Rapid and Sensitive Detection of Shigella spp. and Salmonella spp. by Multiple Endonuclease Restriction Real-Time Loop-Mediated Isothermal Amplification Technique

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Shigella and Salmonella are frequently isolated from various food samples and can cause human gastroenteritis. Here, a novel multiple endonuclease restriction real-time loop-mediated isothermal amplification technology (MERT-LAMP) were successfully established and validated for simultaneous detection of Shigella strains and Salmonella strains in only a single reaction. Two sets of MERT-LAMP primers for 2 kinds of pathogens were designed from ipaH gene of Shigella spp. and invA gene of Salmonella spp., respectively. Under the constant condition at 63°C, the positive results were yielded in as short as 12 min with the genomic DNA extracted from the 19 Shigella strains and 14 Salmonella strains, and the target pathogens present in a sample could be simultaneously identified based on distinct fluorescence curves in real-time format. Accordingly, the multiplex detection assay significantly reduced effort, materials and reagents used, and amplification and differentiation were conducted at the same time, obviating the use of postdetection procedures. The analytical sensitivity of MERT-LAMP was found to be 62.5 and 125 fg DNA/reaction with genomic templates of Shigella strains and Salmonella strains, which was consistent with normal LAMP assay, and at least 10- and 100-fold more sensitive than that of qPCR and conventional PCR approaches. The limit of detection of MERT-LAMP for Shigella strains and Salmonella strains detection in artificially contaminated milk samples was 5.8 and 6.4 CFU per vessel. In conclusion, the MERT-LAMP methodology described here demonstrated a potential and valuable means for simultaneous screening of Shigella and Salmonella in a wide variety of samples.

Keywords: Shigella spp., Salmonella spp., MERT-LAMP, LAMP, LoD

INTRODUCTION

Shigella and Salmonella, recognized as important human pathogens worldwide, are responsible for food-borne gastroenteritis, which cause bacterial diarrhea represent a huge public health problem (Yang et al., 2007). It was estimated that in the word about 1.8 million cases died from diarrheal illnesses, a large proportion of which were attributed to Shigella spp. and Salmonella spp.
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Materials and Methods

Design of the MERT-LAMP Primers

Based on the ipaH gene of Shigella spp. and invA gene of Salmonella spp., two sets of MERT-LAMP primers were designed by PrimerExplorer V4 (Eiken Chemical) according to the mechanism of MERT-LAMP technology. Blast analysis confirmed that two sets of MERT-LAMP primers were specific for Shigella and Salmonella. The dark quenchers used were Black Hole Quencher-1 and Black Hole Quencher-2, and the fluorophores used were HEX and Cy5, which can be monitored in real-time system that was used for carrying out the MERT-LAMP amplifications. All of the oligomers were synthesized and purified by Tian-Yihuiyuan (Beijing, China). The details of target sequences, primer design, primers sequences and locations were shown in Table 1 and Figure 1.

Reagents

The DNA extraction kits (QIAamp DNA Mini Kits) were purchased from Qiagen (Beijing, China), and the Nb.BsrDI was purchased from New England Biolabs (Beijing, China). The Loopamp kits and Loopamp™ Fluorescent Detection Reagent (FD) were purchase from Eiken Chemical (Tokyo, Japan, and Beijing, China).

Bacterial Strains

A total of 54 strains used in this study were shown in Table 2. All strains were stored in 10% (w/v) glycerol broth at −70°C. These strains were refreshed three times on nutrient agar plate at 37°C, and then were used for enrichment and genomic DNA extraction.

Genomic DNA Extraction

According to the manufacturer’s instructions, the genomic DNA templates were extracted from all culture strains using DNA extraction kits (QIAamp DNA minikit; Qiagen, Hilden, Germany). The extracted templates were tested with ultraviolet spectrophotometer at A260/280 and stored under at −20°C before they were used.

