Sensitive and rapid detection of *Escherichia coli* O157:H7 from beef sample based on recombinase aided amplification assisted CRISPR/Cas12a system

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Abstract

Background: Escherichia coli O157:H7, being the cause of hemorrhagic colitis in human, is recognized as one of the most dangerous and widespread foodborne pathogens. A highly specific, sensitive and rapid E. coli O157:H7 detection method need to be developed since the traditional detection methods are complex, costly and time-consuming.

Objective: In this study, a recombinase aided amplification (RAA) assisted CRISPR/Cas12a (RAA-CRISPR/Cas12a) fluorescence platform for specific, sensitive and rapid nucleic acid detection of E. coli O157:H7 was introduced.

Methods: Firstly, the feasibility (components of CRISPR/Cas12a system) of the developed method was evaluated. Then, a total of 34 bacterial strains were used for specificity test, and gradient dilutions of extracted DNA and bacterial solutions of E. coli O157:H7 were prepared for sensitivity test. Thirdly, a real-time PCR assay for detection of the specific wzy gene of E. coli O157: H7 (FDA’s Bacteriological Analytical Manual) was used for sensitivity comparison. Finally, analysis of RAA-CRISPR/Cas12a detection in spiked and 93 real ground beef samples was carried out.

Results: The developed RAA-CRISPR/Cas12a method showed high specificity and the detection could be completed within 30 min (after 4 h enrichment in spiked ground beef samples). The limit of detection (LOD) of bacterial concentrations and genomic DNA was $5.4 \times 10^2$ CFU/mL and $7.5 \times 10^{-4}$ ng/μL, respectively, which exhibited higher sensitivity than RAA-gel electrophoresis and RT-PCR methods. Furthermore, it was shown that E. coli O157:H7 in ground beef samples could be positively detected after 4 h enrichment when the initial bacterial inoculum was $9.0 \times 10^0$ CFU/25 g. The detection results of RAA-CRISPR/Cas12a method were 100% consistent with those of the RT-PCR and traditional culture-based methods while screening the E. coli O157:H7 from 93 local collected ground beef samples.

Conclusions: The developed RAA-CRISPR/Cas12a method showed high specificity, high sensitivity, and rapid positive detection of E. coli O157:H7 from ground beef samples.
The RAA-CRISPR/Cas12a system proposed in this study provided an alternative molecular tool for quick, specific, sensitive and accurate detection of *E. coli* O157:H7 in foods.
Introduction

*Escherichia coli* O157: H7 is one of the most dangerous foodborne pathogens which can cause severe diseases in human beings, such as hemorrhagic colitis, thrombotic thrombocytopenic purpura and hemolytic uremic syndrome (1). It is widely distributed in natural environments and is highly adaptable to stressful environments such as high temperature, low pH and low *a* and *w* (2). These characteristics make it easy for this bacterium to contaminate various foods, such as meat products, fruits and vegetables (3). Therefore, detection methods with specific, sensitive and rapid advantages are highly indispensable for monitoring of *E. coli* O157:H7 for food safety purposes. At present, traditional culture-based method is time-consuming (2~3 days), low-sensitive and manpower dependent (4). Real-time polymerase chain reaction (RT-PCR) detection of specific genes and enzyme-linked immunosorbent assay (ELISA) are rapid and sensitive in comparison with culture-based detection method, but they also have various shortcomings, such as need of specialized equipment and trained personnel, and complicated operation procedures (5, 6). It is vital and challenging to develop an innovative method which can overcome the above shortcomings.

