DEVELOPMENT OF MULTIPLEX PCR PLATFORM FOR SIMULTANEOUS DETECTION OF SELECTED FOODBORNE PATHOGENS

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DEVELOPMENT OF MULTIPLEX PCR PLATFORM FOR SIMULTANEOUS DETECTION OF SELECTED FOODBORNE PATHOGENS

ABSTRACT

Foodborne outbreaks are threatening human population worldwide especially in Malaysia where the occurrence of food poisoning is becoming more prevalent due to contamination caused during food production, food preparation and handling. Most outbreaks are commonly caused by *E. coli*, *Salmonella* sp., *Listeria* sp., *Shigella* spp., *Staphylococcus aureus* and *Yersinia enterocolitica*. Thus, a cost-effective, rapid and sensitive assay is required to find the cause of contamination before such contaminated foods disseminated widely in the market. In this study, a multiplex PCR assay was developed to allow simultaneous detection of six foodborne pathogens. The assay targets species-specific regions namely *phoA, hilA, hyl, ipaH, rpoB* and *yst* respective to *E. coli*, *Salmonella* sp., *Listeria* sp., *Shigella* spp., *Staphylococcus aureus* and *Yersinia enterocolitica*. The specificity and detection limit of the assay was evaluated by using 80 known bacterial cultures and 5 spiked food samples. The primers designed were highly specific except the mphoA primer pair as it is cross-reacted with *E,coli* and *Shigella* strains. Whereas, the detection limit for simultaneous detection of all targeted pathogens was up to 10^4 CFU/ml even though limit of up to 10^1 CFU/ml for *E. coli, Listeria and Shigella*; 10^2 CFU/ml for *Salmonella* and *Yersinia* was obtained respectively. When tested with spiked food samples the detection limit of *E. coli* was 10^1 CFU/ml; *Salmonella*, *Listeria* and *Shigella* was 10^2 CFU/ml in spite the simultaneous detection limit of all the six pathogens was 10^6 CFU/ml. In short, the developed multiplex PCR assay allows rapid and cost-effective simultaneous detection of the six common foodborne pathogens.

Keywords: Multiplex PCR, food-borne pathogens, food samples.

PEMBANGUNAN PLATFORM MULTIPLEKS PCR BAGI PENGESANAN SECARA SERENTAK PATOGEN BAWAAN MAKANAN YANG TERPILIH

ABSTRAK

Keracunan makanan semakin menghantui masyarakat di seluruh dunia terutamanya di Malaysia. Kebanyakan wabak berkaitan makanan selalunya diakibatkan oleh bakteria seperti *E. coli*, *Salmonella enterica*., *Listeria monocytogenes.*, *Shigella* spp., *Staphylococcus aureus* dan *Yersinia enterocolitica*. Oleh itu, dalam kajian ini, sebuah multipleks PCR telah dibentuk untuk mengesan enam bakteria utama secara serentak. Multipleks PCR yang dibentuk menargetkan rantau „region" *phoA*, *hilA*, *hyl*, *ipaH*, *rpoB* dan *yst* yang spesifik kepada *E. coli*, *Salmonella enterica*, *Listeria monocytogenes*, *Shigella* spp., *Staphylococcus aureus* dan *Yersinia enterocolitica* masing-masing. Tahap spesifik dan sensitif multipleks PCR tersebut dikaji dengan menggunakan strain bakteria dan sampel makanan. Setiap set „Primer" yang dibentuk sangat spesifik kepada spesis yang ditarget kecuali „primer" yang manargetkan rantau *phoA* yang sepatutnya spesifik kepada *E. coli* juga menghasilkan garisan positif „band" apabila dikaji dengan *Shigella*. Manakala, tahap pengesanan pula sampai 10^4 CFU/ml untuk pengesanan enam bakteria yang diminati walaupun tahap pengesanan secara individu mencapai 10^1 CFU/ml bagi *E. coli, Listeria* dan *Shigella*; 10^2 CFU/ml bagi *Salmonella* dan *Yersinia*. Tahap pengesanan multiplex PCR apabila dikaji dengan sampel makanan sebenar yang dikontaminasi secara buatan adalah setakat 10^1 CFU/ml bagi E. coli; *Salmonella*, 10^2 CFU/ml bagi *Listeria* dan *Shigella*. Walaubagaimanapun, tahap pengesanan secara serentak bagi keenam-enam bakteria adalah 10^6 CFU/ml. Namun, terbukti bahawa alat pengesanan yang dibentuk dalam kajian ini kos-effektif, spesifik dan sensitif dalam mengesan keenam-enam bakteria berkaitan makanan.

Kata kunci: Multipleks PCR, bakteria berkaitan makanan, sampel makanan.

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LIST OF SYMBOLS AND ABBREVIATIONS

&	:	And
^{O}C	:	Celsius
m	:	Meter
M	:	Molar
$MgCl_2$	:	Magnesium Chloride
µl	:	Microliter
mil	:	Million
min	:	Minutes
ml	:	Milliliter
mm	:	Millimeter
mM	:	Millimolar
%	:	Percentage
pmol	:	Picamole
®	:	Registered
s	:	Seconds
V	:	Voltage
v/v	:	Volume to volume
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base Pair
CFU	:	Colony Forming Unit
ddH_2O	:	Double Distilled Water
DNA	:	Deoxyribonucleic Acid
dNTP	:	Deoxynucleotide Triphosphates
ELISA	:	Enzyme-linked Immunosorbent Assay

etOH	:	Ethanol
HUS	:	Hemolytic Uremic Syndrome
LAMP	:	Loop-Mediated Amplification
LBA	:	Luria-Betani Agar
LOD	:	Limit of Detection
mPCR	:	Multiplex Polymerase Chain Reaction
NASBA	:	Nucleic Acid Sequence Based Amplification
PCR	:	Polymerase Chain Reaction
TBE	:	Tris-Borate-EDTA
TDH	:	Thermostable Direct Hemolysin
TE	:	Tris-EDTA
trnL-F	:	*trnL-F* intergenic spacer
TSA	:	Trypticase Soy Agar
var.	:	Variant

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of Study

Food-borne outbreaks are serious health problem with significant morbidity worldwide (World Health Organization, 2014). Specifically, in less developed countries, diarrheal diseases are the main cause of mortality in children (Carvajal-Vélez et al., 2016). Similarly, in Malaysia, food poisoning has been considered as the major food-borne disease (MOH, 2012). Most of the cases were reported due to mishandling and lack of hygiene in food production processes (Siow et al., 2011). Such foodborne infections also could be due to consumption of food contaminated with pathogenic bacteria, virus or parasites. In general, eggs, meat, dairy products and vegetables are common source of contamination (Pires et al., 2012). *Salmonella enterica, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* O157:H7 and *Shigella flexneri* are commonly causing food poisoning (Chen et al., 2012). Especially, *Salmonella* is responsible for salmonellosis, the most common food-borne disease reported from population-based, active laboratory surveillance in the United States (Mahmoud, 2012). Mainly, enterohaemorrhagic *E. coli* (EHEC) are responsible for haemorrhagic colitis (HC) in humans (Yoon & Hovde 2008) while *E. coli* O157 to be the cause for the most severe cases (Kaper et al., 2004). Other than that, ingestion of *L. monocytogenes*-contaminated foods (Gasanov et al., 2014) especially meats (Martin et al., 2014) can lead to Listeriosis which is often linked to high mortality rate in humans (Mook et al., 2011). Besides that, *Shigella* spp., are responsible for bacillary dysentery or Shigellosis approximately 165 million cases yearly in developing and industrialized countries (Kumar et al., 2010). Whereby, *Staphylococcus aureus* is also another prevalent foodborne pathogen due to the ability to produce staphylococcal enterotoxins (SEs) in foods by enterotoxigenic strains which accompanied with symptoms such as vomiting, abdominal pain, and stomach cramps (Fetsch et al., 2014). Lastly, Yersiniosis which is

caused by *Yersinia enterocolitica* is the third most frequently reported zoonosis in Europe after Camphylobacteriosis and Salmonellosis (EFSA, 2015).

The serious foodborne outbreaks caused by the abovementioned pathogens are reported worldwide. Thus, rapid and specific detection of common food-borne pathogens is highly needed to allow effective detection of pathogen in food so that quicker treatments and remediation can be done. Initially, conventional culturing techniques and biochemical identification are combined for detection of the foodborne pathogens. However, those techniques are time-consuming, laborious and highly prone to exposure of dangerous pathogens. Besides that, the low throughput of techniques does not allow rapid detection of large numbers of food samples (Kawasaki et al., 2009). Thus, nucleic acid-based detection methods especially Polymerase Chain Reaction (PCR) has gained attention in food testing industry due to their high specificity, sensitivity as well as ability to provide unequivocal values. There are various modifications and improvement to the conventional PCR which result in a variety of methods such as Real-time PCR, Reverse-transcriptase PCR, Nested PCR and Broad-Range PCR. Among them, multiplex PCR allows detection of multiple pathogens by targeting multiple regions simultaneously for amplification. This method is not only cost-effective but also rapid as it can detect multiple pathogens in a single test (Xu et al., 2012; Chen et al., 2012).

Therefore, development of multiplex PCR is a subject of considerable attention in Malaysia. In this study, multiplex PCR was developed for simultaneous detection of six common food-borne pathogens namely *Salmonella enterica*, *E. coli*, *Shigella* sp., *Listeria monocytogenes*, *Yersinia enterocolitica* and *Staphylococcus aureus* in food.

1.2 Justification

The development of an assay that allows simultaneous detection of various foodborne pathogens in a rapid, cost-effective and highly sensitive way is much required as foodborne outbreaks becoming more common than ever. There are plenty of tools that have been designed previously to detect foodborne pathogens. However, those are either time consuming or very expensive. This research is an attempt to develop a rapid, sensitive and cost-effective multiplex PCR assay which allows simultaneous detection of six common foodborne pathogens in Malaysia by designing species-specific primers. This assay will be a very helpful tool for various regulatory agencies to detect contamination in food samples.