The Normal LAMP Assay

To test the usability of two sets of LAMP primers, the LAMP approach either for Shigella strains or Salmonella strains was...
TABLE 1 | The primers used in this study.

| Primers name<sup>a</sup> | Sequences and modifications | Length | Genes |
|--------------------------|----------------------------|--------|-------|
| Sal-F3                   | 5′-CGCGGCCCGGATTTTCTCT-3′  | 18 nt  | invA  |
| Sal-B3                   | 5′-GGCAATGCGTCACCTTTTGA-3′ | 19 nt  |       |
| Sal-FIP                  | 5′-CGCGGCCCGGATTTTCTCT-3′  | 40 nt  |       |
| Sal-BIP                  | 5′-CGCGGCCCGGATTTTCTCT-3′  | 39 mer |       |
| Sal-EFIP                 | 5′-CGCGGCCCGGATTTTCTCT-3′  | 46 mer |       |
| Sal-LF                   | 5′-CGCGGCCCGGATTTTCTCT-3′  | 21 nt  |       |
| Sal-LB                   | 5′-AAAGGGAAAGCCACGGTCCTTA-3′ | 22 nt  |       |
| Shi-F3                   | 5′-CGCGGCCCGGATTTTCTCT-3′  | 20 nt  | ipaH  |
| Shi-B3                   | 5′-CGCGGCCCGGATTTTCTCT-3′  | 18 nt  |       |
| Shi-FIP                  | 5′-CGCGGCCCGGATTTTCTCT-3′  | 42 mer |       |
| Shi-EFIP                 | 5′-CGCGGCCCGGATTTTCTCT-3′  | 49 mer |       |
| Shi-BIP                  | 5′-CGCGGCCCGGATTTTCTCT-3′  | 42 mer |       |
| Shi-LF                   | 5′-CGCGGCCCGGATTTTCTCT-3′  | 20 nt  |       |
| Shi-LB                   | 5′-CGCGGCCCGGATTTTCTCT-3′  | 21 nt  |       |

<sup>a</sup>Sal, salmonella; Shi, Shigella; F, forward; B, backward; FIP, forward inner primer; EFIP, the novel forward inner primer; BIP, backward inner primer; LF, loop forward primer; LB, loop backward primer.

FIGURE 1 | Sequence and location of *Shigella* (ipaH) and *Salmonella* (invA) genes used to design MERT-LAMP primers. The nucleotide sequences of the sense strands of ipaH (A) and invA (B) are listed. The sites of primer sequences were underlined. Left arrows and right arrows showed complementary and sense sequences that are used.

performed as the following description. In brief, the reaction was conducted with the Loopamp Kit in a final volume of 25 µl containing 1.6 µM each FIP and BIP primers, 0.8 µM each LF and LB primers, 0.4 µM each F3 and B3 primers, 12.5 µl 2× reaction mix, 1 µl of *Bst* DNA polymerase (8 U), 1 µl FD, and 1 µl DNA template.

The reaction mixtures of normal LAMP were heated for 1 h at 63°C and then at 85°C for 5 min to stop the reaction. Three mainstream techniques were application to monitor the normal LAMP amplification. The positive amplifications could be directly observed color change by FD reagent, and the products were also detected by electrophoresis on 2% agarose gels with ethidium bromide staining. Moreover, real-time monitoring of normal LAMP reactions was conducted by recording the optical density (OD) at 650 nm every 6 s using the Loopamp Real-time Turbidimeter LA-320C (Eiken Chemical Co., Ltd, Japan). A positive result was defined as a threshold value of > 0.1 within 60 min and analysis of each sample (dilution) was determined at least two times.

The Standard MERT-LAMP Assay

To evaluate the feasibility of two sets of MERT-LAMP primers, the amplification mixtures of MERT-LAMP were carried out in a final volume of 25 µl containing 0.8 µM EFIP and FIP primers, 1.6 µM BIP primers, 0.8 µM each LF and LB primers, 0.4 µM each F3 and B3 primers, 12.5 µl 2× reaction mix, 1 µl (8 U) of *Bst* DNA polymerase, 1.5 µl (15 U) of Nb.BsrDI endonuclease, and 1 µl DNA template. The mixture was incubated at 63°C for 55 min and then at 85°C for 5 min to stop the amplification. Mixture without DNA performed as the following description. In brief, the reaction was conducted with the Loopamp Kit in a final volume of 25 µl containing 1.6 µM each FIP and BIP primers, 0.8 µM each LF and LB primers, 0.4 µM each F3 and B3 primers, 12.5 µl 2× reaction mix, 1 µl of *Bst* DNA polymerase (8 U), 1 µl FD, and 1 µl DNA template.

