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TECHNICAL REPORT ON OPTIMIZATION OF A REDUCED PCR REACTION VOLUME AND A SINGLE PCR CONDITION FOR FIVE DIFFERENT FOODBORNE PATHOGENS

By

Amy Atter[1], Kojo Odei Obiri [1], Shadrack Asiedu-Coffie[2], Benjamin Owusu Otu[2], Christopher Galley[1], Evans Agbemafle[1], Ethel Juliet Blessie[1], Wisdom Amoa-Awua[1]

1. Council for Scientific and Industrial Research Ghana- Food Research Institute, Accra, Ghana.
2. Biotechnology Centre, University of Ghana, Legon, Accra, Ghana.

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ABSTRACT

Foodborne pathogens have caused many disease outbreaks globally and more so in developing countries with huge economic effect. Food-borne pathogen identification is an important aspect of health care. The short shelf-life of some food products such as vegetables requires rapid and cost-effective methods for pathogen detection. Therefore this work was conducted to optimize a reduced PCR reaction volume and a single PCR condition that can simultaneously be used to detect five different foodborne pathogens namely *Salmonella spp*, *Escherichia coli* (0157), *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*. After analyzing five protocols from literature which were not able to successfully amplify the target genes of the organisms simultaneously, an in-house method was developed which successfully amplified the genes of the five organisms. The findings of this study suggests that target genes of multiple organisms can be amplified within the shortest period of time using this newly developed in-house method. This approach will also decrease the cost of performing PCR analyses for pathogen identification particularly in developing countries.

INTRODUCTION

The speedy detection of pathogens in food is critical for ensuring the safety of patrons, since the majority of food-borne sicknesses are caused by pathogenic bacteria. Hence, fast, sensitive, low-cost and appropriate approaches to detect food-borne pathogenic bacteria is essential in controlling food safety. Efficient detection of foodborne-related pathogens is ideally achieved using a multifaceted approach combining classical microbiological methods with molecular techniques (Adzitey *et al*., 2013). Traditionally, identification and cataloging of bacteria are based on phenotypic characteristics which are sometimes incapable of distinguishing organisms to specie

level (Ott *et al*., 2004). The method employs plating on to selective agar directly or with prior enrichment steps. Further to this, presumptive colonies of bacteria are confirmed using biochemical testing. These methods are used widely due to the fact that they are cost effective and can only detect viable, relevant bacteria to the tests and separate isolates for further categorization and testing (Adzitey *et al*., 2013). A major disadvantage of the classical microbiology methods of identifying microorganisms is that it is time consuming and labor intensive. To ameliorate this situation, molecular techniques were introduced to reduce the time spent on analyses and churn out more accurate results. In identifying organisms using molecular methods, the polymerase chain reactions (PCR) platform is usually employed. PCR makes use of diverse or multiple primers engraved within a single Polymerase Chain Reaction mixture to identify, detect and differentiate various bacteria (Lee *et al*., 2013). PCR products are then sequenced and the sequences obtained aligned and search against various databases of DNA. Subsequently, specific bacterium can be located and identified.

In a multiplex PCR experiment to simultaneously identification *E. coli* O157:H7, *Salmonella spp.* and *L. monocytogenes* in food, the assay was able to precisely concurrently detect ten colony-forming unit/mL of each pathogen in artificially inoculated samples after enrichment for 12 h. The entire procedure took less than 24 h to complete, indicating that the assay was appropriate for reliable and rapid identification of these three food-borne pathogens (Nguyen *et al*., 2016). Different microorganisms have specific PCR reaction and conditions for molecular identification and detection. These are based on the target gene of interest to be amplified. Literature on reduced PCR reaction volume and single condition that can be used simultaneously to detect different foodborne pathogens is either very limited or nonexistence. Therefore, the aim of this work was to optimize a reduced PCR reaction volume and a single PCR condition that can simultaneously be

used to detect five different foodborne pathogens namely *Salmonella* spp, *Escherichia coli* (0157), *Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus*.