A prospective alternative to existing methods that fulfills the needs of specific and ultrasensitive detection of pathogens is the clustered regularly interspaced short palindromic repeats/associated protein (CRISPR/Cas) system based nucleic acid detection. CRISPR/Cas system is a unique adaptive immune system in prokaryotic cells, which works based on a simple crispr RNA (crRNA)-guided nucleic acid recognition, and the cleavage activity of Cas protein can be triggered when crRNA bounds with complementary target DNA or RNA fragments (7). CRISPR/Cas system has been widely applied in genome editing or biological macromolecule detection due to its ability to cleave foreign genetic components (8, 9). Especially, Cas nucleases such as Cas9, Cas12 and Cas13 have been used to detect nucleic acids of a few important pathogens, showing great application potential in pathogen diagnostics. For instance, Zhang et al. (10) developed a PCR assisted CRISPR/Cas12a assay to detect *Vibrio parahaemolyticus* and achieved visualized endpoint detection by using a mini thermal
cycler. Li et al. (11) created a CRISPR/Cas12a-based fluorescence platform for highly specific and sensitive detection of *Listeria monocytogenes* serotype 4c. Besides, CRISPR/Cas13a was used for detecting *invA* gene of *Salmonella*, providing the limit of detection (LOD) of $10^0$ CFU/mL (12). In addition to bacteria, nucleic acid detection of viruses such as H7N9, HBV and novel coronavirus (SARS-CoV-2) by means of CRISPR/Cas system were reported in recent years (13, 14, 15). Among these nucleases, Cas12a can directly cleave single stranded DNA (ssDNA) with arbitrary sequence while transcription of DNA is not required. Combining with nucleic acid amplification techniques such as PCR, recombinase aided amplification (RAA) and loop-mediated isothermal amplification (LAMP), the CRISPR/Cas12a system can detect specific nucleic acid segment with high sensitivity. RAA is an emerging isothermal nucleic acid amplification technique, which requires three major enzymes including recombinase uvsX (obtained from *E. coli*), single stranded DNA-binding protein (SSB) and DNA polymerase to achieve the detection of target nucleic acids (16). RAA technology has also been used to detect pathogens, such as *E. coli* O157:H7 and *Salmonella* in previous studies (17, 18).

In the present study, RAA technology was integrated into CRISPR/Cas12a system (RAA-CRISPR/Cas12a) for specific, sensitive and rapid detection of 157-bp fragment of the lipopolysaccharide gene (*rfbE* gene) in *E. coli* O157:H7. The cis-cleavage activity to cut the double stranded DNA (dsDNA) of Cas12a was triggered only when crRNA bound with complementary target fragment of *rfbE* gene, and ssDNA-FQ reporter with arbitrary sequence could also be cleaved due to trans-cleavage activity of Cas12a, generating fluorescence signals whose intensity was closely related to the concentration of *rfbE* gene. Subsequently, these fluorescence signals were detected by fluorescence detection equipment (Genie II machine, OptiGene Ltd, England) (Figure 1). Furthermore, the effectiveness of RAA-CRISPR/Cas12a method for the detection of *E. coli* O157:H7 was evaluated against RAA-gel electrophoresis and RT-PCR techniques. Finally, the established RAA-CRISPR/Cas12a detection method was verified by detecting *E. coli* O157:H7 in spiked and real ground beef samples.
**Experimental**

**Bacteria strains and genomic DNA extraction**

A total of 34 bacterial strains were used in this study, including 11 *E. coli* O157:H7, 7 non-O157:H7 *E. coli*, and 16 non-*E. coli* strains (Table 1). All *E. coli* strains were cultured in modified EC Broth (Land Bridge Technology Co., Ltd., Beijing, China), *Vibrio alginolyticus* and *Vibrio parahemolyticus* were cultured in 3% sodium chloride alkaline peptone water (Land Bridge Technology Co., Ltd., Beijing, China), *Campylobacter jejuni* was cultured in Bolton broth (Land Bridge Technology Co., Ltd., Beijing, China), and other strains were grown in brain heart infusion (BHI) broth (Land Bridge Technology Co., Ltd., Beijing, China). All strains were incubated in a shaker with 110 rpm at 37 ± 1°C for 18 h except *Campylobacter jejuni*, which was at 42 ± 1°C for 24 h under microaerobic conditions (5% oxygen, 10% carbon dioxide and 85% nitrogen). Then 1 mL of bacterial cultures were taken for extracting genomic DNA using TIANamp Bacterial DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer’s instructions. The concentrations and purity of genomic DNA were measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Extracted DNA was stored at -20°C for further analysis.

**Primer design and RAA amplification**

In this research, *E. coli* O157:H7-specific *rfbE* gene sequence (GenBank No. S83460.1) was selected as the target sequence for detection. RAA primers were designed manually according to the principles of RAA primer design. RAA primers, crRNA and ssDNA-FQ reporter were all synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Table 2). RAA assay was constructed according to the instructions of the RAA nucleic acid amplification kit (Jiangsu Qitian Gene Biotechnology Co., Ltd., Jiangsu, China). Briefly, each reaction contained 25 μL RAA buffer V, 2 μL of each primer (10 μM), 2 μL DNA template, 16.5 μL nuclease-free water, and 2.5 μL magnesium acetate. The reaction tubes were placed at 39 ± 0.5°C for 20 min to perform RAA reaction. Subsequently, 50 μL of phenol/chloroform (1:1) was added into tubes,
mixed briefly, centrifuged (12000 rpm, 1 min), and finally the purified RAA products were detected by 1.5% agarose gel electrophoresis, which were visualized under an automatic gel imaging machine (Thermo Fisher Scientific Co., Ltd., Shanghai, China).