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TABLE 2 | Bacterial strains used in this study.

| Bacteria          | Serovar/Species | Strain no.(source of strain) | No. of strains |
|-------------------|-----------------|------------------------------|---------------|
| Shigella flexneri |                 | ICDC-NPS001                  | 1             |
|                   |                 | ICDC-NPS002                  | 1             |
|                   |                 | ICDC-NPS003                  | 1             |
|                   |                 | ICDC-NPS004                  | 1             |
|                   |                 | ICDC-NPS005                  | 1             |
|                   |                 | ICDC-NPS006                  | 1             |
|                   |                 | ICDC-NPS007                  | 1             |
|                   |                 | ICDC-NPS008                  | 1             |
|                   |                 | ICDC-NPS009                  | 1             |
|                   |                 | ICDC-NPS010                  | 1             |
|                   |                 | ICDC-NPS011                  | 1             |
|                   |                 | ICDC-NPS012                  | 1             |
|                   |                 | ICDC-NPS013                  | 1             |
|                   |                 | ICDC-NPS014                  | 1             |
| Shigella sonneri   | U               | Isolated strains (ICDC)      | 2             |
| Shigella dysenteriae | U      | Isolated strains (ICDC)      | 2             |
| Shigella boydii    | U               | Isolated strains (ICDC)      | 1             |
| Salmonella         | Choleraesuis    | ICDC-NPSa001                 | 1             |
|                   | Dublin          | ICDC-NPSa002                 | 1             |
|                   | Enteritidis     | ICDC-NPSa003                 | 4             |
|                   | Typhimurium     | ICDC-NPSa004                 | 2             |
|                   | Weltevreden     | ICDC-NPSa005                 | 1             |
| Listeria monocytogenes | 1/2a   | EGD-e                        | 1             |
|                   |                 | ATCC19114                    | 1             |
|                   |                 | ATCC23715                    | 1             |
| Enteropathogenic E. coli | U       | Isolated strains (ICDC)      | 1             |
| Enterotoxigenic E. coli | U       | Isolated strains (ICDC)      | 1             |
| Enteropathogenic E. coli | U       | Isolated strains (ICDC)      | 1             |
| Enterotoxigenic E. coli | U       | Isolated strains (ICDC)      | 1             |
| Enteroinvasive E. coli | U       | Isolated strains (ICDC)      | 1             |
| Enterohemorrhagic E. coli | U   | EDL933                        | 1             |
| Plesiomonas shigelloides | U   | ATCC51903                    | 1             |
| Enterobacter cloacae | U       | Isolated strains (ICDC)      | 1             |
| Enterococcus faecalis | U       | ATCC35667                    | 1             |
| Yersinia enterocolitica | U       | ATCC23715                    | 1             |
| Bontorobacter sakazakii | U       | Isolated strains (ICDC)      | 1             |
| Vibrio cholerae    | U               | Isolated strains (ICDC)      | 1             |
| Vibrio parahaemolyticus | U     | Isolated strains (ICDC)      | 1             |
| Vibrio vulnificus  | U               | Isolated strains (ICDC)      | 1             |
| Staphylococcus aureus | U       | Isolated strains (ICDC)      | 1             |
| Campylobacter jejuni | U       | ATCC33291                    | 1             |
| Pseudomonas aeruginosa | U      | Isolated strains (ICDC)      | 1             |
| Bacillus cereus    | U               | Isolated strains (ICDC)      | 1             |

aU, unidentified serotype; ATCC, American Type Culture Collection; ICDC, National Institute for Communicable Disease Control and Prevention.

The MERT-LAMP reactions were measured by real-time detection.

The Multiplex MERT-LAMP Reaction

For multiplex reactions, the MERT-LAMP approach was conducted as the following system: 25 µl containing 0.4 µM Shi-EFIP and Shi-FIP primers, 0.8 µM Shi-BIP primers, 0.4 µM each Shi-LF and Shi-LB primers, 0.4 µM each Shi-F3 and Shi-B3 primers, 0.6 µM Sal-EFIP and Sal-FIP primers, 1.2 µM Sal-BIP primers, 0.6 µM each Sal-LF and Sal-LB primers, 0.4 µM each Sal-F3 and Sal-B3 primers, 12.5 µl 2× reaction mix, 1 µl (8 U) of Bst DNA polymerase, 1.5 µl (15 U) of Nb.BsrDI endonuclease and 1 µl DNA template DNA each of Shigella strains and Salmonella strains. The MERT-LAMP mixtures were carried out at 63°C for 55 min in a real-time system, and reaction mixtures without the DNA template were used as a negative control. The lowest detectable template amount were examined in triplicate.
In order to determine the optimal amplification temperature of two sets of MERT-LAMP primes, the amplification mixtures of multiplex MERT-LAMP were heated at a constant temperature ranging from 60°C to 67°C for 55 min and then heated at 85°C for 5 min to stop the reaction. Mixtures without DNA template were used as a negative control.

