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Use of Ramification Amplification Assay for Detection of *Escherichia coli* O157:H7 and Other *E. coli* Shiga Toxin-Producing Strains

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***Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC) strains are important human pathogens that are mainly transmitted through the food chain. These pathogens have a low infectious dose and may cause life-threatening illnesses. However, detection of this microorganism in contaminated food or a patient's stool specimens presents a diagnostic challenge because of the low copy number in the sample. Often, a more sensitive nucleic acid amplification method, such as PCR, is required for rapid detection of this microorganism. Ramification amplification (RAM) is a recently introduced isothermal DNA amplification technique that utilizes a circular probe for target detection and achieves exponential amplification through the mechanism of primer extension, strand displacement, and ramification. In this study, we synthesized a circular probe specific for the Shiga toxin 2 gene (stx_2). Our results showed that as few as 10 copies of stx_2 could be detected, indicating that the RAM assay was as sensitive as conventional PCR. We further tested 33 isolates of *E coli* O157:H7, STEC, *Shigella dysenteriae*, and nonpathogenic *E. coli* by RAM assay. Results showed that all 27 STEC isolates containing the stx_2 gene were identified by RAM assay, while *S. dysenteriae* and nonpathogenic *E. coli* isolates were undetected. The RAM results were 100% in concordance with those of PCR. Because of its simplicity and isothermal amplification, the RAM assay could be a useful method for detecting STEC in food and human specimens.**

Escherichia coli O157:H7 and other Shiga toxin-producing *E. coli* (STEC) strains have emerged as significant food-borne pathogens since their early identification in 1982 (7). They can cause severe clinical manifestations, including bloody diarrhea, hemorrhagic colitis, and postinfection hemolytic-uremic syndrome, symptoms associated with high morbidity and mortality. Cytotoxins, Shiga toxin types 1 and 2, produced by *E. coli* O157:H7 and STEC are responsible for these clinical symptoms (6). Infection with *E. coli* O157:H7 and STEC can occur sporadically, in small clusters, or in large outbreaks. The bacteria may be transmitted in a variety of ways, most commonly through food and water. Ruminants have been established as important reservoirs of *E. coli* O157:H7, and consequently, foods derived from or contaminated by these animals and their products are the major vehicles of transmission (5).

A number of methods have been developed for detecting the pathogens in food and clinical specimens, including culture isolation using selective media, such as sorbitol-substituted MacConkey agar and methylumbelliferyl-β-D-glucuronide agar, serological tests to detect O157 and H7 antigens, and immunological detection of Shiga toxins (5). To achieve sensitive, specific, and rapid detection of STEC and *E. coli* O157:H7 strains in clinical specimens and food products, several research teams have employed the PCR technique (1, 2). However, a number

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TABLE 1. Characteristics and genotyping of bacterial isolates by PCR and RAM

Isolate no.	Serotype	Source	Sorbitol fermentation	PCR (stx_1 and/or stx_2)[b]	RAM (stx_2)
1	O5:NM	Sheep	Negative	2	+
2	O22:H8	Ground beef	Positive	2	+
3	O26:H11	Cattle	Positive	1	−
4	O26:H11	Cattle	Positive	1	−
5	O46:H38	Ground beef	Positive	1, 2	+
6	O111:NM	Cattle	Positive	1, 2	+
7	O157:H7	Raw milk	Negative	1, 2	+
8	O113:K75:H21	Human	Positive	2	+
9	O157:H7	Raw milk	Negative	1, 2	+
10	O157:H7	Meat	Negative	1, 2	+
11	O157:H7	Meat	Negative	1, 2	+
12	O157:H7	Meat	Negative	1, 2	+
13	O157:H7	Cattle	Negative	1, 2	+
14	O157:H7	Human	Negative	1, 2	+
15	O157:H7	Human	Negative	1, 2	+
16	O157:H7	Human	Negative	1, 2	+
17	O157:H7	Human	Negative	1, 2	+
18	O157:H7	Cattle	Negative	1, 2	+
19	O157:H7	Human	Negative	1, 2	+
20	O157:H7	Human	Negative	1, 2	+
21	O157:H7	Human	Negative	1, 2	+
22	O157:H7	Human	Negative	1, 2	+
23	O157:H7	Human	Negative	1, 2	+
24	O157:H7	Human	Negative	1, 2	+
25	O157:H7	Cattle	Negative	2	+
26	O157:H7	Chicken	Negative	2	+
27	O157:H7	Goat	Negative	2	+
28	O157:H7	Goat	Negative	2	+
29	O157:H7	Human	Negative	1, 2	+
30–32	Nonpathogenic *E. coli*	Human	ND[a]	Negative	−
33	*Shigella dysenteriae*	Human	ND[a]	1	−