1.3 Objectives

1. To design and develop oligonucleotides for simultaneous detection of six major food-borne pathogens.
2. To optimize conditions of multiplex PCR for detection of selected food-borne pathogens.
3. To evaluate the sensitivity and specificity of the optimized multiplex PCR detection by testing artificially contaminated food samples

CHAPTER 2: LITERATURE REVIEW

2.1 Foodborne Diseases in Global and Malaysian Perspective

Over 250 food-borne diseases have been reported worldwide (CDC, 2017). Food-borne diseases can be defined as diseases caused by consumption of food or water that contaminated with bacteria, toxin, parasites, fungi and virus (Zhao et al., 2014; CDC, 2017). Food poisoning is characterized by symptoms such as diarrhea, vomiting and stomach cramps. The symptoms typically start 4 to 36 hours after consuming the contaminated food (Linscott, 2011). However, symptoms may differ among the different type of foodborne diseases. They can sometimes be severe and can even be life-threatening. Specifically, diarrheal diseases have caused 3% mortality globally (World Health Organization, 2014). Moreover, the risk of foodborne illness has increased markedly over the last 20 years, with nearly a quarter of the population at higher risk for illness today. However, certain people such as young children, older adults, pregnant women and people with suppressed immune system are more prone to foodborne-diseases (Prashanth & Indranil, 2016).

Recently, Norovirus, *Salmonella, Clostridium perfringens, Campylobacter* and *Staphylococcus aureus* are listed as top five foodborne disease-causing pathogens in the United States (Batz et al., 2012). Specifically, some pathogens such as *Clostridium botulinum,* the pathogen that causes botulism; *Listeria*, Shiga toxin-producing *Escherichia coli* (*E. coli*) O157; and *Vibrio* often lead to hospitalization (CDC, 2017). However, different types of bacteria have different incubation periods and duration. Food and water can also be contaminated by viruses such as the Norwalk and hepatitis viruses. Environmental toxins (heavy metals) in foods or water, and poisonous substances in certain foods such mushrooms and shellfish are other causes of food poisoning.

Foodborne infections are caused when the foodborne pathogens allowed to be multiplied. There must be desired conditions that help foodborne microorganisms to multiply. Generally, six conditions namely food high in protein and carbohydrate, acidity, lesser time to multiply, temperature 5 °C to 57 °C, oxygen and moisture are affecting the growth of bacteria (Gkana et al., 2017 & Zeiti et al., 2015). On the other hand, mucus, skin and intestinal micro flora play role as the first barrier to avoid illness during invasion of a pathogen followed by the immune system that protect human (Bezirtzoglou & Stavropoulou, 2011). However, the immune systems and gut microbial communities depend on human diet which is indirectly influenced by socioeconomic status, culture, population growth and agriculture (Kau et al., 2012). This explains the different tolerance level of people towards unhygienic food across different countries. The *E. coli* outbreak in 2011 in Germany reportedly caused US$1.3 billion in losses for farmers and industries (Thomann, 2018).

Similarly, in Malaysia numerous cases of foodborne diseases are associated with outbreaks in academic institutions of Malaysia (Soon et al., 2015) such as food poisoning episodes in schools (62%), in academic institutions (17%) and 8% in community gathering (MOH, 2012). Most of the food poisoning cases in Malaysia are caused by mishandling and lack of hygiene in food production processes (Siow et al., 2011). Other than that, foods are easily contaminated in Malaysia due to the suitable temperature and condition for the growth of most foodborne bacteria. The trends of foodborne diseases in Malaysia vary over the past few years. There was an increase of cholerae, food poisoning and hepatitis A from 2009 to 2011, but a decrease of dysentery. From 2011 to 2013, cases of cholerae, typhoid and hepatitis A decreased but dysentery showed an increment. Furthermore, food poisoning cases showed a decrease in 2012 but immediately increased slightly in 2013 (Abdul-Mutalib et al., 2015). Especially, students are at the highest risk of the population to suffer from food

poisoning cases (New et al., 2017). Up till 2017, 130 students continued to be affected by food poisoning (Malaysian Digest, 2017). However, the actual number of cases could be higher due to under-reporting since food poisoning is usually self-limiting, that is the disease resolves by itself without medical intervention.

Since foodborne diseases are very common in Malaysia due to the suitable temperature and conditions for bacterial growth as well as negligence of hygienic in food production. Thus, foodborne diseases are in the need of attention in Malaysia. More studies are done to reduce food contamination by easily identifying the causative pathogen by utilizing available scientific knowledge.

2.2 Common Foodborne Pathogens and Infections Caused

2.2.1 *Eschericia coli (E. coli)*

E. coli is the predominant facultative anaerobe of the human microbiota, some strains are responsible for enteric disease (Bischoff et al., 2005). Being a natural inhabitant of the intestinal tracts of humans and warm-blooded animals, *E. coli* also acquires antimicrobial resistance faster than any other conventional bacteria (Miranda et al., 2008). However, some *E. coli* are pathogenic as they can cause illness such as diarrhea or even illness outside of the intestinal tract. The types of *E. coli* that can cause diarrhea can be transmitted through contaminated water or food, or through contact with animals or persons (CDC, 2017). In fact, the pathogenic *E. coli* strains are categorized into various pathotypes. Among them, six pathotypes are associated with diarrhea and collectively are referred to as diarrheagenic *E. coli*. (i) Shiga toxin-producing *E. coli* (STEC) also be referred to as Verocytotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC). This pathotype is the one most commonly heard about in the news in association with foodborne outbreaks, (ii) Enteropathogenic *E. coli* (EPEC), (iii) Enterotoxigenic *E. coli* (ETEC), (iv) Enteroaggregative *E. coli* (EAEC), (v) Enteroinvasive *E. coli* (EIEC) and (vi) Diffusely Adherent *E. coli* (DAEC) (Bhavnani et al., 2016)

Many different type of foods have been identified as a potential source of Shiga Toxin-producing *Escherichia coli* (STEC) for which such raw or undercooked foodstuffs get contaminated either during primary production (e.g. slaughtering) or further processing and handling (e.g. cross contamination during processing, human-to-food contamination via food handlers). *E. coli* has been isolated worldwide from poultry meat (Canton et al., 2008; Adesiji et al., 2011).

Around 5–10% of those who are diagnosed with STEC infection develop a potentially life-threatening complication known as hemolytic uremic syndrome (HUS) (Boyer & Niaudet, 2011). Symptoms of HUS include losing pink color in cheeks and inside the lower eyelids, decreased frequency of urination and tiredness. Persons with HUS should be hospitalized because their kidneys may fail and they may develop other serious problems. Most persons with HUS recover within a few weeks, but some suffer permanent damage or die (Gigliucci et al., 2018).

2.2.2 *Salmonella*

Salmonella enterica is a members of the family Enterobacteriaceae and are facultative anaerobic Gram-negative rod-shaped bacteria generally 2 to 5 microns long by 0.5 to 1.5 microns wide and motile by peritrichous flagella (Janda et al., 2015). The European Food Safety Authority indicated that in 2015 a total of 94,625 salmonellosis cases were confirmed, representing a 1.9% increase compared to the previous year (EFSA, 2016). In addition, the most prevalent serovars are *Salmonella* Enteritidis (SE) and *Salmonella Typhimurium* (ST), causing 45.7% and 15.8% of all reported serovars human cases respectively (EFSA, 2015). Among them, serotype *Salmonella* Typhimurium is the most common in food. However, *Salmonella Entertidis* has become a major serovar causing infections in humans since the past decade (Chmielewski et al., 2003, Kottwitz, et al., 2010). Importantly, it has been reported that *S.* enterica serovars, Typhi, Paratyphi A, B, and C, and Sendai are highly adapted to the humans as a host and cause enteric fever (Gal-Mor et al., 2014)

Salmonella may primarily spread through the contaminated water, poor fertilization methods, faeces of wildlife and domestic animals and other agricultural practices. Amazingly, they also can grow and survive in many different food matrices. The behaviour of *Salmonella* in foods is governed by a variety of ecological and

environmental factors including pH, chemical composition, water activity, the presence of natural or added antimicrobial agents, and storage temperature and processing factors (Keerthirathne et al., 2016).

Salmonellosis may be defined as septicemia, gastroenteritis, or enteric fever. Enteric fevers are caused by the human-specific pathogens *S. enterica* serovars Typhi and Paratyphi. Infection severity may vary, depending on the immune system of an individual and the virulence of the *Salmonella* strain. The disease can cause various complications including severe dehydration, shock, collapse, and or septicemia. Symptoms are coon among infants, elderly, and immune-compromised personnel (Scallan et al., 2011). It is known that virulence can be activated by acetic acid stress through the *hilA* gene. Generally, the infective dose depends on the serotype, ranging from $2.0x10^2$ to $1.0x10^6$ CFU/g or mL (Huang, 1999). Therefore, the most important regions of transmission of *Salmonella* are tropical and subtropical regions, as well as places where there is a large concentration of animals and people.

Salmonella may also infect organs other than intestinal tract as *Salmonellae* are able to reach the circulation, they may diffuse extra-intestinal and cause meningitis, osteomyelitis, peritonitis, pyelonephritis, cystitis, endocarditis, pericarditis, arthritis, pneumonia, cholecystitis, vasculitis and other disorders (Gelli, 1995).

High incidence of *Salmonella* in fresh produce poultry sold in wet markets has been reported (Tung et al., 2016). On the other hand, prevalence of *Salmonella* has been reported in other food products in Malaysia. For example, *Salmonella* spp. and *S.* Typhimurium were detected in sliced fruits (such as mango, sapodilla, jackfruit, papaya, watermelon, dragon fruit and honeydew) (Pui et al., 2011), and vegetables (such as cabbage, cucumber, carrot, capsicum, lettuce and tomato) (Elexson et al., 2011). Besides, Najwa et al. (2015) have shown that *Salmonella* spp., *S. Typhimurium* and *S. Enteritidis* were detected in different types of local salad known as *ulam* (such

as *kacang panjang, pegaga nyonya*, *kacang botol,* and *selom*). A major *Salmonella* outbreak in Sekolah Menengah Sains Tapah showed that the food poisoning incident was caused by *salmonella* contamination of the chicken used in the curry. (Malaysian Digest, 2017) Another *Salmonella* outbreak in Kedah, resulted in four deaths and 38 cases of hospitalization due to inappropriate storage of raw chicken, followed by insufficient cooking and the subsequent consumption of contaminated chicken dish ProMed Mail. (2013)

2.2.3 *Listeria monocytogenes*

The genus *Listeria* is a Gram-positive non-spore forming bacilli. Members of the genus *Listeria* are generally aerobes or facultative anaerobes, catalase positive and oxidase negative. *Listeria* is motile with few peritrichous flagella when grown at temperatures below 30°C. The genus includes six species which are *L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri, L. welshimeri* and *L. grayi.* Among them, *Listeria monocytogenes* is being the most concerned as it is causing severe listeriosis infections worldwide (Scallan et al., 2011) and commonly resulting in meningitis, meningoencephalitis, septicemia, abortion, and prenatal infection in individuals with weakened immune systems and immune-compromised individuals (Laksanalamai et al., 2012). The first outbreak of foodborne listeriosis was reported in Canada in 1983, due to contamination of coleslaw (Schlech et al., 1983). It was reported by FoodNet and the European Food Safety Agency (EFSA) that *L. monocytogenes* infections are associated with approximately 12% fatality rate, which is the highest rate among foodborne pathogens (EFSA, 2013; Gilliss et al., 2013). An outbreak of listeriosis from consumption of ice cream was identified in March 2015 as results of regular surveillance. In all, there were nine cases associated with this outbreak (Pouillot et al., 2016). Other than that, thirty-five people were affected due to consumption of contaminated caramel apples (CDC, 2015). Besides that, in March 2018, three

individuals have died after eating rock melon (cantaloupe) contaminated with listeria. It is expected by NSW Health at least 15 people around Australia have been affected, across Victoria, New South Wales and Tasmania (Alison Bevege, 2018).