**Evaluation of the Sensitivity of the MERT-LAMP Assay**

To make a comparative analysis of MERT-LAMP, LAMP, qPCR, and PCR assays by using pure culture, genomic DNA templates were serially diluted. The limit of detection (LoD) of MERT-LAMP, LAMP, qPCR, and PCR assays was confirmed by genomic DNA amount of the template. The *Shigella*-qPCR, *Shigella*-PCR, *Salmonella*-qPCR and *Salmonella*-PCR assays have been develop in previous studies, which were employed to verified the LoD of qPCR and PCR technologies (Novinscak et al., 2007; Ueda and Kuwabara, 2009; Mokhtari et al., 2013).

**Evaluation of the Specificity of the MERT-LAMP Assay**

To assess the specificity of the MERT-LAMP methodology, the multiplex MERT-LAMP reactions were performed under the conditions described above with the genomic DNA templates from 55 strains (Table 2). Analysis of each sample was carried out twice independently.

**Practical Application of MERT-LAMP to Shigella and Salmonella Detection in Food Samples**

In order to test the applicability of MERT-LAMP technology in food sample, *Shigella flexneri* serotype 1d (ICDC-NPS001) and *Salmonella Enteritidis* (ICDC-NPS0a003) were simultaneously added to the pasteurized milk samples, which were purchased from a grocery store in Beijing. The milk samples were confirmed as being *Shigella*- and *Salmonella*-negative by traditional culture assay and PCR. Firstly, to test the minimal detectable colony forming units (CFUs), the cultures with *Shigella flexneri* or *Salmonella Enteritidis* strains were serially diluted (10⁻¹–10⁻⁹), and the aliquots of 100µl appropriate dilution (10⁻⁶) was plated in triplicate on brain heart infusion (BHI). The CFUs were counted after 24 h at 37°C. The following steps, the aliquots of 100µl appropriate dilutions with *Shigella flexneri* and *Salmonella Enteritidis* was simultaneously inoculated into the milk samples, and the number of *Shigella* was adjusted to approximate 2.9 × 10⁵, 2.9 × 10⁴, 2.9 × 10³, 2.9 × 10², 2.9 × 10¹, and
2.9 \times 10^9 \text{ CFU/ml}, \text{Salmonella} \text{ for } 3.2 \times 10^5, 3.2 \times 10^4, 3.2 \times 10^3, 3.2 \times 10^2, 3.2 \times 10^1, \text{ and } 3.2 \times 10^0 \text{ CFU/ml. Simultaneously, aliquots (100 \mu l) of the artificially contaminated milk was used for DNA extraction, and the supernatants (2 \mu l) were used for multiplex MERT-LAMP, LAMP, real-time PCR and PCR detection. Non-contaminated milk sample was selected as negative control. This performance was carried out in triplicate independently.}

**RESULTS**

**Confirmation of Shigella- and Salmonella-LAMP Products**

The color change of positive amplifications in Shigella- and Salmonella-LAMP tubes from light gray to green were directly observed by naked eyes within 1 h incubation periods (Figures 2A,B). The normal LAMP products were also detected by 2% agarose gel electrophoresis, and the typical ladder-like patterns were visual (Figures 2C,D). Thus, two sets of normal LAMP primers for Shigella and Salmonella detection were good candidates for establishing the MERT-LAMP methodologies.

**Sensitivity of Shigella- and Salmonella-LAMP Assay in Pure Culture**

Sensitivity of LAMP reactions on Shigella and Salmonella were examined by analyzing products generated from the serial dilutions (2.5 ng, 250 pg, 25 pg, 2.5 pg, 250 fg, 125 fg, 62.5 fg, and 31.25 fg per microliter) of the Shigella and Salmonella genomic DNA templates in triplicate. As shown in Figures 3A,B, the LAMP amplifications were monitored by real-time turbidity detection; the LoD of Shigella-LAMP approaches was 62.5 fg/reaction (Figure 3A), and the LoD of Salmonella-LAMP assays was 125 fg/reaction (Figure 3B). Moreover, the positive reactions by 2% agarose gel electrophoresis were observed as a ladder-like pattern, the LoD of the agarose gel electrophoresis detection for Shigella- and Salmonella-LAMP reactions was identical with turbidity measurement (Figures 3C,D).

