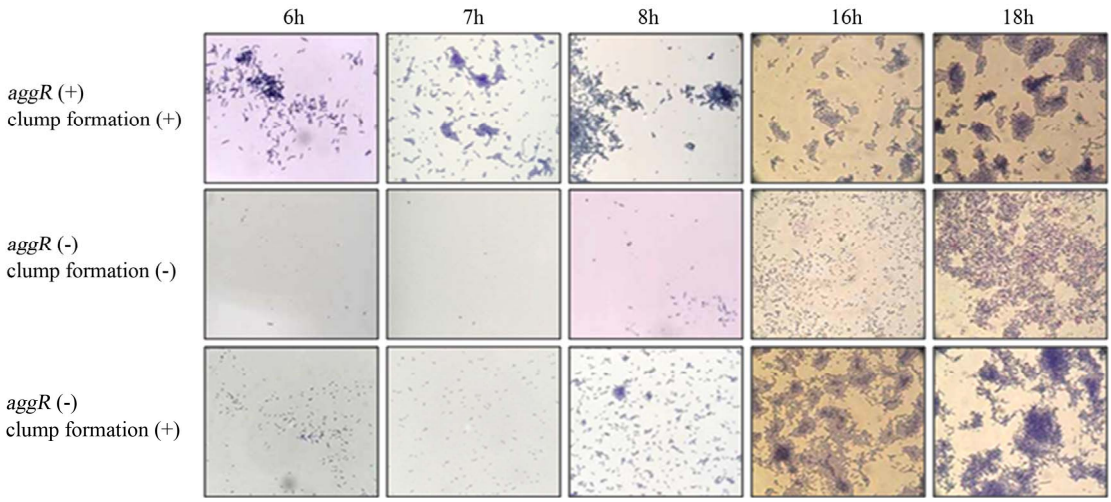

Figure 2. The time course of "stacked-brick configuration of AA". To assess AA was monitored at different times of 6 h to 18 h in this glass slide adherence assay.

usefulness of glass slide adherence assay, 17 *aggR* and/or clump–positive strains were examined (**Table 2**). Among the 13 *aggR*-positive, clump-positive strains showed AA, and 2 *aggR*-positive and 2 *aggR*-negative but clump-positive strains did not show AA (**Figure 3**). The results of the HEp-2 cell adherence assay were found to be similar to those of the glass slide adherence assay (**Figure 4**).

4. Discussion

EAggEC strains cause persistent diarrhea in infants and compromised hosts in developing countries. The main clinical manifestations are watery diarrhea and stomachache, with occasional fever and vomiting. Therefore, it is very important to detect the EAggEC strains as early as possible for a proper treatment [2].

EAggEC strains are currently defined as *E. coli* strains that do not secrete heat-labile (LT) or heat-stable (ST) enterotoxins and that adhere to HEp-2 cells in an AA pattern. The gold standard for EAggEC identification remains the HEp-2 cell adherence assay. However, the test requires specialized facilities and equipment, and confirmation of the specific gene still remains to be done by PCR. Albert *et al.* [11] reported bacterial clump formation as a rapid, convenient, and useful test to screen for EAggEC strains. In this study, we used a combination of the PCR assay, clump formation test, glass slide adherence assay, and HEp-2 adherence assay to develop a rapid detection method for clinical application.

Of the total 683 *E. coli* strains examined by PCR, 15 strains were found to be *aggR*/*pCVD*432-positive. Some researchers believe that a typical EAggEC possesses both *aggR* and *pCVD*432 genes that are present in the virulence plasmid pAA [17,18]. Kimata *et al.* [19] identified a few *pCVD*432-positive and *aggR*-negative strains, and