Not only that, *Listeria monocytogenes* infections also responsible for the highest hospitalization rates (91%) amongst known food-borne pathogens (Jemmi & Stephan, 2006). *Listeria monocytogenes* had been isolated from feces of animals, food, and food processing plants (Ruckerl et al., 2014). *L. monocytogenes* have been commonly reported to contaminate raw and undercooked meats, raw vegetables and fruits, unpasteurized milk and soft cheeses (Martin et al., 2014). *L. monocytogenes* also can be isolated from marine water, animal feeds vegetation, sewage and causing final seafood products to be contaminated (Buchanan et al., 2017).

2.2.4 *Shigella* spp.

Shigella spp. are fastidious Gram-negative organisms which can be subdivided into four serogroups - *S. sonnei*, *S. boydii*, *S. flexneri* and *S. dysenteriae* and humans are the principal reservoir of infection. The infectious dose of *Shigella* is as low as 10 bacterial cells (Germani & Sansonetti, 2006) and the transmission of infection occurs through the faecal-oral pathway. Thus, causes bacillary dysentery or shigellosis caused by *Shigella* spp. becomes endemic throughout the world. It is responsible for approximately 165 million cases annually, of which 163 million are in developing countries and 1.5 million in industrialized countries (Kumar et al., 2010). The symptoms of *Shigella* infection range from mild watery diarrhoea normally in case of *S. sonnei* to severe bacillary dysentery with fever, abdominal pain, blood and mucus in stool samples caused mainly by strains of *S. dysenteriae* 1 (Kumar et al., 2006). *S. flexneri* and *S. boydii* can cause either mild or severe illnesses. However, resistance to the oral antimicrobial medications ampicillin and trimethoprim/sulfamethoxazole is common among *shigellae*

in the United States, and resistance to fluoroquinolones is increasing among *shigellae* globally (CDC, 2015).

2.2.5 *Staphylococcus aureus*

Staphylococcus aureus is an important food-borne pathogen due to the ability of enterotoxigenic strains to produce staphylococcal enterotoxins (SEs) in food samples (Fetsch et al., 2014). A clinical estimate also reported that *S. aureus* has caused more than 94,000 serious infections and more than 18,000 deaths in the United States since 2005 (Schmelcher et al., 2012).This organism has emerged as a major pathogen for both nosocomial and community acquired infections. *S. aureus* does not form spores but can cause contamination of food products during food preparation and processing. *S. aureus* can grow in a wide range of temperature (7 ºC to 48.5 ºC; optimum 30 ºC to 37 ºC), sodium chloride concentration up to 15% NaCl and pH (4.2 to 9.3; optimum 7 to 7.5),. It is also a desiccation tolerant organism thus can survive in potentially dry and stressful environments, such as the human nose and on skin (Chaibenjawong et al., 2010).

The most common symptoms of staphylococcal food poisoning are sudden vomiting, abdominal pain, and stomach cramps (Hennekinne et al., 2012). Eventually, it can be severe to warrant hospitalization particularly among the group of young, old, pregnant, immunosuppressed person (Murray, 2005). Foods that usually favor the growth of *Staphylococcus aureus* are animal origin food with high protein content such as milk products, meat, meat products and salads, bakery products, particularly cakes and cream-filled pastries (Hennekinne et al., 2012). *S. aureus* also was detected most frequently in 20%, 23.1% and 83.9% of the exported fresh, organic vegetables analyzed, respectively, as in a report (Nguz et al., 2005). Moreover, the incidence of *S. aureus* in vegetable dishes was found to be much higher than *L. monocytogenes* and *Salmonella* spp. (Sospedra et al., 2013). It shows that *Staphylococci* are ubiquitous in the

environment and can be found in the environmental surfaces, air, dust, sewage, water, humans and animals (Hennekinne et al., 2012).

It is also likely to be carried by food handlers and pose significant risk to consumers (Dagnew et al., 2012). Infected food handlers are often implicated in outbreaks of known or suspected viral or bacterial etiology and might well have been the cause of many of these outbreaks.

2.2.6 *Yersinia enterocolitica*

Y. enterocolitica is a Gram-negative zoonotic enteropathogenic bacterium responsible to yersiniosis. *Y. enterocolitica* belongs to the family Enterobacteriaceae and exhibits 10 – 30% of DNA homology with other genera of this family (Golubov et al., 2003). The ability of *Y. enterocolitica* to survive at low temperatures makes it an important pathogen associated with foodborne infections. Reports have shown survival and propagation of *Y. enterocolitica* in vacuum-packed foods or foods at refrigeration temperature (Lindqvist & Lindblad, 2009).

Humans commonly become infected with *Y. enterocolitica* through the consumption of raw or undercooked pork (Saraka et al., 2017), as slaughtered pigs are considered the principal reservoir for pathogenic *Y. enterocolitica* (Rosner et al., 2013). Touching contaminated surfaces is also likely to cause infection instead of food ingestion. It is well known that *Yersinia* tend to form biofilms on surfaces to survive hostile environments (Flemming et al., 2007; Eurosurveillance-Editorial, 2015).

Yersiniosis is known as the third most commonly reported zoonosis in Europe (EFSA, 2015). It is associated with clinical symptoms range from mild gastroenteritis to invasive syndromes like terminal ileitis (Bottone, 1999). Consumption of contaminated food or water could lead to the infection. Following ingestion, the bacteria colonize the lumen of the intestine and cross the intestinal tissue barrier by invading M cells (Schulte

et al., 2000; Kim et al., 2017). This may result in dissemination of the bacteria to the mesenteric lymph nodes and extra-intestinal sites such as spleen, liver or lungs. However, all *Y. enterocolitica* are not pathogenic for human. The species is divided into six biotypes at which the biotype 1A generally regarded as nonpathogenic while the pathogenic biotypes are BT1B, BT2, BT3, BT4, BT5 (Le Guern et al., 2016).

2.3 Detection Methods of Foodborne Pathogens

2.3.1 Conventional Culture-based Detection

The conventional methods for detecting the foodborne bacterial pathogens present in food are based on culturing the microorganisms on selective media agar plates followed by standard biochemical identifications (Mandal et al., 2011). Conventional methods are simple and inexpensive. However, these methods are time consuming as they depend on the ability of the microorganisms to grow in different culture media such as pre-enrichment media, selective enrichment media and selective plating media (Law et al., 2015). Those methods usually require 2 to 3 days for preliminary identification and more than a week for confirmation of the species identification (Zhao et al., 2014). The culture-based methods are also laborious as they require the preparation of culture media, inoculation and colony counting (Mandal et al., 2011). Moreover, conventional methods considered to have low sensitivity (Lee et al., 2014). This is because false negative results may occur due to viable but non-culturable (VBNC) pathogens. Eventually, the failure to detect foodborne pathogens would increase the risk of disease transmission.

Thus, there are various culture-independent rapid methods developed to complement the culture methods with improvements in terms of rapidity, sensitivity, specificity and suitability for *in-situ* analysis and distinction of the viable cell (Zhao et al., 2014). The alternative rapid methods are the immunology-based, molecular-based, sequence-based and biosensors. However, each of the rapid detection methods has its own advantages and disadvantages.

2.3.2 Immunology-based Detection

The detection of foodborne pathogens by immunological-based methods is done based on highly specific antibody-antigen interactions. This is possible when a particular antibody binds to its specific antigen. The binding strength of an antibody to antigen decides the sensitivity and specificity of the assay. Besides that, polyclonal and monoclonal antibodies are also utilized immunological-based methods as described in a review by Zhao et al. (2014). Enzyme-linked Immunosorbent Assay (ELISA) and lateral flow immune assay are also included. ELISA is the most widely used method especially the Sandwich ELISA. It involves interaction of the complex consisting antigen sandwiched between two antibodies and detection can be done by adding a colorless substrate (Zhang, 2013); Kumar et al. (2011) performed the detection of pathogenic *Vibrio parahaemolyticus* in seafood via sandwich ELISA using monoclonal antibodies against the TDH-related hemolysin (TRH) of pathogenic *Vibrio parahaemolyticus*. The detection limit of the assay was 10^3 cells of pathogenic *Vibrio parahaemolyticus*. Other than that, there are ELISA test kits also available for detection of *Salmonella* in food products. The detection limit of this kit was reported to be 10 CFU/25g sample with minimum four of the 20 food matrix tested (Bolton et al., 2000). ELISA is also used to detect toxins such as *Clostridium perfringens* α, β, and ε toxin, staphylococcal enteroxins A, B, C, and E, botulinum toxins and *Escherichia coli* enterotoxins in food samples (Aschfalk & Mülller, 2002).

Other than that, high-throughput and automated ELISA systems such as VIDAS (BioMerieux) are also available for the detection of foodborne pathogens (Glynn et al., 2006). Several studies used VIDAS for detection of (i) *Salmonella* in pork sample, fruits and vegetables (Vieira-Pinto et al., 2007; Gómez-Govea et al., 2012), (ii) *Listeria monocytogenes* in fish samples, beef, pork, fruits and vegetables (Vaz-Velho et al., 2000; Meyer et al., 2011; Gómez-Govea et al., 2012), (iii) *Escherichia coli* O157:H7 in cheese,

fruits and vegetables(Gómez-Govea et al., 2012; Carvalho et al., 2014), (iv) *Campylobacter* spp. in fruits and vegetables (Gómez-Govea et al., 2012), and staphylococcal enterotoxin in milk cheese (Cremonesi et al., 2007).

Besides that, lateral flow immune assay that employs mono-disperse latex, colloidal gold, carbon and fluorescent tags are also utilized to detect foodborne pathogens (Zhao et al., 2014). For example, immuno-chromatographic strip was developed by Jung et al. (2005) to detect *Escherichia coli* O157 with detection limit of 1.8×10^5 CFU/mL and 1.8 CFU/mL without and with enrichment respectively (Niu et al., 2014). Another study by Xu et al. (2013) employed immuno-chromatographic test strip for the detection of *Staphylococcus aureus* with detection limit of 10^3 CFU/mL. Besides that, foodborne pathogens such as *Listeria* spp. and *Salmonell*a also have been detected using this method (Kim et al., 2007; Shukla et al., 2011).