**Confirmation and Detection of MERT-LAMP Products in Nonreal-Time Format**

To validate the utility of two sets of MERT-LAMP primers, reaction systems were conducted in the presence or absence genomic DNA templates according to the standard MERT-LAMP condition. The color change of positive MERT-LAMP reactions in ipaH- and invA-MERT-LAMP tubes from light gray to green were directly seen by naked eyes within 1 h incubation periods (Figures 4A,B). Furthermore, the positive products were also detected by 2% agarose gel electrophoresis, and the typical ladder-like patterns were visible but not in negative control (Figures 4C,D). The results indicated the ipaH- and invA-MERT-LAMP primers were feasible for Shigella and Salmonella detection.

**Analytical Sensitivity of MERT-LAMP Detection for a Single Target**

In this study, we evaluated MERT-LAMP amplification in a single target format by using separate detection of ipaH (Shigella spp.-specific gene) and invA (Salmonella spp.-specific gene) from an Shigella and Salmonella genomic DNA templates with EFIP in each reaction. As expected, the release of quenching was observed as a robust increase of Cy5 and Hex signals, and positive results were obtained in \sim 12 \text{ min} (Figures 5A,B). The LoD of MERT-LAMP methodology for independently detecting ipaH and invA genes was 62.5 and 125 fg of genomic DNA templates per reaction, respectively (Figures 5A,B). Furthermore, the positive amplifications by 2% agarose gel electrophoresis were seen as the typical ladder-like patterns were visible but not in negative control, and the LoD of the agarose gel electrophoresis detection for Shigella- and Salmonella-LAMP amplifications was identical with real-time measurement (Figures 5C,D).

- The LoD of MERT-LAMP, LAMP, qPCR, and PCR assays on Shigella was 62.5 fg, 62.5 fg, 2.5 pg and 25 pg, and on Salmonella.
FIGURE 5 | Sensitivity of the ipaH- and invA-MERT-LAMP assays using serially genomic DNA with Shigella strains and Salmonella strains as templates. Sensitivity of ipaH-MERT-LAMP (A) and invA-MERT-LAMP (B) for Shigella and Salmonella detection was monitored by real-time format, and signals 1, 2, 3, 4, 5, 6, 7, 8, and 9 represent DNA levels of 2.5 ng, 250 pg, 25 pg, 2.5 pg, 250 fg, 125 fg, 62.5 fg, and 31.25 fg per vessel and negative control. The LoD of ipaH-MERT-LAMP assay was 62.5 fg per tube, and the invA-MERT-LAMP for 125 fg per reaction. Sensitivity of ipaH-MERT-LAMP (C) and invA-MERT-LAMP (D) for Shigella and Salmonella detection was analyzed by 2% agarose gel electrophoresis, and the positive amplifications were observed as a ladder-like pattern on 2% agarose gel electrophoresis analysis. Lane 1, DL 100-bp DNA marker.

TABLE 3 | The LoD and time for single MERT-LAMP targeting ipaH and invA genes compared with that of qPCR and conventional PCR approaches.

| Assays          | Isothermal regions recognized | Multiplex detection | LoD for Shigella spp./Salmonella spp. (no./reaction) | Fastest time (minutes) | LoD time (minutes)* |
|-----------------|-------------------------------|---------------------|------------------------------------------------------|------------------------|----------------------|
| MERT-LAMP       | +                             | +                   | 62.5 fg/125 fg                                       | 12                     | 26                   |
| LAMP            | +                             | –                   | 62.5 fg/125 fg                                       | 18                     | 28                   |
| qPCR            | –                             | 3                   | 2.5 pg/2.5 pg                                        | 32                     | 56                   |
| PCR             | –                             | 2                   | 25 pg/25 pg                                          | 150                    | 150                  |

*The LoD values are the lowest genomic DNA level that was positively amplified in triplicate. The positive results of qPCR were obtained as c_{T} values, which converted to time for detection. MERT-LAMP, multiple endonuclease restriction real-time loop-mediated isothermal amplification, qPCR, quantitative real-time PCR, LoD, limit of detection.

The LoD was 125 fg, 125 fg, 2.5 pg, and 25 pg, respectively (Table 3, Figures 3, 5). The results indicated that the sensitivity of MERT-LAMP technique for detecting a single target was identical with normal LAMP approach, whereas was at least 10- and 100-fold more sensitive than that of qPCR and PCR assays, respectively.