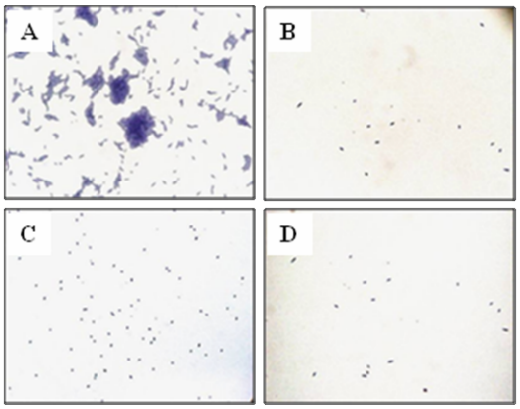

Figure 3. Confirmation of AA in glass slide adherence assay. (A) *aggR* (+) and clump formation (+) strain; (B) *aggR* (+) and clump formation (–) strain; (C) *aggR* (–) and clump formation (+) strain; (D) *aggR* (–) and clump formation (–) strain.

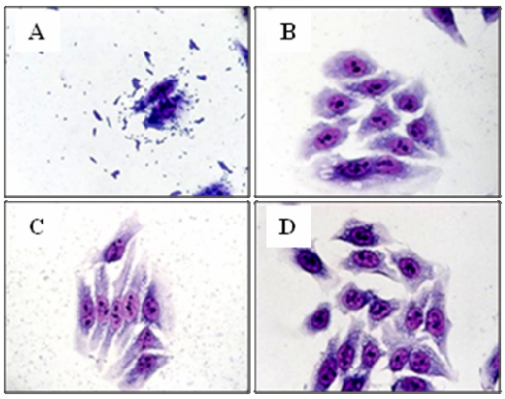

Figure 4. Confirmation of AA in HEp-2 cell adherence assay. (A) *aggR* (+) and clump formation (+) strain; (B) A*: aggR* (+) and clump formation (–) strain; (C) *aggR* (–) and clump formation (+) strain; (D) *aggR* (–) and clump formation (–) strain.

Table 2. The results of using *aggR* and/or clump formation-positive strains for glass slide adherence assay and HEp-2 cell adherence assay in this study.

adherence assay	No. of *aggR* and/or clump-positive strains (n = 17)		
	aggR (+) Clump (–) (n = 2)	*aggR* (–) Clump (+) (n = 2)	*aggR* (+) Clump (+) (n = 13)
Glass slide	0	0	13
HEp-2 cell	0	0	13

vice versa. But in this study, no such strains could be detected. On the other hand, 13 of the *aggR*-positive strains and 2 of 668 *aggR*-negative strains demonstrated clump formation in this study. Although all of the 13 *aggR*-positive and clump-positive strains showed AA, none of the 668 *aggR*-negative strains or the 2 *aggR*-positive except clump-negative strains displayed any AA. Therefore, even though the *aggR*-negative strains were clump-positive, they did not demonstrate AA.

AA was characterized by the prominent autoagglutination of bacterial cells as well as adherence to glass cover slips without HEp-2 cells. The *sine qua non* of AA, however, was the characteristic layering of the bacteria, best described as a stacked-brick configuration. To observe AA in the glass slide adherence and HEp-2 cell adherence assay, we examined the *E. coli* strains after 6 - 18 h of incubation. After 6 h, the *E. coli* strains did not show sufficient enrichment; however, after 8 h of incubation, the strains overgrew, and adherences were not clearly visible. Therefore, the optimal incubation time required to observe AA was chosen as 7 h.

EAggEC strains characteristically enhance mucus secretion from the mucosa, trapping the bacteria in a bacterium-mucus biofilm. Nataro *et al.* [2] reported that the role of excess mucus production in EAggEC pathogenesis was unclear. However, the formation of a heavy biofilm may be related to the diarrheagenicity of the organism and, perhaps, to its ability to cause persistent colonization and diarrhea. Therefore, the demonstration of AA is very useful for the rapid diagnosis of EAggEC. From all these results, we make the following suggestion for the identification of EAggEC: the *E. coli* strains should first be screened using the clump formation test, which should then be followed by the glass slide adherence assay for confirmation.

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