[a] ND, not determined.
[b] 1, stx_1; 2, stx_2.

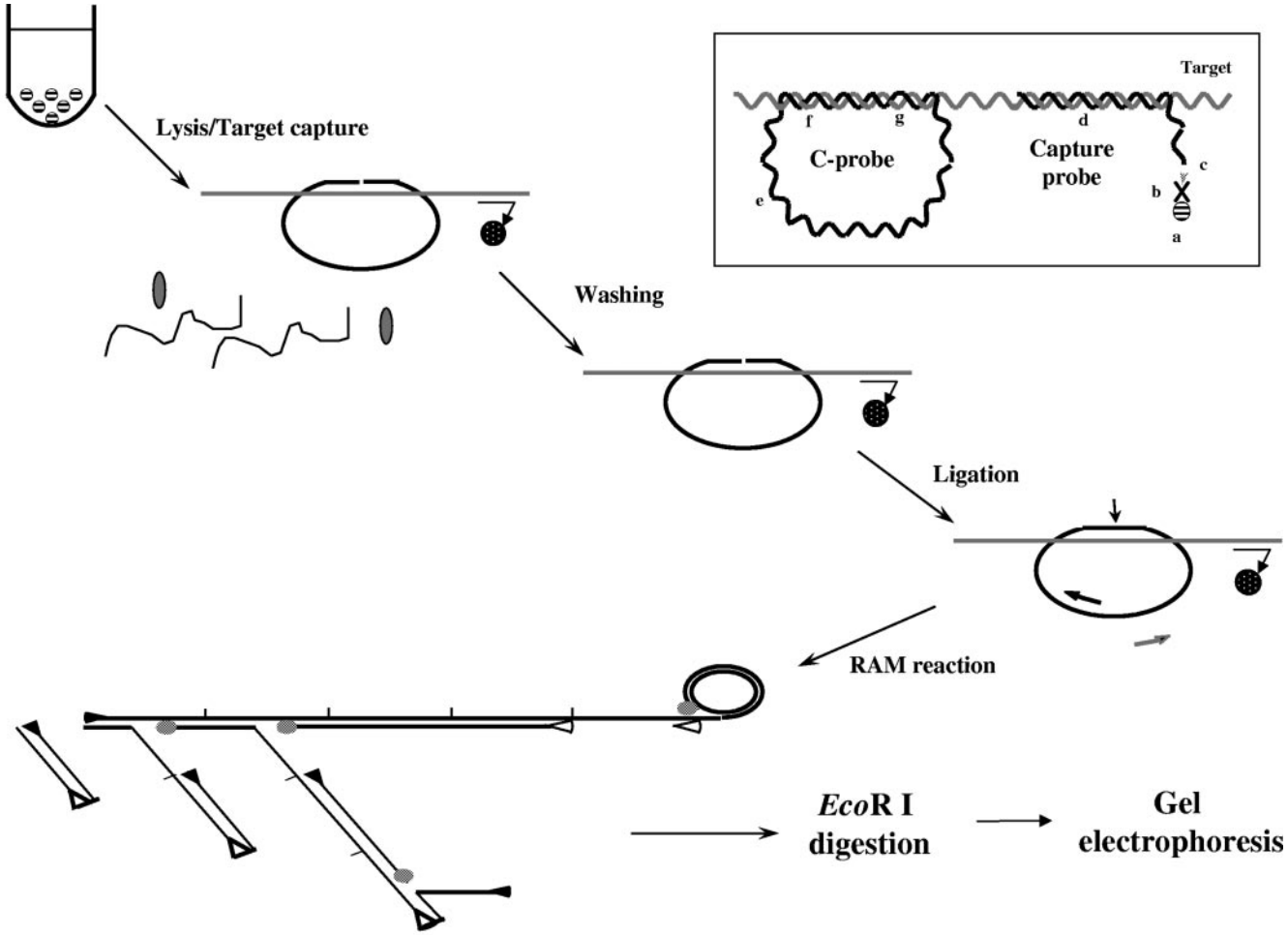

FIG. 1. Schematic representation of RAM assay. Target DNA, capture probe, C-probe, and paramagnetic bead are added to hybridization buffer to allow the formation of a hybrid complex. The hybrid is captured on a paramagnetic bead, allowing extensive washing to remove unbound C-probe and cellular components. The C-probe aligned on the target is linked together by a DNA ligase. RAM amplification is then carried out by the addition of forward (▶) and reverse (▷) primers and DNA polymerase (●). The forward primer bound to the C-probe is extended by the polymerase and continues after one round of synthesis by displacing the bound forward primer and its extended product, generating a long single-stranded DNA (ssDNA) with repeated sequence. With the reactions taking place, multiple reverse primers can bind to the nascent ssDNA as their binding sites become available. Each bound reverse primer will be extended and displace the downstream primers and their extended products. The forward primer binding sites of the displaced ssDNA are then available for the forward primer to bind and extend in a similar fashion, thus forming a large ramifying DNA complex. Finally, the RAM products are examined by gel electrophoresis after EcoRI digestion. (Inset) The C-probe hybridizes to the target through their complementary regions (f and g), and the sequence at the noncomplementary region (e) is generic for the binding of primers. The C-probe–target hybrid is captured on a paramagnetic bead (a) through the binding of the biotin moiety (c) on the capture probe (d) to the streptavidin (b) that the beads were coated with.

of drawbacks associated with such a PCR approach have limited its routine use in many laboratories (2).