**CRISPR/Cas12a detection**

The CRISPR/Cas12a-based detection mixture (20 μL) contained 2 μL Cas12a-ssDNA Buffer (10×) (Novoprotein Scientific Co., Ltd., Shanghai, China), 2 μL purified RAA products, 1 μM crRNA, 10 μM ssDNA-FQ reporter, 2.5 μg/μL LbaCas12a (Novoprotein Scientific Co., Ltd., Shanghai, China) and nuclease-free water. Blank controls were carried out by using nuclease-free water to replace RAA products. The reaction was monitored at 42 ± 0.2°C for 10 min on an isothermal amplification platform (Genie II machine, OptiGene Ltd, England) with fluorescence intensity measurements taken every 15 s.

**Specificity and sensitivity detection of RAA-CRISPR/Cas12a method**

In order to test the specificity of the proposed method, 11 *E. coli* O157:H7 and 23 non-O157:H7 *E. coli* strains were used (Table 1). Briefly, RAA amplified genomic DNA of the above 34 bacteria strains was added into the CRISPR/Cas12a system to detect fluorescence. The sensitivity was evaluated by using gradient dilutions of DNA (ranging from 7.5×10^{-1} ng/μL to 7.5×10^{-5} ng/μL) and bacterial solutions (ranging from 5.4×10^{7} CFU/mL to 5.4×10^{1} CFU/mL) of *E. coli* O157:H7 (CICC 10907).

**RT-PCR detection**

For sensitivity comparison, the primer/probe sequences for specific detection of *E. coli* O157:H7 from Chapter 4A of FDA’s Bacteriological Analytical Manual (BAM): Diarrheagenic *Escherichia coli* was used (19). The detection assay was performed on ABI 7500 Fast system (Thermo Fisher Scientific Co., Ltd., the United States). The primer and probe sequences (Table 2) of *wzy* gene for RT-PCR detection were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Components of 25 μL of
reaction mixture contained 12.5 μL RT-PCR premix (Takara Biomedical Technology Co., Ltd., Beijing, China), 1 μL of each primer (10 μM), 0.6 μL of the probe (10 μM), 2 μL DNA template and nuclease-free water up to 25 μL. The RT-PCR reaction conditions were as follows: 95°C pre-denaturation for 2 min, followed by 40 cycles of 95°C denaturation (5 s) and 60°C annealing for 34 s.

**Analysis of RAA-CRISPR/Cas12a detection in ground beef samples**

For spiked samples analysis, fresh ground beef samples were purchased from local supermarkets (Shanghai, China), which were tested negative for *E. coli* O157:H7 according to China national standard method (20). In order to test the effectiveness of RAA-CRISPR/Cas12a method for detecting *E. coli* O157:H7, two parallel samples of 25 g ground beef were prepared, one was spiked with 9.0×10⁶ CFU/25 g of *E. coli* O157:H7 and the other non-treated was used for negative control. The 225 mL mEC + n broth (modified *E. coli* broth with novobiocin) was added to each sample, followed by incubation at 37 ± 1°C for 8 h. 1 mL of the enrichment solution was separately sampled and centrifugated at 12000 rpm for 2 min at 1, 2, 4, 6 hrs for genomic DNA extraction, which was then analyzed by RAA-CRISPR/Cas12a system. The above experiment was repeated three times at different times.

To evaluate the practical application of RAA-CRISPR/Cas12a platform, 93 real ground beef samples were bought from local markets (Shanghai, China). 25 g of each sample was added into 225 mL mEC + n broth, followed by incubation at 37 ± 1°C for 18 h. Subsequently, all samples were detected by using RAA-CRISPR/Cas12a system, RT-PCR and traditional culture-based method for comparison purpose.