In spite of their shorter assay time compared to traditional culture techniques, immunology-based detection still lacks the ability to detect microorganisms in "real-time". Immunology-based methods coupled with other methods for pathogen detection, like immune-magnetic separation on magnetic beads is coupled with matrix-assisted laser desorption ionization-time of flight mass spectrometry for detection of staphylococcal enterotoxin B (Schlosser et al., 2007), combination of immunomagnetic separation with flow cytometry for detection of *L. monocytogenes* (Hibi et al., 2006; Jung et al., 2003).

2.3.3 Molecular-based Detection

Molecular-based methods are carried out by detecting the species-specific DNA or RNA sequences in the target pathogen. This is done by hybridizing the target nucleic acid sequence to a synthetic oligonucleotide (probes/ primers). The primer sequence is complementary to the target sequence and allows amplification of particular region

upon annealing or hybridization (Zhao et al., 2014). There are many foodborne pathogens such as *Clostridium botulinum, Vibrio cholerae, Staphylococcus aureus,* and *Escherichia coli* O157 which produced toxins that cause diseases (Singh et al., 2001; Fusco et al., 2011; Radu et al., 2014). Those toxin-related genes in the pathogens can be detected via molecular-based methods (Zhao et al., 2014). Other than that, pathogens that exhibit ambiguous phenotypic characteristics can be identified through the molecular-based methods (Adzitey et al., 2012). As these methods allow detection of specific genes of pathogen, ambiguous or wrongly interpreted results can be avoided. The recent methods fall under this category are the simple polymerase chain reaction (PCR), multiplex polymerase chain reaction (mPCR), real-time/quantitative polymerase chain reaction (qPCR), nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP) and microarray technology.

2.3.3.1 Conventional Polymerase Chain Reaction (PCR)

PCR employs detection of a single bacterial pathogen that present in food by detecting a specific target DNA sequence (Velusamy et al., 2010). PCR also enables amplification of specific target DNA in a cyclic three steps process namely denaturation, annealing and extension. (Mandal et al., 2011). PCR mainly involves the polymerization process whereby the primers complementary to the single-stranded DNA are extended with the presence of deoxyribonucleotides (dNTPs) and a thermostable DNA polymerase.

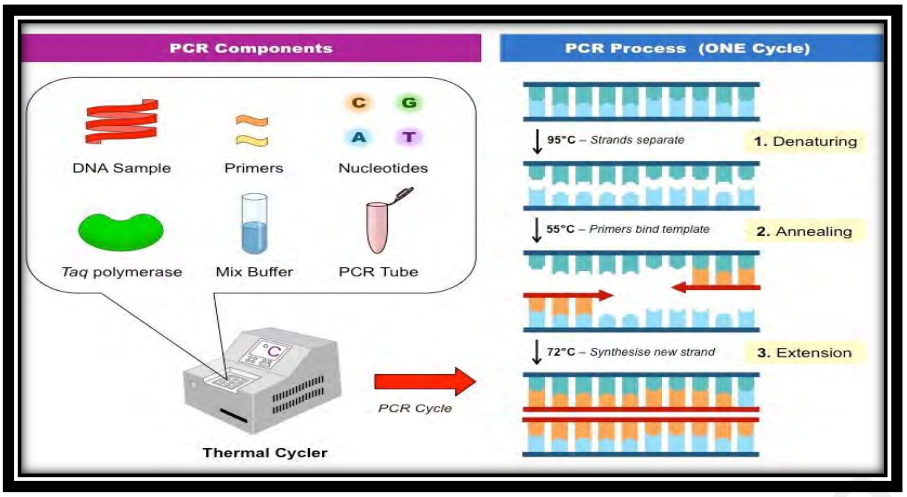


Figure 2.1: Polymerase Chain Reaction (PCR) Reagents and Steps Involved. Figure retrieved from http://ib.bioninja.com.au/standard-level/topic-3-genetics/35-genetic-modification-and/pcr.html

Then, the PCR amplification products are visualized on electrophoresis gel as bands by staining with ethidium bromide (Zhao et al., 2014). Foodborne pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus, Campylobacter jejuni, Salmonella* spp. and *Shigella* spp. have been detected using PCR (Cheah et al., 2008; Lee et al., 2008; Alves et al., 2012; Chiang et al., 2012; Zhou et al., 2013).

The main advantage of PCR is that it is very sensitive method. The DNA of interest can be amplified with the DNA from just one cell (Wassenegger, 2001). Thus, very small amounts of starting material can be used. Also, old or degraded DNA very often yields enough starting material to amplify the DNA of interest (Chen et al., 2012).

The sensitivity of PCR itself is a major disadvantage since very small amounts of contaminating DNA (from a different sample) can also be amplified. Thus, the person conducting the run must be skillful (Velusamy et al., 2010).

2.3.3.2 Real-time PCR

Real-time PCR or quantitative PCR (qPCR) is different from conventional PCR in which agarose gel electrophoresis is not required to view the PCR products. Instead, the method monitors amplification of PCR product continuously by measuring the fluorescent signals. The fluorescence intensity is proportional to the amount of PCR amplicons (Zhao et al., 2014). Among the developed fluorescence for qPCR SYBR green, TaqMan probes and molecular beacons are the commonly used ones. SYBR green is a double-stranded DNA (dsDNA)-binding fluorescent dye (Gomes et al., 2017). Eventually, TaqMan probes and molecular beacons started to alternate SYBR green (Rodriquez et al., 2012). The detection of *Salmonella* in fresh-cut fruits and vegetables by molecular beacon qPCR targeting the invasion associated gene (*iagA*) was first reported by Liming and Bhagwat (2004) with a detection limit 4 CFU/25g of upon enrichment. Besides that, Tyagi et al. (2009) developed a highly sensitive SYBRgreen qPCR assay for the detection of pathogenic *tdh*-positive *Vibrio parahaemolyticus* in tropical shellfish with a detection limit of 10^2 CFU/ml for shrimp. Moreover, detection of enterotoxin gene cluster (*egc*) corresponding to *Staphylococcus aureus* in raw milk at which 10^3 CFU/mL, 10^4 CFU/ml was detected by SYBRgreen and TaqMan qPCR respectively (Fusco et al., 2011).

Although, qPCR possess a lot advantages there are also drawbacks such as (i) difficult for multiplex real-time PCR assay, (ii) affected by PCR inhibitors, (iii) difficult to distinguish between viable and non-viable cells, (iv) required trained personnel (Park et al., 2014; Law et al., 2015). It also more expensive than conventional PCR and technical expertise is needed (Law et al., 2015).

2.3.3.3 Multiplex PCR

Multiplex PCR (mPCR) offers a more rapid detection as compared to simple PCR through the simultaneous amplification of multiple gene targets. The basic principle of mPCR is similar to conventional PCR. However, several sets of specific primers are used in mPCR assay whereas only one set of specific primers are used in conventional PCR assay. Primer design is crucial for the development of mPCR, as the primer sets should have similar annealing temperature (Zhao et al., 2014). Besides, the concentration of primers is also important in mPCR because interaction may occur between the multiple primer sets in mPCR that results in primer dimers (Zhao et al., 2014). Other important factors for a successful mPCR assay include the PCR buffer concentrations, the balance between magnesium chloride and deoxynucleotide concentrations, the quantities of DNA template, cycling temperatures and Taq DNA polymerase (Khoo et al., 2009).

Initially, two to three genes only were targeted. Eventually, more genes were incorporated to develop various mPCR. For instance, Chen et al. (2012) developed a mPCR that can detect five pathogens simultaneously which are *Salmonella* Enteritidis*, Staphylococcus aureus, Shigella flexneri, Listeria monocytogenes,* and *Escherichia coli* O157:H7 using five pairs of primers targeting invasion protein(*invA*), 16SrDNA, invasion plasmid antigen H(*ipaH*), listeriolysine O (*hlyA*) and intimin (*eaeA*) gene respectively. Besides that, Ryu et al. (2013) developed a PCR to differentiate 6 species of *Listeria*. The limit of detection of the developed assay was $7.58X10^4$CFU/ml for mixed genomic DNA. Other than that, another study utilized GeXP-mPCR for detection of six foodborne pathogens namely *Salmonella enterica, Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Shigella* spp. and *Campylobacter jejuni* with detection limit of 420 CFU/ml, 310 CFU/ml, 270 CFU/ml, 93 CFU/ml, 85 CFU/ml and 66 CFU/ml respectively (Zhou et al., 2013). In the study, capillary

electrophoresis was used instead of gel electrophoresis for visualization at which even closer bands can be identified easily. Recently, Propidium monoazide PMA-mPCR assay was developed to detect viable *Cronobacter sakazakii*, *Staphylococcus aureus* and *Bacillus cereus* in infant food products (Li et al., 2016). The stated assay was able to detect as low as 10^1 CFU/g for *C. sakazakii* and *S. aureus,* and 10^0 CFU/g for *B. cereus* in spiked infant food products.

2.3.3.4 Loop-mediated Amplification (LAMP)

LAMP is a molecular-based amplification method developed by Notomi et al., (2000) which provides a rapid, sensitive and specific detection of foodborne pathogens. LAMP is based on auto-cycling strand displacement at which *Bst* DNA polymerase is utilized instead of Taq polymerase as in PCR. Besides that, LAMP also differs from PCR as it only requires isothermal conditions between 59 °C and 65 °C for 60 min. In LAMP, four primers targeting six specific regions of target DNA are used. Thus, LAMP is able to amplify products three times faster than PCR. The amplicons of LAMP can be visualized by agarose gel electrophoresis which appear as a ladder of DNA fragments or SYBR Green dye similar to PCR (Wang et al., 2008; Zhao et al., 2014).