We have recently developed a novel isothermal DNA amplification technology, termed ramification amplification or RAM (8). In this study, we developed a detection assay by combining magnetic bead-based DNA isolation, DNA amplification by RAM, and real-time fluorescence detection (9). The technique uses a circularizable probe to detect the target with subsequent amplification of the circular probe generated by a target-dependent ligation through a mechanism of primer extension, strand displacement, and ramification to achieve a billionfold amplification under isothermal conditions (Fig. 1) (11). The objective of this study was to determine the analytical sensitivity and specificity of the RAM assay for detecting the Shiga toxin 2 gene (stx_2) and its feasibility for detecting *E. coli* O157:H7 and other STEC strains isolated from food and human specimens.

MATERIALS AND METHODS

Sample preparation. Bacterial isolates were obtained from the University of Maryland (18 isolates) and Center for Disease Control, China (12 isolates). All *E. coli* isolates were characterized by culture on sorbitol-substituted MacConkey agar and serologically typed for O and H antigens (Table 1). The presence of Shiga toxin genes (stx_1 and stx_2) was determined by PCR for all isolates. Of the 32 *E. coli* isolates, 23 were sorbitol negative and 22 were serologically determined to be *E. coli* O157:H7. Seven isolates were serologically determined to be non-O157 strains, of which six were sorbitol fermenters and only one was a nonfermenter. Three nonpathogenic *E. coli* isolates and one *Shigella dysenteriae* isolate obtained from the Clinical Microbiology Laboratory, Mount Sinai Hospital, were included as controls in this study.