**Results**

**Optimal design of RAA-CRISPR/Cas12a detection system**

In the present study, the *rfbE* gene of *E. coli* O157:H7 was amplified by RAA and detected by CRISPR/Cas12a system. In this detection platform, crRNA, Cas12a, ssDNA-FQ reporter and target DNA were important factors affecting the fluorescence
signal readout, which had further effects on the specificity and sensitivity of the
detection (21, 22). First, the feasibility of RAA primers and components of
CRISPR/Cas12a system was evaluated (Figure 2). Genomic DNA of gradient dilutions
of bacterial cultures (ranging from 5.4×10^7 CFU/mL to 5.4×10^1 CFU/mL) was
extracted to perform RAA reaction, and RAA products were then analyzed by 1.5%
agarose gel electrophoresis. Results showed that a fragment at 157 bp length of rfbE
gene was successfully amplified by RAA technique and indicated that the LOD of
RAA-gel electrophoresis method was 5.4×10^4 CFU/mL (Figure 2a). Furthermore, the
ssDNA-FQ reporter could only be efficiently cleaved by Cas12a protein when there
were target DNA and Cas12a/crRNA complex in the system (Figure 2b). These results
indicated that the constructed RAA-CRISPR/Cas12a system in this research could be
applied for detecting the rfbE gene of E. coli O157:H7. To achieve the optimal
fluorescence intensity, different concentrations of three factors (crRNA, Cas12a and
ssDNA-FQ reporter) on their efficiency for the detection were explored. As shown in
Figure 3, the maximal fluorescence value was reached when the concentrations of
crRNA, ssDNA-FQ reporter and Cas12a were at 1 μM, 10 μM and 2.5 μg/μL,
respectively. Consequently, a 20 μL reaction mixture consisting of 2 μL Cas12a-ssDNA
Buffer (10×), 2 μL purified RAA products, 1 μL crRNA (1 μM), 0.5 μL ssDNA-FQ
reporter (10 μM), 0.5 μL LbaCas12a (2.5 μg/μL) and 14 μL nuclease-free water was
used for subsequent experiments.

Specificity and sensitivity of RAA-CRISPR/Cas12a detection method

In order to evaluate the specificity of the RAA-CRISPR/Cas12a detection method,
34 bacterial strains consisting of 11 E. coli O157:H7, 7 non-O157:H7 E. coli, and 16
non-E. coli were tested (Table 1). As shown in Table 1, only 11 E. coli O157:H7 strains
exhibited strong positive fluorescence signals, the rest (non-target strains: non-
O157:H7 E. coli, non-E. coli) showed extremely low fluorescence values, being similar
to fluorescence values illustrated by the negative controls (nuclease-free water). These
results demonstrated that RAA-CRISPR/Cas12a method had high specificity for the
detection of *E. coli* O157:H7.

In order to estimate the sensitivity of RAA-CRISPR/Cas12a detection system, gradient dilutions of extracted DNA (ranging from $7.5 \times 10^1$ ng/µL to $7.5 \times 10^{-5}$ ng/µL) and bacterial solutions (ranging from $5.4 \times 10^7$ CFU/mL to $5.4 \times 10^1$ CFU/mL) of *E. coli* O157:H7 (CICC 10907) were prepared and analyzed accordingly. Results showed that the LOD of bacterial cultures and genomic DNA was $5.4 \times 10^2$ CFU/mL and $7.5 \times 10^{-4}$ ng/µL, respectively (Figure 4), and sensitivity assay were achieved within 30 min (including 20 min of RAA amplification and 10 min of CRISPR/Cas12a detection). Furthermore, the LOD of RAA-gel electrophoresis and RT-PCR methods was also tested, and sensitivity was compared for the three methods. As depicted in Figure 2a and Figure 5, the LOD of bacterial solutions for RAA-gel electrophoresis and RT-PCR methods was $5.4 \times 10^4$ CFU/mL and $5.4 \times 10^3$ CFU/mL, respectively. Therefore, RAA-CRISPR/Cas12a method significantly improved the detection sensitivity of *E. coli* O157:H7, contributing to practical application.

**Detection of *E. coli* O157:H7 in ground beef samples**

Ground beef samples were spiked with a low level of *E. coli* O157:H7 ($9.0 \times 10^0$ CFU/25 g) and then subjected to RAA-CRISPR/Cas12a detection, the results of one experiment were shown in Figure 6. Beef samples inoculated with *E. coli* O157:H7 after 4 h enrichment have already shown strong detectable fluorescence signals, which are much higher than baseline and negative controls. After 6 h and 8 h enrichment, the fluorescence signals from inoculated samples kept increasing. All negative controls and baseline were indistinguishable. The results of the remaining two replicated experiments were consistent. These results demonstrated that the established RAA-CRISPR/Cas12a detection platform in this study could detect *E. coli* O157:H7 from ground beef products after 4 h enrichment.