Previously, LAMP was used to detect *stxA*2 gene in *Escherichia coli* O157:H7 (Maruyama et al., 2003). A number of studies have reported that the specificity and sensitivity of LAMP assay were higher than PCR as far as foodborne pathogens detection is concerned due to the utilization of four sets of primers (Ohtsuka et al., 2005; Wang et al., 2008; Yamazaki et al., 2008; Xu et al., 2012). There are commercial LAMP assay kits available for detection of *Listeria, Salmonella, Campylobacter,Legionella,* and verotoxin-producing *Escherichia coli* (Mori & Notomi, 2009). For example, the Loopamp detection kit (Eiken Chemical) is commercially available for the detection of foodborne pathogens. Many LAMP assays are also developed for *Salmonella enterica*

(Ohtsuka et al., 2005), *Shigella* (Song et al., 2005), enteroinvasive *Escherichia coli* (Song et al., 2005), verotoxigenic *Escherichia coli* O157 and O26 (Hara-Kudo et al., 2008) and *Campylobacter* (Yamazaki et al., 2009). Another LAMP assay was developed for the detection of *Yersinia enterocolitica* isolates in both pure bacterial cultures and pork meat with primers corresponding to the *gyrB* gene. A sensitivity level of 65 CFU/mL was recorded (Gao et al., 2009).

Similar to PCR, a lot of modifications were done to LAMP such as multiplex LAMP, reverse-transcription LAMP, real-time LAMP and *in situ* LAMP (Law et al., 2015). Most importantly, LAMP allows visualization of amplification product by measuring the turbidity.

2.3.4 Biosensor-based Detection

Biosensor is an analytical device that consists of two main elements: a bioreceptor and a transducer. The bioreceptor recognizes the target analyte which can be either (i) Biological material: enzymes, antibodies, nucleic acids and cell receptors, or (ii) Biologically derived materials: aptamers and recombinant antibodies or (iii) Bio-mimic: imprinted polymers and synthetic catalysts. The transducer converts the biological interactions into a measurable electrical signal which can be optical, thermometric, micromechanical, electrochemical, mass- based, or magnetic (Velusamy et al., 2010; Zhao et al., 2014).

Biosensors are easy to operate and they do not require sample pre-enrichment, unlike nucleic-acid based methods and immunological methods which require sample pre-enrichment (Singh et al., 2013). The recent biosensors that commonly used for the detection of foodborne pathogens are optical, electrochemical and mass-based biosensors (Zhang, 2013; Zhao et al., 2014).

As far as detection of foodborne pathogens are concerned, optical biosensor were used for detection of *Salmonella enterica* Thyphimurium, *Listeria monocytogenes*, *Campylobacter jejuni, E. coli* 0157:H7 (Taylor et al., 2006) with a sensitivity level of 10^4 CFU/ml, 10^3 CFU/ml, 10^5 CFU/ml and 10^4 CFU/ml respectively. In another study a detection level of 10^3 CFU/ml was obtained for detection of *Campylobacter jejuni* (Wei et al., 2007). Besides that, optical biosensor also used to detect *E.coli* in cucumber and ground beef samples at a detection limit of 10^3 CFU/ml (Wang et al., 2013). Other than that, *Listeria monocytogenes* has been detected by using electrochemical biosensors and obtained a sensitivity of 10^3 CFU/ml in lettuce, milk and ground beef samples (Kanayeva et al., 2012). In 2005, Varshney et al. used electrochemical biosensor to detect *E. coli* at which the detection limit was 10^7 cells/ ml without enrichment and 10^1cells/ml with enrichment.

However, the drawback of this method is there are difficulties in producing inexpensive and reliable sensors, the storage of biosensors, the stabilization of biosensors, methods of sensor calibration and total integration of the sensor system (Velasco-Garcia & Mottram, 2003).

Table 2.1: Summary of rapid detection methods of food-borne pathogens

Rapid Detection Method	Targeted Pathogens	Limit of Detection	Tested On	Reference
Multiplex PCR	Shiga-toxin producing *E. coli*	5 x 10^1 CFU/ml	Ground beef	Fratamico et al., 2016
	Vibrio parahaemolitycus *Vibrio cholarae* *Vibrio vulnificus*	1 x 10^1 CFU/tube For each targeted pathogens. In spiked foods 1 x 10^2 CFU/ml after enrichment	Seafood samples	Neogi et al., 2010
	Salmonella enterica Enteriditis *Staphylococcus aurues* *Shigella flexneri* *Listeria monocytogenes* *E. coli 0157:H7*	Low level (1 x 10^1-1.7 x 10^1 CFU/g of sample) High level (1.2 x 10^3- 1.7x 10^3 CFU/g of sample) after 24 h enrichment.	Lean pork samples	Chen et al., 2012
	Listeria grayi *Listeria innocua* *Listeria ivanovii* *Listeria monocytogenes* *Listeria seeligei* *Listeria welshimeri*	7.58 x 10^4 copies/ml	Processed foods	Ryu et al., 2013
	Vibrio Genus and pathogenic five *Vibrio* sp	Between 5 x 10^3 and 5x 10^2 copies of genomic DNA in a 25-cycle PCR Between 5x 10^2 and 5 x 10^1 copies of genomic DNA in a 30-cycle PCR	Bacterial Strains	Kim et al., 2015

Table 2.1, continued.

Rapid Detection Method	Targeted Pathogens	Limit of Detection	Tested On	Reference
Multiplex Real-time PCR with Melting Curve Analysis	*Staphylococcus aurues* *Listeria monocytogenes* *Salmonella enterica* *Vibrio parahaemolitycus* *Shigella* spp.	0.82 x 10^1 pg for *Staphylococcus aureus*, 4 x 10^1 pg for *Listeria monocytogenes*, 0.62 x 10^1 pg for *Salmonella enterica*, 0.25 x 10^1 pg for *Vibrio parahaemolyticus* and 3.9 x 10^{-1} pg for *Shigella* spp. of the extracted genomic DNA	Food samples	He et al., 2016
	Vibrio parahaemolitycus *Vibrio cholarae* *Vibrio vulnificus*	0.1x 10^1 CFU/ gram of food homogenate. After 8h of enrichment	Seafood samples (Oyster, crab meat and raw fish)	Kim et al., 2012
Target-enriched mPCR (Tem-PCR)	*Salmonella enterica* *Staphylococcus aurues* *E. coli* *Listeria monocytogenes* *Shigella* spp.	<1.1 x 10^2 CFU/ml	Poultry meat, raw pork, raw milk, egg, sausage, raw beef, milk powder, and frozen meat	Xu et al., 2015
Dual-priming oligonucleotide system-based Multiplex PCR	*Salmonella* spp. *Listeria monocytogenes* *Shigella* spp. *Staphylococcus aurues* *Campylobacter jejunii* *Yersinia enterocolitcus*	1 x 10^2-1x 10^3 CFU/ml	Pure cultures and Artificially contaminated food samples	Xu et al., 2017

Table 2.1, continued.

Rapid Detection Method	Targeted Pathogens	Limit of Detection	Tested On	Reference
Propidium monoazide (PMA)-mPCR Assay	*Cronobacter sakazakii* *Staphylococcus aureus* *Bacillus cereus*	0.1 x 10^1 - 1 x 10^1 CFU/g viable cells after 12 h enrichment.	Infant food products	Li et al., 2016
Loop-mediated Amplification (LAMP)	*E.coli* 0157:H7 *Salmonella enterica* Thyphimurium *Vibrio parahaemolyticus*	3.8 x 10^2 copies/ml	Bacterial strains	Oh et al., 2016
	E. coli 0157:H7	0.5 x 10^1 CFU/ reaction tube in pure bacterial culture. 1 x 10^3 CFU/ml without pre-enrichment 1 x 10^1 CFU/ml after 4h pre-enrichment	Ground beef	Ravan H et al., 2016
	Listeria spp. *Listeria monocytogenes*	0.2 x 10^1 CFU/ reaction tube in pure bacterial culture	Chicken samples	Tang et al., 2011
	Listeria monocytogenes	0.5 x 10^1 CFU/g	Dairy products	Tirloni et al., 2017
Loop-mediated Amplification (LAMP)	*E.coli* 0157:H7 *Salmonella enterica* Thyphimurium *Vibrio parahaemolyticus*	3.8 x 10^2 copies/ml	Bacterial strains	Oh et al., 2016

Table 2.1, continued.

Rapid Detection Method	Targeted Pathogens	Limit of Detection	Tested On	Reference
Loop-mediated Amplification (LAMP)	*E. coli* 0157:H7	0.5x 10^1 CFU/ reaction tube in pure bacterial culture. 1x 10^3 CFU/ml without pre-enrichment 0.1 x 10^1 CFU/ml after 4h pre-enrichment	Ground beef	Ravan H et al., 2016
	Listeria spp. *Listeria monocytogenes*	0.2 x 10^1 CFU/ reaction tube in pure bacterial culture	Chicken samples	Tang et al., 2011
	Listeria monocytogenes	0.5 x 10^1 CFU/g	Dairy products	Tirloni et al., 2017
Microfluidic Lab-On-Disk integrated LAMP	*Salmonella enterica*	5 x 10^{-3} ng/ul DNA	Tomatoes	Sayad et al., 2016
Biosensor-based Detection Biosensor using double-layer capillary-based immunomagnetic separation & nanocluster-based amplification	*E.coli* 0157:H7	7.9 x 10^1 CFU/ml	Milk products	Huang F. et al., 2017

CHAPTER 3: METHODOLOGY

3.1 Revival of Bacterial Cultures

A total of 80 bacterial strains were tested in the study. The list of strains is provided in Table 3.1. All the 80 strains were revived from stab cultures and glycerol stocks from laboratory collection. All the strains were propagated on Luria-Betani Agar (LBA) plates overnight. However, the *Listeria* colonies that cultured on LBA were too small causing difficulty in collecting single colony for DNA extraction purpose. Thus, the *Listeria* strains were propagated on Trypticase Soy Agar (TSA) instead to allow formation of relatively larger and well-isolated colonies.

In addition, *Yersinia enterocolitica* strains took longer incubation period than the rest of the bacterial strains to produce colonies. Revival of *Yersinia enterocolitica* strains took 48 h while the others took about 12 h incubation to form visible colonies. The list of tested strains is shown in Table 3.1.

Table 3.1: Strains used in the study.

No	Strain ID
1	*E. coli* FE138EC4EC109
2	*E. coli* P141E4EC110
3	*E. coli* P141EC2EC111
4	*E. coli* P136EC4EC101
5	*E. coli* V137EC1EC102
6	*E. coli* EC0157
7	*E. coli* ATCC25923
8	*E. coli* V137EC3EC104
9	*E. coli* FE138EC3EC108
10	*E. coli* FE138EC1EC106
11	*E. coli* J144EC3EC144
12	*E. coli* FE138EC4EC109
13	*Salmonella enterica* Typhii H22i
14	*Salmonella enterica* Enteriditis MOB 2054/05
15	*Salmonella enterica* Albany MOB 1549/05

No	Strain ID
16	*Salmonella enterica* Paratyphi *A* 3/2/04
17	*Salmonella enterica* Typhimurium ATCC13311
18	*Salmonella enterica* Typhimurium ATCC9251
19	*Salmonella enterica* Enteritiditis Sal 1/9/02
20	*Salmonella enterica* Enteritiditis 2/9/02
21	*Salmonella enterica* ATCC6539
22	*Salmonella enterica* ATCC13070
23	*Salmonella enterica* STM071
24	*Salmonella enterica* STM048
25	*Salmonella enterica* SEH12
26	*Salmonella enterica* Sal 9/05

Table 3.1, continued.