**The Optimal Amplification Temperature of ipaH- and invA-MERT-LAMP Assays**

In order to examine the optimal detection temperature of multiplex MERT-LMAP approach, the multiplex MERT-LMAP amplifications, which simultaneously contained the Shigella flexneri and Salmonella enteritidis genomic DNA templates at the level of 2.5 ng per tube, were performed at distinct temperatures (60°C–67°C) according to the multiplex MERT-LAMP reaction. The results were analyzed by means of real-time format, and the typical kinetics graphs were produced (Figure 6). Each detection temperature generated a robust signal corresponding to Cy5 and Hex channel, with the faster amplification observed for assay temperature of 62°C–65°C, which were considered as the standard temperature for multiplex MERT-LAMP detection. The detection temperature of 63°C was used for the rest of multiplex MERT-LAMP reaction conducted in this study.
Analytical Sensitivity of MERT-LAMP Detection for Multiple Targets in a Reaction

In order to simultaneously identify multiple targets in a single reaction, we slightly modified the amount of the primers on the base of standard MERT-LAMP reaction, and the multiplex MERT-LAMP systems were also performed at 63°C for 55 min. Two different fluorescence curves were simultaneously yielded from multiplex MERT-LAMP reactions containing two complete primer sets and their corresponding genomic DNA templates (Figure 7). The MERT-LAMP assay successfully detected *Shigella* and *Salmonella* in a single reaction, and simultaneously presented two sets of robust signals for two targets. The LoD of MERT-LAMP technology for simultaneously differentiating *ipaH* and *invA* genes was 62.5 and 125 fg of each genomic DNA per tube, respectively (Figure 7). No difference of sensitivity was seen between detecting multiple targets and a single target in a MERT-LAMP system.

Analytical Specificity of the Multiplex MERT-LAMP Methodology

To evaluate the specificity of multiplex MERT-LAMP technique, a total of 54 strains, including 19 *Shigella* and 15 *Salmonella* and 20 non-*Shigella* and non-*Salmonella*, were subjected to MERT-LAMP reaction. By observation, positive results...
FIGURE 6 | The optimal temperature for multiplex MERT-LAMP assay. Two sets of MERT-LAMP primers targeting *ipaH* and *invA* genes were used in the same reaction tube, (A,B) were simultaneously obtained from Cy5 (labeling Shi-EFIP of *ipaH*) and Hex (labeling Sal-EFIP of *invA*) channels, respectively. The multiplex MERT-LAMP amplifications were detected by means of real-time format, and the corresponding curves of DNA concentrations were listed. Signal 1 indicates *Shigella flexneri* strains in Cy5 channel (A), *Salmonella Enteritidis* strain for Hex channel (B), and signal 2 indicates negative control. Eight kinetic graphs (1–8) were generated at different amplification temperature (60°C–67°C) with *Shigella* genomic DNA at the level of 2.5 ng in Cy5 channel (A); another eight kinetic graphs (1–8) were yielded at different detection temperature (60°C–67°C) with *Salmonella* genomic DNA at the level of 2.5 ng in Hex channel (B). The graphs from 62°C to 65°C show robust amplification.

could be generated only when genomic DNAs of *Shigella* strains and *Salmonella* strains were used as templates in multiplex MERT-LAMP assay, and the target pathogens were correctly distinguished (Figure 8). In contrast, negative control, non-*Shigella* strains and non-*Salmonella* strains tested by multiplex MERT-LAMP technology were negative after 55-min incubation period. These results showed that the multiplex MERT-LAMP assay presented here was specific to target sequence identification.

**Multiplex MERT-LAMP Technology for Artificially Contaminated Milk Sample**

To ascertain the applicability the MERT-LAMP technology as a surveillance tool for *Shigella* and *Salmonella* in food, the
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FIGURE 7 | The sensitivity of multiplex MERT-LAMP assay for simultaneously detecting two target pathogens. Two sets of MERT-LAMP primers targeting *ipaH* and *invA* genes were simultaneously added to a reaction tube. [A, B] were simultaneously generated from Cy5 (labeling Shi-EFIP of *ipaH*) and Hex (labeling Sal-EFIP of *invA*) channels, respectively. Sensitivity of multiplex-MERT-LAMP for simultaneously detecting *Shigella* (A) and *Salmonella* (B) was monitored by real-time format, and signals 1, 2, 3, 4, 5, 6, 7, 8, and 9 represent DNA levels of 2.5 ng, 250 pg, 25 pg, 2.5 pg, 250 fg, 125 fg, 62.5 fg, and 31.25 fg per vessel and negative control. The LoD of multiplex MERT-LAMP assay for *Shigella* detection was 62.5 fg per tube, and the LoD of multiplex MERT-LAMP for *Salmonella* detection was 125 fg per reaction.