To evaluate the practical application of the developed RAA-CRISPR/Cas12a method, *E. coli* O157:H7 detection in 93 real ground beef samples were analyzed. The results demonstrated that three ground beef samples were detected as positive, and the rest 90
samples were negative (Table 3), which were in consistent with RT-PCR and traditional culture-based methods.

**Discussion**

*E. coli* O157:H7 has been considered as one of the most dangerous pathogenic microorganisms causing human illness and is a serious challenge to food safety monitoring. In recent years, CRISPR/Cas-based detection system has attracted wide attention for detecting various clinical and foodborne pathogens. This technology has the potential to become another powerful tool in the category of highly sensitive and rapid nucleic acid detection for pathogens (23).

The *rfbE* gene was used for specific detection of *E. coli* O157:H7 in previous studies (24, 25, 26, 27). The isothermal Genome Exponential Amplification Reaction (GEAR) assay based on *rfbE* gene developed by Jothikumar et al. (25) was found to be specific for O157:H7 through a panel of 96 non-target bacteria including 10 non-O157:H7 *E. coli* and 86 other bacterial species. Hu et al. (26) developed a recombinase polymerase amplification (RPA) combined with triple-labeled nucleotide probe assay using *rfbE* as the target and obtained highly specific results (100%) for O157:H7 using five *E. coli* O157:H7, 11 *E. coli* of non-O157, and 18 other bacterial species. Wang et al. (27) reported 100% specificity for O157:H7 detection from both RT-PCR and Crispr assays by targeting *rfbE* through testing four O157:H7, 10 *E. coli* of non-O157 and 7 other species. In the present study, results demonstrated that our newly designed RAA primers, crRNA and ssDNA-FQ reporter sequences were also highly effective for detecting *E. coli* O157:H7 from pure cultures and ground beef samples (Table 1, Table 3 and Figure 6). RAA-CRISPR/Cas12a system in this study was highly specific (100%) and the detection procedures were completed within 30 min (after 4 h enrichment in spiked ground beef samples).

The RAA-CRISPR/Cas12a system described in the present study had the low LOD of $5.4 \times 10^2$ CFU/mL for *E. coli* O157:H7 in pure cultures, exhibiting higher sensitivity than RAA-gel electrophoresis and RT-PCR methods ($5.4 \times 10^4$ CFU/mL and $5.4 \times 10^3$...
CFU/mL, respectively) (Figure 2 and Figure 5). Sun et al. (28) developed an SDA-rolling circle amplification (RCA) method with CRISPR/Cas9 system for fluorescence detection of *E. coli* O157:H7 and obtained the high sensitivity with LOD at 4.0×10¹ CFU/mL. However, the SDA-RCA amplification technique required approximately 2 h of reaction time. Moreover, compared with cis-cleavage activity of Cas9 protein, Cas12a selected in our research was capable of giving higher turnovers due to trans-cleavage activity (16). In addition, an assay for detecting *rfbE* gene of *E. coli* O157:H7 within 1 h based on CRISPR/Cas12a system was reported by Wang et al. (27). Their LOD at 6.5×10⁴ CFU/mL of pure culture was higher than that in our study (5.4×10² CFU/mL). Similar to our results, the sensitivity of the proposed CRISPR/Cas12a detection platform in their study was higher than (multienzyme isothermal rapid amplification) MIRA-gel electrophoresis and RT-PCR methods. In a study with *Listeria monocytogenes*, the sensitivity of the RAA-CRISPR/Cas12a fluorescence detection platform was improved by more than 100-fold in comparison with real-time RAA method (11).

Currently, in order to detect *E. coli* O157:H7 with very low levels of contamination in foods, the enrichment step is indispensable (29). In the present study, results showed that RAA-CRISPR/Cas12a system could rapidly detect *E. coli* O157:H7 (9.0×10⁰ CFU/25 g) from ground beef samples after 4 h enrichment. This result is similar to the that of a previous study, which obtained positive results for *E. coli* O157:H7 after 5 h of enrichment in ground beef samples with the initial inoculation of 14 CFU/mL (26). Piskernik et al. (30) reported a RT-PCR assay which positively detected *E. coli* O157:H7 in ground beef samples after 6 h enrichment with the original contamination level of 1.6×10¹ CFU/g. Furthermore, Wang, Jiang and Ge (31) reported that positive detection occurred at 6 h enrichment by using LAMP assays when ground beef samples were spiked with *E. coli* O157:H7 (10~20 CFU/25 g). In addition, the RAA-CRISPR/Cas12a method accurately identified three samples containing *E. coli* O157:H7 among the 93 ground beef samples, which was in good correlation with the results of RT-PCR and traditional culture-based methods. Evidently, the newly
designed RAA-CRISPR/Cas12a method has improved the sensitivity and has great potential in the detection of *E. coli* O157:H7 from beef samples.