No	Strain ID
27	*Listeria monocytogenes* LM15
28	*Listeria monocytogenes* LM31
29	*Listeria monocytogenes* LM34
30	*Listeria monocytogenes* LM44
31	*Listeria monocytogenes* LM50
32	*Listeria monocytogenes* LM60
33	*Listeria monocytogenes* LM150
34	*Listeria monocytogenes* LM161
35	*Listeria monocytogenes* LM162
36	*Listeria monocytogenes* LM163
37	*Listeria monocytogenes* LM164
38	*Listeria monocytogenes* LM177
39	*Listeria monocytogenes* LM171
40	*Listeria monocytogenes* LM191
41	*Listeria monocytogenes* LM192
42	*Listeria monocytogenes* LM197
43	*Listeria monocytogenes* LM85
44	*Listeria monocytogenes* LM178
45	*Listeria monocytogenes* LM186
46	*Shigella flexneri* 2a TH10/07
47	*Shigella sonnei* TC2/97
48	*Shigella flexneri* 2a TH6/01
49	*Shigella flexneri* 2a TH6/07
50	*Shigella flexneri* 3a TH5/09
51	*Shigella sonnei* ATCC11060
52	*Shigella sonnei* TH20/97
53	*Shigella sonnei* TH3/01
54	*Shigella sonnei* TH3/00
55	*Shigella sonnei* TH1300
56	*Shigella sonnei* TH5/00

No	Strain ID
57	*Shigella sonnei* TH4/00
58	*Shigella sonnei* TH2/00
59	*Shigella dysentrise* TH26/98
60	*Staphylococcus aureus* CB37SA1
61	*Staphylococcus aureus* IK9SA1
62	*Staphylococcus aureus* ILI29SA1
63	*Staphylococcus aureus* TC29SA3
64	*Staphylococcus aureus* FH5SA2
65	*Staphylococcus aureus* FH81SA3
66	*Staphylococcus aureus* FH68SA1
67	*Staphylococcus aureus* TCSA2
68	*Staphylococcus aureus* FH1SA1
69	*Staphylococcus aureus* FH62SA1
70	*Yersinia enterocolitica* ATCC9610
71	*Yersinia enterocolitica* PCM3K42318
72	*Yersinia enterocolitica* PCM3K13
73	*Yersinia enterocolitica* PCM3K12
74	*Yersinia enterocolitica* PCM1K52418
75	*Yersinia enterocolitica* PCM1K4
76	*Yersinia enterocolitica* PCM1K1
77	*Yersinia enterocolitica* PCM1K5
78	*Yersinia enterocolitica* PCM1K12
79	*Yersinia enterocolitica* PCM1K13
80	*Yersinia enterocolitica* a-C-04

3.2 Primer Designation

First of all, the DNA sequences of *E. coli, Salmonella enteric, Listeria monocytogenes, Shigella* sp., *Staphylococcus aureus and Yersinia enterocolitica* were downloaded from National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Genbank/). These genome sequences were then aligned using Basic Local Alignment Search Tool (BLAST) to look for common conserved regions that can be used for amplification. Primer design was done in accordance to guidelines given for best result when conducting agarose gel electrophoresis. Length of amplicons should be less than 1000 bp while the primer itself should be around 18-22 bp with GC content 30-80 %. Whereby, the optimal annealing temperature of the primers should be around 55 ºC to 60 ºC.

Then, Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primers targeting species-specific genes and to ensure the primers have no non-specific amplification. Design of more than one pair of primers was also cross-matched using Primer-BLAST to avoid occurrence of non-specific amplification among different combination of forward and reverse primers. This was done in order to get the best primer sequence before synthesis. The amplicon size and sequence as well as primer dimerization was checked out using *In-silico* PCR (http://insilico.ehu.es/PCR/). The selected primer sequences were then sent to Integrated DNA Technologies, Inc. for synthesis.

3.3 Crude Genomic DNA Extraction

Briefly, a well isolated colony from an overnight culture on LB agar was inoculated into a microfuge tube containing 100 µl of sterile water and the cell suspension was boiled at 99 °C for 5 min, snapped cooled on ice for 10 min. The cell lysate was then centrifuged for 5 min at 13,400 rpm. Then, an aliquot of 80 µl supernatant was transferred to a fresh tube to be used as DNA template in PCR. The concentration of crude DNA is measured and recorded by using Nanodrop.

3.4 Primer Specificity Test

To evaluate the specificity of designed primers, a monoplex Polymerase Chain Reaction (PCR) was done with each primer pair and tested with bacterial strains of the six pathogens. Once the specificity was confirmed, then all six pairs of primer sets were pooled and tested with targeted bacterial strains.

3.5 Purification and Validation of PCR Product

The validity of the PCR was carried by DNA sequencing of the amplicons amplified by the species-specific primers. The PCR products of representative targeted bacterial strains were purified prior to sequencing by using MEGAquick-spin™ Total fragment DNA Purification Kit. 20 µl of PCR product was mixed with 5 volume of BNL buffer (100 µl) and incubated for 1 min. Then, the mixture was transferred to MEGAquick-spin™ column (blue color) and centrifuged for 1 min to allow binding of DNA. Once the flow through discarded, 700 µl of wash buffer added and centrifuged at 13,000 rpm for 1 min. This step was repeated twice. Finally, the MEGAquick-spin™ column was transferred to a clean 1.5 ml micro-centrifuge tube in which 30 µl of the elution buffer

was added and incubated at room temperature for 1 min followed by centrifugation for 1 min at 13,000 rpm. The micro-centrifuge tube containing the eluted DNA was then stored at -20 ℃ prior to sending to a commercial facility for sequencing to confirm the identity.

3.6 Multiplex Polymerase Chain Reaction (mPCR) Optimization

The optimization was done by adjusting 2 parameters namely annealing temperature and primer concentration. First of all, monoplex PCR was carried out for every primer pair individually to optimize annealing temperature. In a total reaction volume of 25 µl, including 1X PCR buffer, 2.25 µM $MgCl_2$, 0.12 µM dNTP, and 60 ng/ml Taq DNA Polymerase (Promega, Madison, WI) and 5 µl DNA. The detailed recipe of PCR reagents for each monoplex PCR is shown in Table Appendix A.

The annealing temperature was optimized at which the same reaction mixture as stated in Appendix A was subjected to gradient PCR with seven different annealing temperature which were 51.5 °C, 53.4 °C, 55.8 °C,58.3 °C, 61 °C, 63.7 °C and 66.1 °C. The cycling conditions for the monoplex gradient PCR is as shown in Table 3.2. Once PCR is completed, the PCR products were electrophoresed on 2.0 % (w/v) agarose gel. The temperature at which bands with best intensity formed was rounded off and fixed as optimal annealing temperature.

Once optimal temperature is fixed, the concentration of each primer pair was adjusted eventually to increase specificity and to reduce primer dimers. The similar cycling condition as described in Table 3.2 was used for multiplex PCR.

Table 3.2: Cycling condition for monoplex gradient PCR.

Stage	**Temperature**	**Duration**	
Pre-denaturation	95 ºC	5 min	
Cyclic Denaturation	95 ºC	1 min	30 cycles
Cyclic Annealing	51.5 °C,53.4 °C, 55.8 °C,58.3 °C, 61 °C, 63.7 °C and 66.1 °C. **(Gradient)**	30 sec	
Cyclic Extension	72 ºC	1 min	
Final Extension	72 ºC	8 min	

3.7 Sensitivity Evaluation of mPCR Assay Using Bacterial Strains

Bacterial suspension of 0.5 McFarland (10^8 CFU/ml) from each targeted bacterium was prepared. The suspension was serially diluted with TE buffer to give a range from 10^6,10^4, 10^2, 10^1 and 10^0 CFU/ml. Crude DNA was extracted from each diluted cell suspension as previously described. The supernatant was collected as DNA to be proceeded with multiplex PCR. The PCR products were viewed using a 2.0 % agarose gel.

3.8 Application of mPCR Assay Using Artificially Contaminated Food Samples

A bacterial cocktail containing each targeted bacteria strains was prepared by mixing 1ml each (10^8 CFU/ml). The mixture was well mixed by gentle vortex. One ml of bacterial cocktail suspension was then spiked into 10 g of different food matrix (chicken, leafy vegetables, dairy products and ready-to-eat foods). The spiked foods were allowed to sit at room temperature for 30 min. The food samples were

homogenized with 100 ml buffered peptone water and incubated at 37 ºC for two hours. One ml of the food homogenate was then serially diluted with TE buffer (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-9}, 10^{-10}). Each diluted suspension was subjected to DNA extraction followed by PCR testing.

3.9 Application of mPCR Assay Using Naturally Contaminated Food Samples

Food samples (chicken, leafy vegetables and dairy product) were purchased from various local retails. Then, 10 g of each food sample was weighed and kept at 4 ºC. Then, the food samples were homogenized with 100 ml (1:10) buffered peptone water and incubated at 37 ºC for two hours. 1 ml of each food homogenate was subjected to DNA extraction followed by PCR testing. At the same time, 1 ml of the same food homogenate was stored to be plated on selective media in case of positive detection.

3.10 Visualization of PCR Products via Agarose Gel electrophoresis

The PCR amplicons were visualized by using agarose gel electrophoresis. A 2.0 % (w/v) agarose gel stained with GelRed™ (BiotiumInc, CA, USA). The PCR products were also visualized under UV and analyzed using a GelDoc system (Biorad, CA, USA).

CHAPTER 4: RESULTS

4.1 Revival of Bacterial Cultures

A total of 80 bacterial strains were tested in the study. The list of strains is provided in Table 3.1 previously. All the 80 strains were revived from stab cultures and glycerol stocks from laboratory collection. All the strains were propagated on Luria-Betani Agar (LBA) plates overnight. However, the *Listeria* colonies that cultured on LBA were too small causing difficulty in collecting single colony for DNA extraction purpose. Thus, the *Listeria* strains were propagated on Trypticase Soy Agar (TSA) instead to allow formation of relatively larger and well-isolated colonies.

In addition, *Yersinia enterocolitica* strains took longer incubation period than the rest of the bacterial strains to produce colonies. Revival of *Yersinia enterocolitica* strains took 48 h while the others took about 12 h incubation to form visible colonies.