METHODOLOGY

Some PCR conditions tried

A number of trials were carried out with certified reference pathogenic cultures obtained from CSIR-Food Research Institute. The reference cultures were *Escherichia coli* NCCB100282, *Staphylococcus aureus* NCCB 100294, *Listeria monocytogenes* NCCB 100286**,** *Bacillus cereus* NCCB 100292 and *Salmonella* spp 20B-1410 all obtained from Health Protection Agency (HPA) Culture Collection, UK. Reduced PCR reaction volume of 12.5 µL and not the usual 50 µL or 25 µL with only the specific primers changing were used for all the trials.

Again, different reported PCR conditions were experimented for all the pathogens at a single run in the thermal cycler. There were some of the conditions that only worked for one or two particular pathogen but not all the different bacteria pathogens in a single run.

The following were some of the unsuccessful PCR conditions tried:

- **Condition 1: Holland et al., 2000**

Reaction Mix	Volume (µL)
Master Mix	6.75
Template	1.5
Primer F (20µM)	0.25
Primer R (20µM)	0.25
NFH_2O	3.75
	12.5

PCR Programme

94°C	2min	
94°C	30’	x30 cycles
56°C	30’	
72°C	30’	
72°C	10min	
	4°C as holding temp	

- **Condition 2: Clermont et al., 2000**

Reaction Mix	Volume (µL)
Master Mix	6.75
Template	1.5
Primer F (20µM)	0.25
Primer R (20µM)	0.25
NFH_2O	3.75
	12.5

PCR Programme

94°C	5min	
94°C	30’	x30 cycles
55°C	30’	
72°C	30’	
72°C	7min	
	4°C as holding temp	

- **Condition 3: Wang et al., 2018**

Reaction Mix	Volume (µL)
Master Mix	6.75
Template	1.5
Primer F (20µM)	0.25
Primer R (20µM)	0.25
NFH_2O	3.75
	12.5

PCR Programme

94°C	6min	
94°C	30’	x 30 cycles
54°C	30’	
72°C	35’	
72°C	10min	
	4°C as holding temp	

- **Condition 4: Hoque et al., 2018**

Reaction Mix	Volume (µL)
Master Mix	6.75
Template	1.5
Primer F (20µM)	0.25
Primer R (20µM)	0.25
NFH_2O	3.75
	12.5

PCR Programme

95°C	10min	
94°C	5min	x 35 cycles
55°C	0.5min	
72°C	1.5min	
72°C	3.5min	
	4°C as holding temp	

- **Condition 5: Hansen et al., 2001**

Reaction Mix	Volume (µL)
Master Mix	6.75
Template	1.5
Primer F (20M)	0.25
Primer R (20µM)	0.25
NFH_2O	3.75
	12.5

PCR Programme

94°C	15’	
94°C	5min	x 30 cycles
63°C	45’	
72°C	2min	
72°C	3.5min	
	4°C as holding temp	

- **The best laboratory developed PCR condition**

Reaction Mix	Volume (µL)
Master Mix	6.75
Template	1.5
Primer F (20µM)	0.25
Primer R (20µM)	0.25
NFH_2O	3.75
	12.5

PCR Programme

95°C	5’	
94°C	30’	x40 cycles
55°C	1min	
72°C	3min	
72°C	10min	

4°C as holding temp.

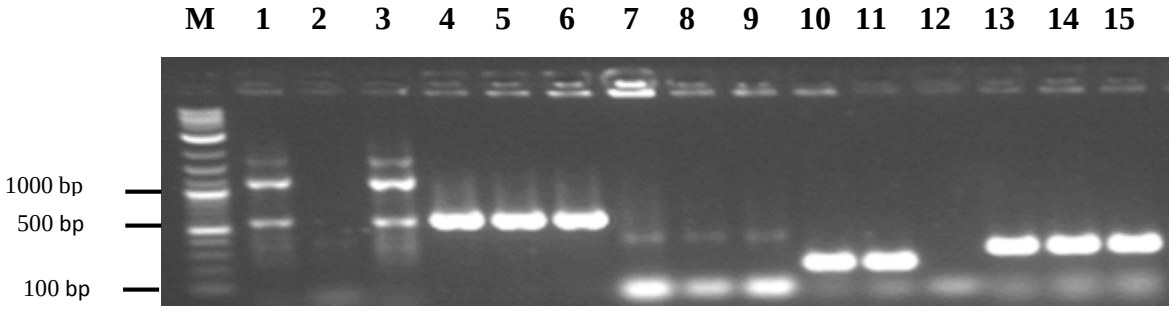

Figure 1. PCR amplification of reference samples using primers for optimization. Lanes: M = Ladder; 1-3 = *Bacillus* cereus; 4-6 = *Escherichia coli*; 7-9 = *Salmonella* spp; 10-12 = *Listeria monocytogenes*, 13-15 = *Staphylococcus* aureus.