There are numerous methods that have been established in the field of *E. coli* O157:H7 diagnostics. Among these techniques, molecular biology detection methods, such as PCR and RT-PCR, have high specificity and sensitivity, however specialized equipment, trained personnel and complicated operation procedures were required. Isothermal nucleic acid amplification techniques don’t have the disadvantages. Ravan, Amandadi and Sanadgol (32) developed a LAMP assay with six special primers to detect Z3276 gene of *E. coli* O157:H7 and achieved the specific detection within 1 h. However, compared to RAA-CRISPR/Cas12a method, LAMP technique still has some limitations such as complex primer design, high constant reaction temperature (65°C) and long reaction time (1 h). Strand displacement amplification (SDA) and helicase-dependent amplification (HDA) assays were also evaluated to detect *E. coli* O157:H7, but these methods were also time-consuming (90~120 min) (33, 34). In the present study, RAA-CRISPR/Cas12a detection platform significantly shortened the total detection time (within 30 min).

This novel RAA-CRISPR/Cas12a method developed in the present study only needs a simple fluorescent device with temperature range of 39~42°C, such as Genie II machine or any other instrument with similar functions. Compared with traditional RT-PCR machine, the isothermal amplification machine is smaller, lighter, cheaper, and more energy-saving. This assay system is not only rapid and sensitive but also portable, therefore it can be used for on-site detection of *E. coli* O157:H7 from food and environmental samples and has great potential in foodborne pathogen diagnostics in both the laboratory and the field.

**Conclusions**

In summary, a RAA-CRISPR/Cas12a fluorescence platform for rapid, specific and sensitive nucleic acid detection of *E. coli* O157:H7 was developed in this study. By integrating RAA into CRISPR/Cas12a system, the established method showed high
specificity and could be completed within 30 min. Compared with RAA-gel electrophoresis assay targeting the same *rfbE* gene and a referenced FDA’s RT-PCR method, RAA-CRISPR/Cas12a method had higher sensitivity. More importantly, the detection of *E. coli* O157:H7 in spiked and real food samples demonstrated that the proposed method achieved the satisfactory accuracy results in real ground beef samples and could rapidly detect ground beef samples spiked with low level of *E. coli* O157:H7 (4 h enrichment). In addition, the instrument used for this detection system is portable. Therefore, the RAA-CRISPR/Cas12a method presented in this research provided another specific, sensitive, and rapid molecular tool in foodborne pathogen diagnostics which could be used in both the laboratory and the field.
Acknowledgments

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Conflict of Interest

All authors declare that they have no conflict of interest.
Reference


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<td>ATCC 10792</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 29213</td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td><em>Bacillus cereus</em></td>
<td>ATCC 33019</td>
<td>-</td>
</tr>
<tr>
<td>28</td>
<td><em>Vibrio alginolyticus</em></td>
<td>ATCC 33787</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
<td><em>Vibrio parahaemolyticus</em></td>
<td>ATCC 33847</td>
<td>-</td>
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<tr>
<td>30</td>
<td><em>Klebsiella oxytoca</em></td>
<td>ATCC 700324</td>
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<td>31</td>
<td><em>Enterobacter pyogenes</em></td>
<td>ATCC 49399</td>
<td>-</td>
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<td>32</td>
<td><em>Cronobacter sakazakii</em></td>
<td>ATCC 45401</td>
<td>-</td>
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<td>33</td>
<td><em>Campylobacter jejuni</em></td>
<td>ATCC 33560</td>
<td>-</td>
</tr>
<tr>
<td>34</td>
<td><em>Shigella dysenteriae</em></td>
<td>ATCC 13313</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) CICC, China Center of Industrial Culture Collection.
b ATCC, American Type Culture Collection.

“+/−”, indicates positive and negative detection results.