4.2 Development of Primers based on Selected Genes

The DNA sequences of the housekeeping gene *phoA*, as well as virulence genes *hilA, hylA, ipaH, rpoB* and *yst* corresponding to *E. coli, Salmonella enterica, Listeria monocytogenes, Shigella* sp., *Staphylococcus aureus and Yersinia enterocolitica* respectively were successfully retrieved from GenBank and aligned using BLAST program. Then, a pair of specific and compatible primers was designed successfully for each targeted pathogens as described in 3.2. The primer sequence available for *E. coli, Salmonella enterica* and *Yersinia enterocolitica* were modified from previous publication to acquire product size below 1000 bp for easier visualization using agarose gel electrophoresis. The sequence of each primer and size of PCR product is shown in Table 4.1 as follows:

Table 4.1: Primer sequences and product sizes used in this study (*m= modified).

No	Bacteria	Target Gene	Primers	Product size (bp)
1	***E. coli***	*phoA*	*mphoA-F	**903**
			*mphoA-R	
2	***Salmonella enterica***	*hilA*	*mhil-1	**784**
			*mhil-2	
3	***Listeria monocytogenes***	*hylA*	LM1	**702**
			LM2	
4	***Shigella* sp.**	*ipaH1*	ShipaH1-F	**272**
			ShipaH1-R	
5	***Staphylococcus aureus***	*rpoB*	SArpoB-1	**202**
			SArpoB-2	
6	***Yersinia enterocolitica***	*yst*	*myst 1	**145**
			*myst 2	

The details of the primer sequence are not provided in this report because of proprietary reasons.

As shown in Table 4.1, all the criteria expected in the primer length and product size were fulfilled successfully. All the expected product sizes were below 1000 bp so that a 100 bp DNA ladder can be used for further investigations in the study. All the product sizes were far enough from one another to enable visualization in 2 % agarose gel electrophoresis under UV.

In addition, *In-silico* PCR was done to check for occurrence of primer dimers or unspecific bands. The results showed that all the designed primers were specific to the corresponding targeted bacteria except the mphoA primers designed to amplify *E. coli* strains. This primer also amplified the DNA of *Shigella* spp. strains. This is due to very close evolutionary relationships between the two bacteria. However, the primer pair designed for *Shigella* spp. was highly specific to *Shigella* spp. exhibiting clear difference between *Shigella* spp. and *E. coli*. The results of In-silico for each primer pair is shown in Appendix C.

4.3 Efficiency of Crude Genomic DNA Extraction

The described boiling method to extract crude genomic DNA of bacteria was very efficient. The boiling method allowed the bacterial colonies to lyse upon boiling and eventually release the crude DNA to be used for PCR testing. The concentration of DNA extracted from representative strains are shown in Table 4.2.

Table 4.2: The DNA concentration of the representative bacterial strains.

Representative Strain	Concentration of DNA (ng/ul)
Eschericia coli (EC110)	69.2
Salmonella enterica (ATCC 13311)	98.7
Listeria monocytogenes (LM163)	91.2
Shigella sp. (ATCC 11060)	92.5
Staphylococcus aureus (TC1SA1)	73.4
*Yersinia enterocolitica (*ATCC 9610)	82.8

4.4 Specificity of Primers

To evaluate the specificity of designed primer sets, each primer pair was tested individually (monoplex PCR) to distinguish targeted bacteria from other strains. All the 80 strains were tested for specificity with each primer sets. A summary of the results is shown in Table 4.3. The specificity of each primer set of representative strains is shown in Figure 4.1 to 4.6.

Table 4.3: Summary of specificity test**.**

NO	Primer Set / Bacterial Strains	mphoA F&R	mhilA F&R	LM 1&2	hipAH 1 F&R	rpoB 1&2	myst F&R
1	*E. coli* FE138EC4EC109	+					
2	*E. coli* P141E4EC110	+					
3	*E. coli* P141EC2EC111	+					
4	*E. coli* P136EC4EC101	+					

Table 4.3, continued.

NO	Primer Set / Bacterial Strains	mphoA F&R	mhilA F&R	LM 1&2	hipAH1 F&R	rpoB 1&2	myst F&R
5	*E. coli* V137EC1EC102	+					
6	*E. coli* EC0157	+					
7	*E. coli* ATCC25923	+					
8	*E. coli* V137EC3EC104	+					
9	*E. coli* FE138EC3EC108	+					
10	*E. coli* FE138EC1EC106	+					
11	*E. coli* J144EC3EC144	+					
12	*E. coli* FE138EC4EC109	+					
13	*Salmonella enterica* Typhii H22i		+				
14	*Salmonella enterica* Enteriditis MOB 2054/05		+				
15	*Salmonella enterica* Albany MOB 1549/05		+				
16	*Salmonella enterica* Paratyphi A 3/2/04		+				
17	*Salmonella enterica* Typhimurium ATCC13311		+				
18	*Salmonella enterica* ATCC9251		+				
19	*Salmonella enterica* Sal 1/9/02		+				
20	*Salmonella enterica* Sal 2/9/02		+				
21	*Salmonella enterica* ATCC6539		+				
22	*Salmonella enterica* ATCC13070		+				

Table 4.3, continued.

NO	Primer Set / Bacterial Strains	mphoA F&R	mhilA F&R	LM 1&2	hipAH1 F&R	rpoB 1&2	myst F&R
23	*Salmonella enterica* STM071		+				
24	*Salmonella enterica* STM048		+				
25	*Salmonella enterica* SEH12		+				
26	*Salmonella enterica* Sal 9/05		+				
27	*Listeria monocytogenes* LM15			+			
28	*Listeria monocytogenes* LM31			+			
29	*Listeria monocytogenes* LM34			+			
30	*Listeria monocytogenes* LM44			+			
31	*Listeria monocytogenes* LM50			+			
32	*Listeria monocytogenes* LM60			+			
33	*Listeria monocytogenes* LM150			+			
34	*Listeria monocytogenes* LM161			+			
35	*Listeria monocytogenes* LM162			+			
36	*Listeria monocytogenes* LM163			+			
37	*Listeria monocytogenes* LM164			+			
38	*Listeria monocytogenes* LM177			+			

Table 4.3, continued.

NO	Primer Set / Bacterial Strains	mphoA F&R	mhilA F&R	LM 1&2	hipAH1 F&R	rpoB 1&2	myst F&R
39	*Listeria monocytogenes* LM171			+			
40	*Listeria monocytogenes* LM191			+			
41	*Listeria monocytogenes* LM192			+			
42	*Listeria monocytogenes* LM197			+			
43	*Listeria monocytogenes* LM85			+			
44	*Listeria monocytogenes* LM178			+			
45	*Listeria monocytogenes* LM186			+			
46	*Shigella flexneri* 2a TH10/07	+			+		
47	*Shigella sonnei* TC2/97	+			+		
48	*Shigella flexneri* 2a TH6/01	+			+		
49	*Shigella flexneri* 2a TH6/07	+			+		
50	*Shigella flexneri* 3a TH5/09	+			+		
51	*Shigella sonnei* ATCC11060	+			+		
52	*Shigella sonnei* TH20/97	+			+		
53	*Shigella sonnei* TH3/01	+			+		
54	*Shigella sonnei* TH3/00	+			+		
55	*Shigella sonnei* TH1300	+			+		
56	*Shigella sonnei* TH5/00	+			+		
57	*Shigella sonnei* TH4/00	+			+		

Table 4.3, continued.

NO	Primer Set / Bacterial Strains	mphoA F&R	mhilA F&R	LM 1&2	hipAH1 F&R	rpoB 1&2	myst F&R
58	*Shigella sonnei* TH2/00	+			+		
59	*Shigella dysentrise* TH26/98	+			+		
60	*Staphylococcus aureus* CB37SA1					+	
61	*Staphylococcus aureus* IK9SA1					+	
62	*Staphylococcus aureus* ILI29SA1					+	
63	*Staphylococcus aureus* TC29SA3					+	
64	*Staphylococcus aureus* FH5SA2					+	
65	*Staphylococcus aureus* FH81SA3					+	
66	*Staphylococcus aureus* FH68SA1					+	
67	*Staphylococcus aureus* TCSA2					+	
68	*Staphylococcus aureus* FH1SA1					+	
69	*Staphylococcus aureus* FH62SA1					+	
70	*Yersinia enterocolitica* ATCC9610						+
71	*Yersinia enterocolitica* PCM3K42318						+
72	*Yersinia enterocolitica* PCM3K13						+
73	*Yersinia enterocolitica* PCM3K12						+
74	*Yersinia enterocolitica* PCM1K52418						+
75	*Yersinia enterocolitica* PCM1K4						+
76	*Yersinia enterocolitica* PCM1K1						+

Table 4.3, continued.

NO	Primer Set / Bacterial Strains	mphoA F&R	mhilA F&R	LM 1&2	hipAH1 F&R	rpoB 1&2	myst F&R
77	*Yersinia enterocolitica* PCM1K5						+
78	*Yersinia enterocolitica* PCM1K12						+
79	*Yersinia enterocolitica* PCM1K13						+
80	*Yersinia enterocolitica* a-C-04						+

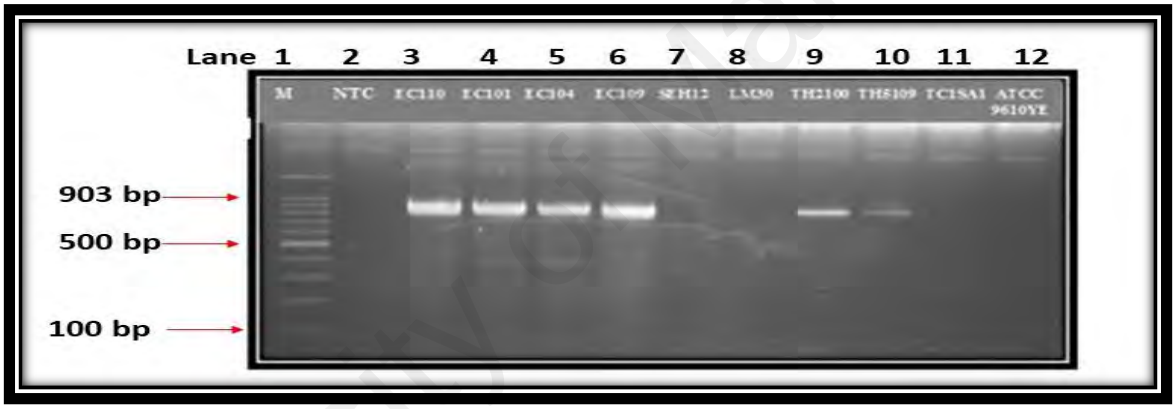


Figure 4.1: Specificity test of mphoA primers for to *E. coli* (903 bp) (Lane 1: 100 bp DNA ladder, Lane 2: negative control, Lanes 3-6: different *E. coli* strains & Lanes 7-12: different non-*E. coli* strains such as *Salmonella enterica*, *Listeria monocytogenes*, *Shigella* spp., *Staphylococcus aureus* and *Yersinia enterocolitica* respectively)