TABLE 2. Sequences of circular probe, capture probe, RAM and PCR primers, and synthetic target sequence

Probe (no. of nucleotides)	Sequence (5′–3′)[a]
C-probe (124)	GGAATAGGATACCGAAGAAAAACCCAGTGTCTGTGTATCTGCTA**ACCAAGAGCAACTACACgaattc**TC
	G**ATTAGGTTACTGCGATTAGCACAAG**CGGTCGAAAAGTCTATCGTAAACTCCCG
Capture probe (43)	Biotin-TACCACTGAACTCCATTAACGCCAGATATGATGAAACC
RAM primer 1 (25)	CTTGTGCTAATCGCAGTAACCTAAT (forward)
RAM primer 2 (23)	ACCAAGAGCAACTACACGAATTC (reverse)
stx_2 target (97)	CTGGGTTTTTCTTCGGTATCCTATTCC|GCCCTCAAATGCTATCTGAAAAGCTGGGTTGGTTTCATC
	ATATCTGGCGTTAATGGAGTTCAGTGGTA
stx_1 PCR primer 1 (20)	ACACTGGATGATCTCAGTGG (forward)
stx_1 PCR primer 2 (20)	CTGAATCCCCCTCCATTATG (reverse)
stx_2 PCR primer 1 (21)	CCATGACAACGGACAGCAGTT (forward)
stx_2 PCR primer 2 (20)	CCTGTCAACTGAGCACTTTG (reverse)

[a] Single underlining indicate sequences complementary to stx_2 genes of pathogenic *E. coli*; lowercase letters indicate the restriction enzyme EcoRI recognition site (GAATTC); underlining and boldface type indicate the binding region for RAM primer 1; dotted underlining and boldface type indicate the binding region for RAM primer 2. For the stx_2 target, single underlining indicates the C-probe binding region; the vertical bar indicates contact sites of the 3′ and 5′ ends of the C-probe, and double underlining indicates the capture probe binding region.

The bacteria were inoculated onto a blood agar plate and incubated at 37°C overnight. A single colony was picked and suspended in water in a centrifuge tube. For the RAM assay, the bacteria were washed twice with saline and lysed in 100 μl of 5 M guanidium thiocyanate (GTC; Sigma, St. Louis, MO), 0.5% bovine serum albumin (Sigma), 80 mM EDTA, 400 mM Tris-HCl (pH 7.5), and 0.5% sodium-*N*-lauroylsarcosine (Sigma) (9). The lysates were incubated at 100°C for 10 min and then at 60°C overnight. The lysed specimens were stored at −20°C until later use. For PCR assay, the bacteria were resuspended in 200 μl of distilled water, heated to 99°C for 10 min, and then centrifuged for 2 min at 12,000 rpm in an Eppendorf centrifuge. The resulting supernatant was used for PCR assay.

For quantitative analysis of *E. coli* O157:H7, a bacterial colony was picked and dissolved in saline. The bacterial density was determined by densitometry, and the concentration was determined by comparing the optical density value with those of standards of known bacterial concentrations. The bacteria were diluted with saline in a series of 10-fold dilutions, starting from 10^5 to 10 bacteria/μl. One microliter of suspension was inoculated onto MacConkey agar, and the number of colonies formed was determined to confirm the number of bacteria in initial dilutions. The supernatant was removed, and 5 M GTC was added to each tube, boiled at 100°C for 10 min, and incubated at 60°C overnight.

PCR assay. PCR was carried out in a 50-μl reaction mixture composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM concentrations of each deoxynucleoside triphosphate, 10 pmol of each primer (Table 2), 1 unit of *Taq* DNA polymerase (Roche, Indianapolis, IN), and 10 μl of bacterial lysate (2). The reaction was performed in a thermal cycler (GeneAmp 2700 PCR system; Perkin Elmer, Boston, MA) for 30 cycles of 94°C, 55°C, and 72°C for 1 min each. Fifteen microliters of PCR products was analyzed by gel electrophoresis using a 2.0% agarose gel and visualized after staining with 0.5 μg/ml of ethidium bromide. The expected PCR products are 614 bp for stx_1 and 779 bp for stx_2.