Table 2. Specific DNA sequences of all primers and probes used in this project

<table>
<thead>
<tr>
<th>Methods</th>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAA</td>
<td>rfbE-F</td>
<td>TTGTCACGAATGACAAAAACACTTTATGACC</td>
</tr>
<tr>
<td></td>
<td>rfbE-R</td>
<td>TCAGCTTGTTCTAACTGGGCTAATCCTATAGC</td>
</tr>
<tr>
<td>CRISPR/Cas12a</td>
<td>crRNA</td>
<td>AAUUUAUACUGUUGUAGAUAGACCCGUUGU</td>
</tr>
<tr>
<td></td>
<td>reporter</td>
<td>UUACAUUUUAAAG</td>
</tr>
<tr>
<td></td>
<td>FAM-CACCGACGCCAGACCAGACTTT-TAMARA</td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>wzy-F</td>
<td>CTCGATAAAATTGCGCATTCTATTTC</td>
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<tr>
<td></td>
<td>wzy-R</td>
<td>CAATACGGAGAGAAAGACCAA</td>
</tr>
<tr>
<td></td>
<td>reporter</td>
<td>6FAM-CTTACCGTGGGAATGCATTGCACCCG-BHQ1</td>
</tr>
</tbody>
</table>

Table 3. Detection of *E. coli* O157:H7 in 93 real ground beef samples by the RAA-CRISPR/Cas12a, RT-PCR and traditional culture-based methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAA-CRISPR/Cas12a</td>
<td>3/93 90/93</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>3/93 90/93</td>
</tr>
<tr>
<td>Culture-based method</td>
<td>3/93 90/93</td>
</tr>
</tbody>
</table>

“+/−”, indicates positive and negative detection results.
Figure Captions

Figure 1. Flow chart of RAA-CRISPR/Cas12a system for the detection of *E. coli* O157:H7

![Flow chart of RAA-CRISPR/Cas12a system](image)

- DNA extraction
- RAA at 39°C for 20 min
- Activated CRISPR/Cas12a
  - crRNA
  - Cas12a
  - Reporter
- Fluorescence detection at 42°C for 10 min

Figure 2. Feasibility analysis of RAA-CRISPR/Cas12a system for the detection of *E. coli* O157:H7.

(a): Visualization of RAA amplicons by 1.5% agarose gel electrophoresis (M: 2000 DNA Marker; lanes 1-7: 5.4×10⁷ CFU/mL to 5.4×10¹ CFU/mL of bacterial solutions; lane 8: nuclease-free water); (b): Effects of different components of RAA-CRISPR/Cas12a system on fluorescence analysis. Only the combination of “cas12a/crRNA + ssDNA + target DNA” gave positive result.
Figure 3. Effects of different concentrations of three factors (crRNA, Cas12a, and ssDNA-FQ reporter) on the effectiveness of CRISPR/Cas12a for the detection of *E. coli* O157:H7. (a): crRNA; (b): Cas12a; (c): ssDNA-FQ reporter.

Figure 4. Sensitivity analysis of RAA-CRISPR/Cas12a detection system. (a): Real-time fluorescence curves for different amounts of genomic DNA of *E. coli* O157:H7; (b): Real-time fluorescence curves for different concentrations of *E. coli* O157:H7.
Figure 5. Real-time fluorescence curves of standard RT-PCR method. A: $5.4 \times 10^7$ CFU/mL; B: $5.4 \times 10^6$ CFU/mL; C: $5.4 \times 10^5$ CFU/mL; D: $5.4 \times 10^4$ CFU/mL; E: $5.4 \times 10^3$ CFU/mL; F: $5.4 \times 10^2$ CFU/mL; G: $5.4 \times 10^1$ CFU/mL; H: nuclease-free water.

Figure 6. Fluorescence intensity of *E. coli* O157:H7 spiked beef samples at different detection time by using RAA-CRISPR/Cas12a system (one repeat was shown). 1 h: 1 h-enrichment for spiked sample; 1 h-negative: 1 h-enrichment for negative control sample; 2 h: 2 h-enrichment for spiked sample; 2 h-negative: 2 h-enrichment for negative control sample; 4 h: 4 h-enrichment for spiked sample; 4 h-negative: 4 h-enrichment for negative control sample; 6 h: 6 h-enrichment for spiked sample; 6 h-negative: 6 h-enrichment for negative control sample; 8 h: 8 h-enrichment for spiked sample; 8 h-negative: 8 h-enrichment for negative control sample.