FIGURE 8 | The specificity of multiplex MERT-LAMP detection for different strains. The multiplex MERT-LAMP reactions were conducted using different genomic DNA templates and were analyzed by means of real-time format. [A, B] were simultaneously yielded from Cy5 and Hex channels. Signals 1–8, *Shigella flexneri* strains of serovar 1d (ICDC-NPS001), 4a (ICDC-NPS002), 5a (ICDC-NPS003), 2b (ICDC-NPS004), 1b (ICDC-NPS005), *Shigella boydii*, *Shigella sonneri* and *Shigella dysenteriae*; signals 9–13, *Salmonella Choleraesuis* (ICDC-NPSa001), *Salmonella Dublin* (ICDC-NPSa002), *Salmonella Enteritidis* (ICDC-NPSa003), *Salmonella Typhimurium* (ICDC-NPSa004), *Salmonella Wattevreden* (ICDC-NPSa005); signals 14 and 15, two *Salmonella* strains of unidentified serotype; signals 16–35, *Listeria monocytogenes* stains of serovar 1/2a (EGD-e), *Listeria monocytogenes* stains of serovar 4a (ATCC19114), *Enteropathogenic E. coli*, *Enterotoxigenic E. coli*, *Enteraggregative E. coli*, *Enteroinvasive E. coli*, *Enterohemorrhagic E. coli*, *Plesiomonas shigelloides*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Bacteroides fragilis*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, and *Bacillus cereus*, signal 36, negative control.

MERT-LAMP assay was tested by the artificially inoculating *Shigella* strain and *Salmonella* strain into milk sample. The multiplex MERT-LAMP approach could yield positive amplification when the contaminated numbers of *Shigella* and *Salmonella* were more than $2.9 \times 10^2$ CFU/ml ($\sim 5.8$ CFU/tube) and $3.2 \times 10^2$ CFU/ml ($\sim 6.4$ CFU/tube), respectively, and the two target pathogens were simultaneously detected in a single MERT-LAMP reaction (Figure 9, Table 4). The non-contaminated milk sample was found to be negative.

The detection limit of multiplex MERT-LAMP technology was consistent with that of normal LAMP detection only for *Shigella* or *Salmonella*, respectively (Table 4). Comparatively, the qPCR and PCR assays produced positive results when the contaminate numbers of *Shigella* amounted to more than $2.9 \times 10^3$ CFU/ml ($\sim 58$ CFU/tube) and $2.9 \times 10^4$ CFU/ml ($\sim 580$ CFU/tube), *Salmonella* for $3.2 \times 10^3$ CFU/ml ($\sim 64$ CFU/tube), and $3.2 \times 10^4$ CFU/ml ($\sim 640$ CFU/tube), respectively. The results revealed that the sensitivity of multiplex MERT-LAMP assays was 10- and 100-fold more sensitive than that of qPCR and PCR methodologies (Table 4).

DISCUSSION

Previous studies have reported that *Shigella* and *Salmonella* could contaminate similar types of samples, thus it would be significant to simultaneously identify the two pathogens (Li and Mustapha, 2004; MacRitchie et al., 2014). Culture-based techniques and biochemical are the most common detection assays, while require a long enrichment time and subsequent identification (Mokhtari et al., 2012; Soria et al., 2013). PCR-based technologies have
TABLE 4 | Comparison of MERT-LAMP, LAMP, qPCR, and PCR assay for detection of Shigella spp. and Salmonella spp. in artificially contaminated milk samples.

| Detection methodsa | Multiplex detection | Shigella spp. detection | Salmonella spp. detection |
|--------------------|---------------------|-------------------------|--------------------------|
| MERT-LAMP          | +                   | 5.8 CFU ~ (2.7 × 10^2 CFU/ml) | 6.4 CFU ~ (3.2 × 10^2 CFU/ml) |
| LAMP               | –                   | 5.8 CFU ~ (2.7 × 10^2 CFU/ml) | 6.4 CFU ~ (3.2 × 10^2 CFU/ml) |
| qPCR               | –                   | 58 CFU ~ (2.7 × 10^3 CFU/ml) | 64 CFU ~ (3.2 × 10^3 CFU/ml) |
| PCR                | –                   | 580 CFU ~ (2.7 × 10^4 CFU/ml) | 640 CFU ~ (3.2 × 10^4 CFU/ml) |

aMERT-LAMP, multiple endonuclease restriction real-time loop-mediated isothermal amplification; LAMP, loop-mediated isothermal amplification.

been successfully developed to detect Shigella and Salmonella from various food products and environment samples (Okamura et al., 2009; Liew et al., 2014). However, these techniques require a sophisticated apparatus to denature the template DNA, which significantly limit its wider application in under-resourced settings and in field laboratories (Wang et al., 2014). In recent years, the LAMP methodology was widely applied to detect Shigella and Salmonella due to the simplicity and rapidity, while it was restricted to identify only a single target in a vessel.

