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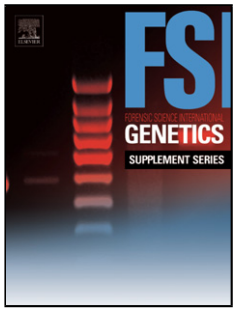

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ACCEPTED MANUSCRIPT

Heptaplex-direct PCR assay for simultaneous detection of foodborne pathogens

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Abstract. Foodborne pathogens pose significant problems for public health and economy. The gold standard, cultivation, is time-consuming and costly. In this study, a heptaplex-direct PCR assay for simultaneous detection of seven foodborne pathogens without DNA extraction and enrichment was developed and validated. Seven virulent genes of target strains were amplified and found that the assay provided the expected PCR fragment of 583, 490, 415, 343, 224, 209, and 105 bp for *Shigella* spp., Shiga toxin-producing *Escherichia coli* (STEC), *Streptococcus pyogenes*, *Campylobacter jejuni*, *Salmonella* Typhi, *Listeria monocytogenes*, and *Staphylococcus aureus*, respectively. Validation study showed that the assay was highly reproducible, specific and sensitive (10^6-10^0 CFU/ml of detection limit). Moreover, assay application on 22 artificially- and 100 naturally-contaminated food samples provided a statistically equivalent efficiency to the culture method. A heptaplex-direct PCR assay thus can be used in microbial forensic science.

Keywords: multiplex PCR, direct PCR, Foodborne pathogen, bacterial identification

1. Introduction

Pathogenic bacteria and their toxin pose a significant problem for public health and the economy. World Health Organization (WHO) lists the most common and virulent foodborne pathogens: *Salmonella* spp., *Shigella* spp., *E. coli* O157:H7, *L. monocytogenes*, *C. jejuni*, *S. aureus*, and *S. pyogenes* [1-2]. Contamination of raw and processed food can occur both inadvertently or deliberately. Therefore, microbial forensics plays important role in bacterial identification of alleged criminal acts.

Bacterial culture, the gold standard for bacterial identification, is time-consuming and costly. Multiplex PCR combined with direct protocol can overcome these problems with high specificity, sensitivity and simultaneous detection of several species simultaneously without DNA extraction process [1]. Thus, we aimed to develop the heptaplex-direct PCR for simultaneous detection of these seven pathogens. The developed assay was also validated for its reproducibility, specificity, sensitivity, and mock-case testing.

2. Materials and methods

Seven pathogen-specific primers were used for assay development; six were modified from previous reports [1-6] and one for *Shigella* spp. was designed in this study. All candidate primers were checked to avoid secondary structures using OligoCalc and AutoDimer. Direct amplification was performed using pre-PCR solution protocol. To do this, bacterial colonies were suspended in 20 µl of PBS buffer and 0.5 µl proteinase K before incubating at 98°C for 2 min. One microliter of the pre-PCR solution was then used as DNA template in PCR reaction using Q5 High Fidelity PCR kit (New England BioLabs, UK). Primer concentrations were 1.25 µM for shigella (shi2), stx2, spy and hipo primers, 1.0 µM for lm and S primers, and 0.75 µM of sTyphi primers. The PCR condition were 30 cycles of denaturation at 98°C for 5 s, annealing at 67°C for 10 s, extension at 72°C for 20 s, and a final extension at 72°C for 2 min. PCR products were separated on 4% agarose gel electrophoresis and detected under Gel Doc™ EZ System.

Reproducibility test of the developed assay was performed using nineteen samples of seven target species. Specificity test was performed with seven target species and other nine bacterial species commonly found in food. Sensitivity was also conducted by making ten-fold serial dilution of target species ranging from 10^{10}-10^0 CFU/ml. Mock-case testing was done with 22 artificially spiked food and 100 food samples randomly chosen from local market and supermarket.

3. Results and discussion

The heptaplex-direct PCR assay was successfully developed to simultaneously detect the seven target pathogens and provided the expected PCR products for *Shigella* spp., STEC, *S. pyogenes*, *C. jejuni*, *S.*Typhi, *L. monocytogenes*, and *S. aureus*, (Fig. 1). The important keys for success are the use of modified DNA polymerase, additives in the PCR buffer, pre-PCR treatment, and

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assay optimization. Moreover, all seven species-specific primer pairs were modified or designed to have narrow melting temperature ranges to facilitate multiplexing.

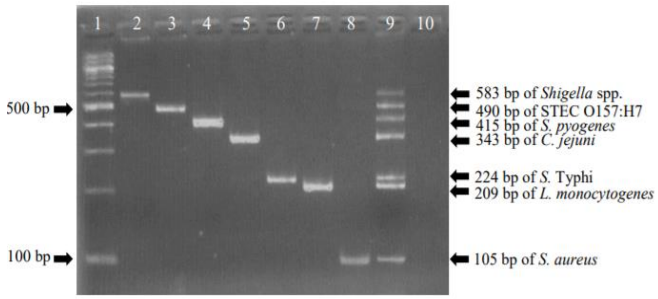

Fig. 1. 4% agarose gel electrophoresis of heptaplex-direct PCR. Lane 1 DNA ladder, lane 2 *Shigella* spp., lane 3 STEC, lane 4 *S. pyogenes*, lane 5 *C. jejuni*, lane 6 *S.* Typhi, lane 7 *L. monocytogenes*, lane 8 *Staph. aureus*, lane 9 multiplex PCR of seven strains (prepared by pooling pre-PCR samples in PBS) and lane 10 negative control.

Reproducibility test showed that all seven target strains were consistently amplified. Specificity test indicated that the assay was specific to the seven target species and all non-target strains failed to amplify. This could be due to selection of unique virulent genes of target pathogens. The detection limit (in CFU/ml) of the assay was 10^0 for STEC and *L.monocytogenes*, 10^1 for *S. pyogenes* and *S.* Typhi, 10^5 for *C. jejuni*, and 10^6 for *Shigella* spp. and *S. aureus*. These detection limits are sufficiently sensitive to detect infectious doses of target pathogens. Artificial contamination demonstrated that the developed assay was significantly better than the culture method at detecting *Shigella* spp. but worse in *L. monocytogenes*. No statistical differences were found for the rest (Table 1). Only one food sample was identified as naturally STEC-contaminated by the developed assay but negative by culture method (data not shown). This could be due to the tolerance of STEC to cooking conditions.

Table 1. The number of artificially spiked samples detected with the multiplex PCR and standard culture method (N = 22).

Bacterial strain	PCR	Culture
Shigella spp. *	15	4
STEC	15	22
S. pyogenes	6	12
C. jejuni	16	22
S. Typhi	21	22
L. monocytogenes *	10	22
S. aureus	22	22

* indicates significant difference

4. Conclusion

The heptaplex-direct PCR assay for simultaneous detection of seven foodborne pathogens was successfully developed and validated. The assay is reliable, rapid and high sensitivity compared to gold standard. It can be the alternative tool for screening microbial contamination in food.

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Conflict of interest

None

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