Primer sequence

Bc1 F: GAG TTA GAG AAC GGT ATT TAT GCT GC
Bc2 R: CTA CTG CCG CTC CAT GAA TCC

Ec3 F: GCG CTG TCG AGT TCT ATC GAG C
Ec4 R: CAA CGG TGA CTT TAT CGC CAT TCC

Sm5 F: GGG TGG GCG GAA AAC TAT TTC
Sm6 R: CGG CAC GGC GGA ATA GAG CAC

Lm4 F: GCA GTT GCA AGC GCT TGG AGT GAA
Lm5 R: GCA ACG TAT CCT CCA GAG TGA AGT GAA

Sa1 F: GCA AGC GTT ATC CGG ATT T
Sa2 R: CTT AAT GAT GGC AAC TAA GC

- **The second best laboratory developed PCR reaction and condition that came close but produced faint bands was**:

Reaction Mix	Volume (µL)
Master Mix	6.75
Template	1.5
Primer F (20µM)	0.25
Primer R (20µM)	0.25
NFH_2O	3.75
	12.5

PCR Programme

95°C	5′	
94°C	30′	x40 cycles
53°C	1min	
72°C	3min	
72°C	10min	

4°C as holding temp.

DISCUSSION

The common food-borne pathogenic bacteria which are responsible for most of the food-borne disease outbreaks are *L. monocytogenes*, *E. coli* O157:H7, *Staphylococcus aureus*, *S. enterica*, *Bacillus cereus*, *Vibrio spp*., *Campylobacter jejuni*, *Clostridium perfringens* and *Shiga* toxin-producing *E coli* (STEC) (Scallan *et al*., 2011; Zhao *et al.*, 2014). While the conventional methods used to detect food-borne pathogen are inefficient and arduous, the molecular methods for detection are becoming more essential in many food analyses (Díaz-López, 2011).

Therefore, in this study, an attempt was made to simultaneously use a single PCR condition with reduced reaction volume to identify four of these pathogenic organisms. Reduced PCR reaction volume of 12.5 µL was found to be appropriate for the assays. A number of PCR reaction protocols from literature were used to amplify the various target genes of the bacteria. The results showed that none of the protocols sourced from literature worked as desired. Subsequently an in house protocol was developed to analyze the samples.

The protocol from Holland and colleagues evaluated one in-house method and three commercially available kits for their ability to extract *E. coli* O157:H7 DNA directly from stool specimens for

PCR (Holland *et al*., 2000). The PCR method used in their work was modified and used in the current study to amplify the target genes of the four pathogenic organisms. In their study they were able to successfully amplify the *E. coli* O157:H7 gene. In this study only the E.coli gene was amplified. The target genes for the other organisms were not successfully amplified. The second PCR protocol modified from Clermont *et al.,* (2000) was only able to amplify *E.coli* target gene as the protocol was designed specifically for this purpose. Similar observations were made when protocol 3 to 5 were used to amplify the target genes of the organisms. The best protocol that was able to amplify the target genes of interest of the five organisms as shown in figure 1 was developed in-house and was able to amply the genes of all the five pathogens. PCR has become a very quick and dependable tool for the molecular biology-based analyses of a variety pathogens in food samples. Therefore the development of this protocol is very pivotal in the routine testing of food-borne pathogens in Africa. In countries where resources are limited the reduced PCR volume developed in this study will enable laboratories to save cost on reagents. The ability to amplify multiple target genes of interest at the same time is also an added advantage as a lot more results can be achieved within the shortest possible time.

CONCLUSION

This finding will go a long way to reduce the cost of detecting these five foodborne pathogens simultaneously from a food sample because it's cheaper as compared to detecting these pathogens individually. Secondly, the time for amplification is drastically reduced as they can all be run on the thermal cycler concurrently. This method can be further researched into involving other pathogens and can become an alternative method for laboratories in developing countries.

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