As a novel isothermal detection technology, MERT-LAMP assay was recently devised and the working principle was successfully validated (Wang et al., 2015a). Under the isothermal condition, the MERT-LAMP methodology permitted real-time detection of multiple targets in a single reaction, and the amplification and detection were conducted simultaneously within 55 min. In this study, the MERT-LAMP technique for the simultaneous differentiation of Shigella and Salmonella was successfully developed and evaluated. The MERT-LAMP amplifications were carried out at only one isothermal step, obviating the use of a thermal cycling equipment, and did not further pyrosequencing and agarose gel detection. Therefore, the multiplex MERT-LAMP approach significantly reduced reagents, materials and effort used, and identification and amplification were performed at the same time, alleviating the use of further procedures. Moreover, the reaction vessels were kept closed in the whole course of the experiment, which effectively eliminated any carryover contamination.

Two species-specific genes (ipaH and invA) present in both Shigella and Salmonella were used as target sequences for designing MERT-LAMP primers (Vantarakis et al., 2000; Hara-Kudo et al., 2005). The specificity of multiplex MERT-LAMP reactions was successful examined, and the positive results were generated in the assay of Shigella strains and Salmonella strains but not for non-Shigella strains and non-Salmonella strains. Furthermore, the MERT-LAMP established here can simultaneously detect and correctly distinguish the target pathogens in a sample, which can be completed in only one isothermal detection step with easily interpretable results. Therefore, the MERT-LAMP assay combined with hand-held diagnostic devices can be as a potential tool for detecting target pathogens, which can be widely applied to various fields, such as point-of-care diagnosis, field testing, and more.

The established multiplex MERT-LAMP assay detected as little as 62.5 and 125 fg of Shigella and Salmonella DNA per tube in the pure cultures, which was consistent with that of normal LAMP and singleplex MERT-LAMP reactions.
However, the normal LAMP technology was limited to detect only one target template (Yan et al., 2014). The analytical sensitivity of multiplex MERT-LAMP assay indicated that it could screen marginal amounts of target sequences and was at least 10- and 100-fold more sensitive than that of qPCR and PCR approaches, respectively. With regard to assay speed and sensitivity level obtained in our study, the MERT-LAMP methodology performed better than qPCR and PCR techniques, thus significantly shortening the total detection time (Table 3). Therefore, the MERT-LAMP assay presented here was recommended as a potential diagnostic tool for screening Shigella strains and Salmonella strains in a variety of samples.

To test the practical application of MERT-LAMP detection of Shigella strains and Salmonella strains in food products, artificially contaminated milk samples were analyzed by conventional PCR, qPCR, LAMP, and multiplex MERT-LAMP assays. The detection limit of multiplex MERT-LAMP for Shigella strains and Salmonella strains detection in artificially contaminated milk samples was 5.8 and 6.4 CFU per tube, which was also identical with normal LAMP analysis. The two target pathogens could be simultaneously screened within 40 min with only isothermal incubation step and the positive results were presented in real-time format (Figure 9). Moreover, the LoD of multiplex MERT-LAMP technology for artificially contaminated milk samples was 10- and 100-fold more sensitive than qPCR and conventional PCR assays. Herein, the multiplex MERT-LAMP approach was more suitable than LAMP, qPCR, and PCR techniques for multiplex, rapid, specific and sensitive detection of Shigella strains and Salmonella strains in food samples.

In conclusion, a rapid, multiplex, specific, and sensitive MERT-LAMP assay for simultaneous detection of Shigella strains and Salmonella strains was established by targeting ipaH and invA genes, and the specificity, sensitivity and practical application were successfully evaluated. Thus, the novel MERT-LAMP assay could be used as a potential tool for facilitating simultaneous monitoring of Shigella strains and Salmonella strains in variety of samples.

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**Disclosures:** Yi Wang, Yan Wang and Changyun Ye have filed for a patent from the State Intellectual Property Office of China, which covers the novel method and sequences included in this manuscript (application number CN201510792208.6).

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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