RAM assay. The RAM assay consists of steps, including hybridization of C-probe and capture probe to a target, capture of the hybrid onto magnetic beads, washing of the beads to remove unbound probes and cellular components, ligation of the 3′ and 5′ ends to form a closed C-probe, and amplification by primer extension, strand displacement, and ramification (Fig. 1) (8). Hybridization of C-probes and capture probes to targets was carried out in an 80-μl reaction mixture containing 2 M GTC, 0.5% bovine serum albumin (Sigma), 80 mM EDTA, 40 mM Tris-HCl (pH 7.5), 0.5% sodium-*N*-lauroylsarcosine (Sigma), 50 nM phosphorylated C-probe (Table 2), 2 μM capture probes, and 2 μl of lysed specimens or synthetic target DNA. The reaction mixture was incubated at 55°C for 2 h to allow complete hybridization. Then, 2 μl of magnetic beads (10 mg/ml; Dynal, Lake Success, NY) in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 M NaCl was added, and the mixture was incubated to allow the beads coated with streptavidin to capture the biotin on the capture probe. The beads with bound complex were then washed twice with 400 μl of TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA) at room temperature to remove unhybridized C-probes and other cellular components. Twenty microliters of ligase mixture containing 20 mM Tris-HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol, 1 mM NAD, 0.1% Triton X-100, and 12 units of *Taq* DNA ligase (New England Biolabs, Boston, MA) was added to the bead pellet and incubated at 60°C for 10 min. Ligation of the 3′ and 5′ ends of the C-probe produced a closed circular DNA that was locked onto the target. After ligation, the RAM reaction was initiated by adding 50 μl of RAM reaction mixture containing 20 mM Tris-HCl buffer (pH 8.8), 300 μM deoxynucleoside triphosphate, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 1.2 μM concentrations of each forward and reverse primer (Table 2), 6.4 units of *Bst* DNA polymerase large fragment (New England Biolabs), 4 ng T_4 Gene 32 protein (USB Biochemicals, Cleveland, OH), and 6% dimethyl sulfoxide (Sigma). This mixture was then incubated at 63°C for 1 h to start the ramification reaction, followed by heating at 95°C for 5 min to inactivate the *Bst* DNA polymerase. Fifteen microliters of the RAM products was transferred to a 5-μl mixture containing 50 mM Tris-HCl (pH 7), 15 units of EcoRI (Boehringer Mannheim, Mannheim, Germany), 100 mM $MgCl_2$, and 1 mM dithioerythritol, and the reaction mixture was incubated at 37°C for 3 h. Fifteen microliters of the digested products was analyzed on a 2% agarose gel.

Real-time RAM. The RAM reactions were carried out as described above except that 2.5 μl of 1:5,000 diluted SYBR Green I (Roche) was added to each reaction mixture. SYBR Green I is a fluorochrome that, upon binding to the minor groove of double-stranded DNA, emits an intense green fluorescent signal which can be readily detected using a fluorometer. The reactions were monitored at 37°C for 2 h in a SmartCycler (Cepheid, Sunnyvale, CA).

RESULTS AND DISCUSSION

RAM technology employs a circular probe for target detection and amplification, which offers several unique features

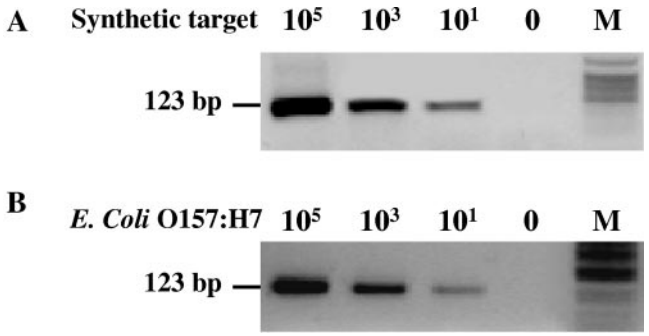

FIG. 2. Sensitivity of RAM assay for detecting Shiga toxin target. (A) The RAM reactions were initiated with the synthetic Shiga toxin 2 targets of 10^5, 10^3, and 10 copies. The RAM products were examined on a 2% agarose gel following EcoRI digestion. The results showed that 10 copies of Shiga toxin 2 targets were detected. Lane 0, no target present; lane M, molecular marker (pBR 322/MspI; New England Biolabs). (B) Sensitivity was confirmed using *E. coli* O157:H7 that was diluted to 10^5, 10^3, and 10 copies. As few as 10 bacteria of *E. coli* O157:H7 can be detected. Lane 0, no target present; lane M, molecular marker (pBR 322/MspI; New England Biolabs).

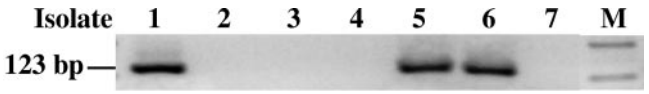

FIG. 3. Specificity of RAM assay for detecting Shiga toxin genes in bacteria. Seven bacterial isolates were tested with the RAM assay. Our results showed that the Shiga toxin 2 gene was present only in pathogenic *E. coli*. Lane 1, *E. coli* O157:H7; lanes 2 to 4, nonpathogenic *E. coli*; lane 5, *E. coli* O46:H38; lane 6, *E. coli* O111:NM; lane 7, *S. dysenteriae*.

(Fig. 1) (10, 11). The formation of a closed C-probe requires target-specific ligation of the C-probe; the 5′ end of the C-probe must align perfectly with its 3′ end on the target DNA for ligation to occur (3, 4). The C-probe can then be amplified with a set of generic primers that bind to the loop region of the C-probe (3), achieving an exponential amplification with a power similar to that of PCR. However, since no temperature cycling is required, the reaction can be carried out at a constant temperature, obviating the use of an expensive thermocycler.

We initially determined the analytical sensitivity of the RAM assay using a synthetic stx_2 DNA target (Table 2). The DNA was diluted in 100-fold serial dilutions from 10^5 to 10^3 to 10^1 molecules/2 μl and was used to initiate the RAM reaction. The lowest number of targets detected by RAM assay was 10 molecules (Fig. 2A), and the reactions were confirmed by finding the correct products (124 bp) in each lane after digestion with EcoRI, establishing the C-probe as their source. In the absence of a target molecule, no DNA was produced, validating a target-dependent amplification of the C-probe. The assay sensitivity was further determined using an *E. coli* O157:H7 strain. The bacterial concentration was determined by densitometry. The bacteria were lysed in 5 M GTC, diluted to 10^5, 10^3, and 10^1 copies/2 μl, and then used to initiate the RAM reaction. The results in Fig. 2B showed that the assay was able to detect as few as 10 bacteria, a sensitivity comparable to that of PCR.

To determine the assay specificity, we tested several bacterial strains, including *E. coli* O157:H7, *E. coli* O46:H38, *E. coli* O111:NM, three nonpathogenic *E. coli* isolates, and *S. dysenteriae* by RAM assay to determine the assay specificity. As expected, *E. coli* O157:H7, *E. coli* O46:H38, and *E. coli* O111:NM were positive for the stx_2 gene, while *S. dysenteriae* and the three nonpathogenic *E. coli* isolates were negative (Fig. 3). These results evidently confirmed the specificity of the RAM assay.

We then tested 29 pathogenic *E. coli* isolates from human and food samples for the presence of *stx* genes (Table 1). The presence of *stx* genes was determined by PCR using primers specific for stx_1 and stx_2 (Table 2). Since the C-probe was designed specifically to recognize stx_2, it was expected that the presence of stx_2 would give a positive result by RAM assay. All 27 Shiga toxin 2-producing *E. coli* isolates were positive by RAM assay, irrespective of their serological types (Table 1). Two pathogenic *E. coli* isolates containing only stx_1 were negative by RAM assay, thus confirming the specificity of the C-probe.

It will be desirable to detect the RAM reaction by real-time monitoring instead of gel electrophoresis. We applied SYBR Green I dye in our RAM reactions. The initial experiment was performed using a lysed *E. coli* O157:H7 sample. Our results showed that as few as 10 bacteria could be detected and that the time needed for the emergence of a detectable signal was dependent on the target concentration (Fig. 4A). Additionally, we have employed this method for the detection of bacterial isolates, and Fig. 4B shows an example of real-time RAM assay of three isolates. This study showed that real-time RAM assay can be developed for diagnostic use, which can significantly shorten the assay time and eliminate the possibility of carryover contamination.

This study demonstrated that RAM assay could be another DNA amplification method to detect STEC. The high sensitivity and specificity of the RAM assay coupled with its ease of application encourage further investigation and improvement of this technique. Future study will focus on designing several C-probes to target other virulence genes, such as the stx_1, *hly*, and *eae* genes, for multiplex RAM assay. We also hope, in the near future, to conduct a larger clinical study to determine

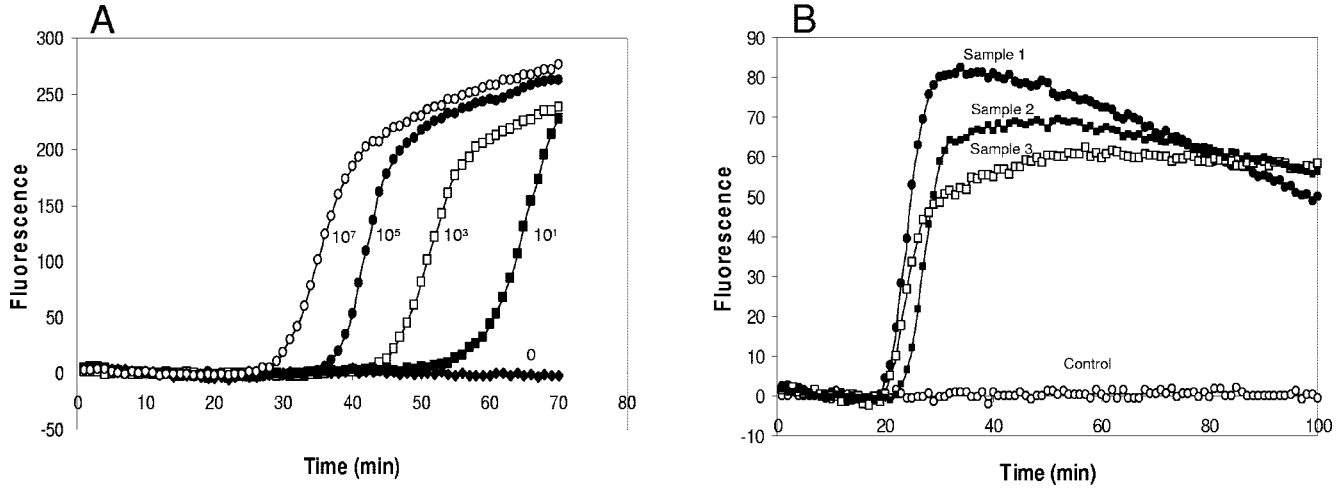

FIG. 4. Real-time RAM assay. (A) RAM assays were initiated with 10^7, 10^5, 10^3, and 10 cells of *E. coli* O157:H7 in the presence of SYBR Green I; (B) RAM reactions were monitored using a SmartCycler. Three isolates were assayed with RAM in the presence of SYBR Green I, and RAM products were detected as the intensity of the fluorescent signal increased.

assay sensitivity and specificity for complex clinical samples such as stool and food.

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