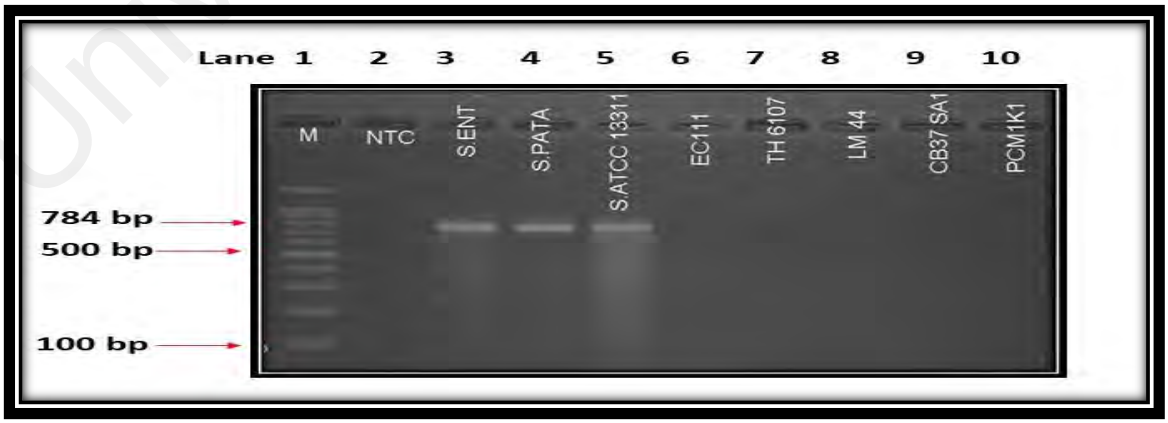


Figure 4.2: Specificity test of mhilA primers for *Salmonella enterica* (784 bp) (Lane 1: 100 bp DNA ladder, Lane 2: negative control, Lanes 3-5: different *Salmonella enterica* strains & Lanes 6-10: non-*Salmonella* strains such as *E. coli*, *Shigella* spp., *Listeria monocytogenes*, *Staphylococcus aureus* and *Yersinia enterocolitica* respectively)

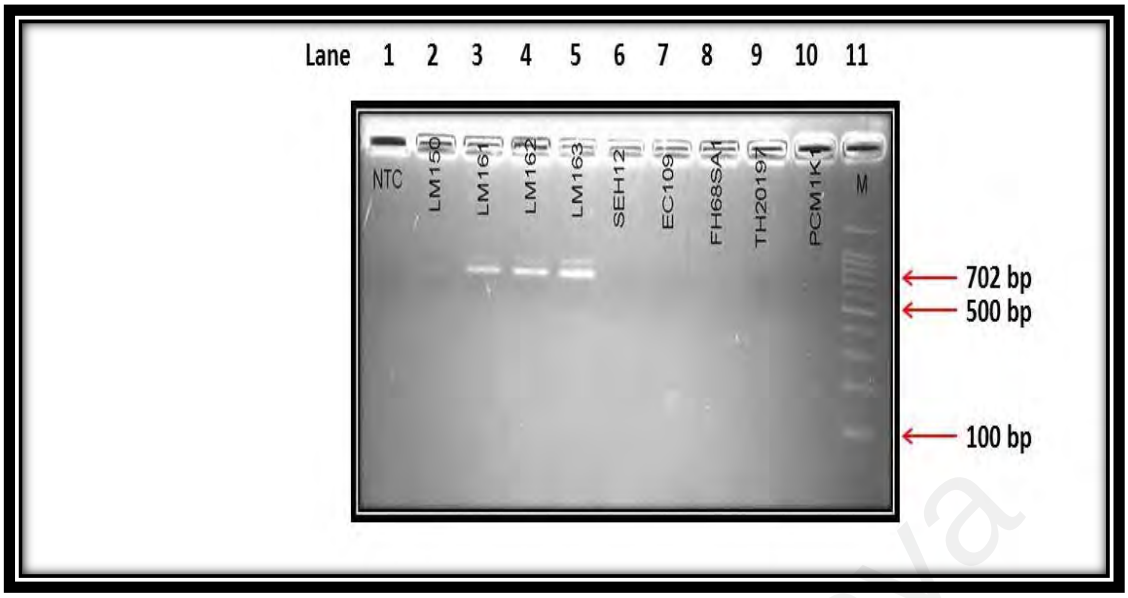


Figure 4.3: Specificity test of LM primers for *Listeria monocytogenes* (702 bp) (Lane 1: negative control, Lanes 2-5: different *Listeria monocytogenes* strains, Lane 6-10: non-*Listeria monocytogene* strains such as *Salmonella enterica*, *E. coli*, *Staphylococcus aureus*, *Shigella* spp. and *Yersinia enterocolitica* respectively & Lane 11: 100 bp DNA ladder)

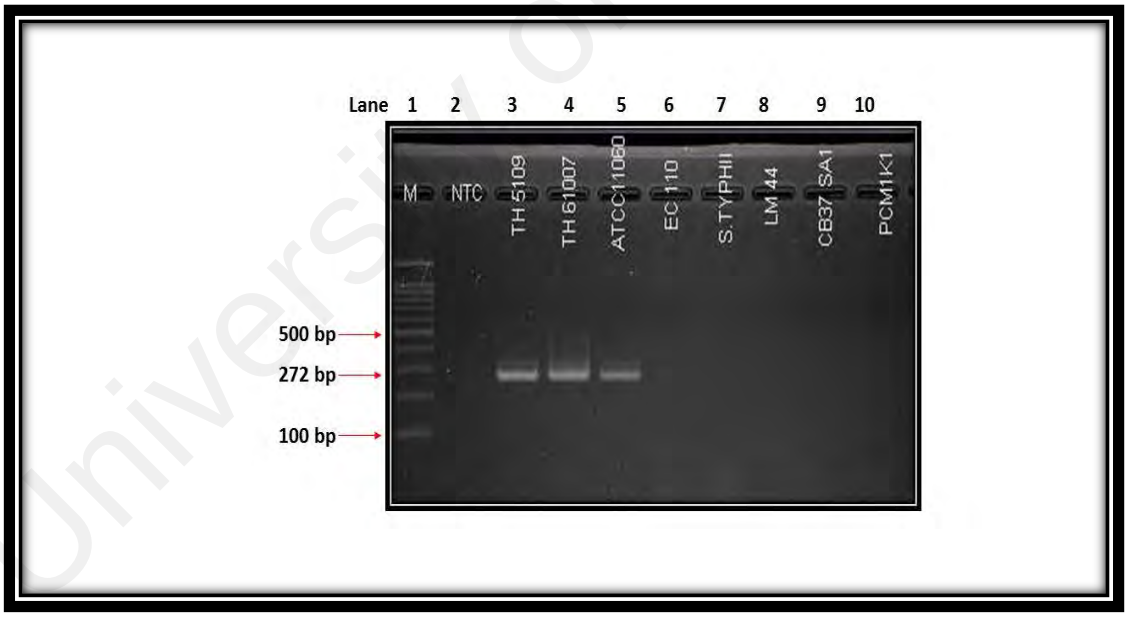


Figure 4.4: Specificity test of ipaH primers for *Shigella* spp. (272 bp) (Lane 1: 100 bp DNA ladder, Lane 2: negative control, Lanes 3-5: different *Shigella* spp. strains & Lanes 6-10: non-*Shigella* spp. strains such as *E. coli*, *Salmonella enterica*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Yersinia enterocolitica* respectively)

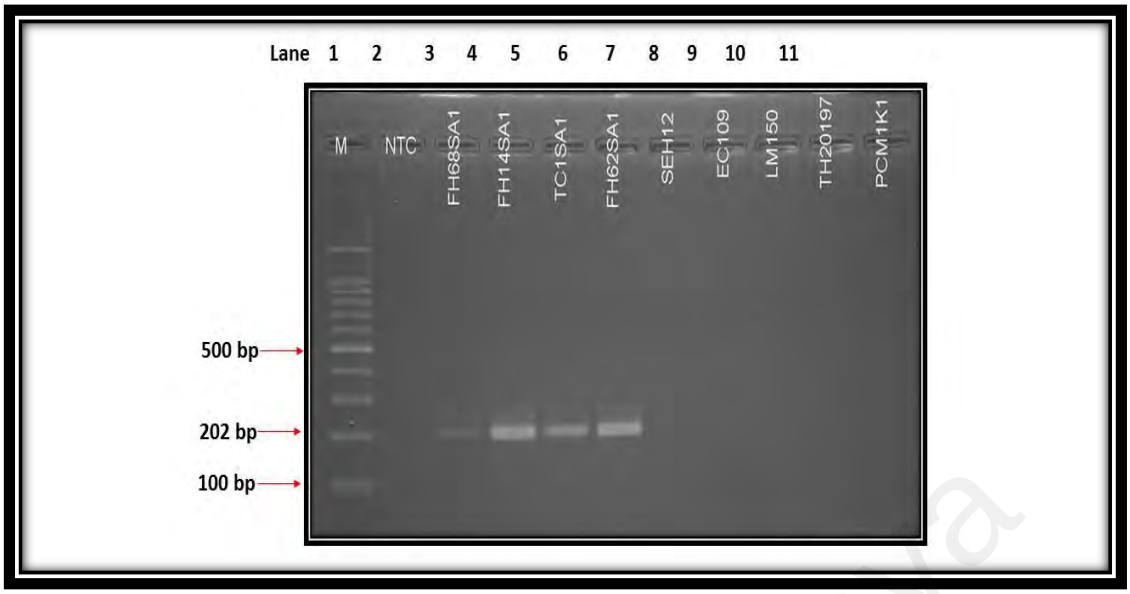


Figure 4.5: Specificity test of rpoB primers for *Staphylococcus aureus* (202 bp) (Lane 1: 100bp DNA ladder, Lane 2: negative control, Lanes 3-6: different *Staphylococcus aureus* strains & Lanes 7-11: non-*Sta*phylococcus *aureus* strains such as *Salmonella enterica*, *E. coli*, *Listeria monocytogenes*, *Shigella* spp., and *Yersinia enterocolitica* respectively)

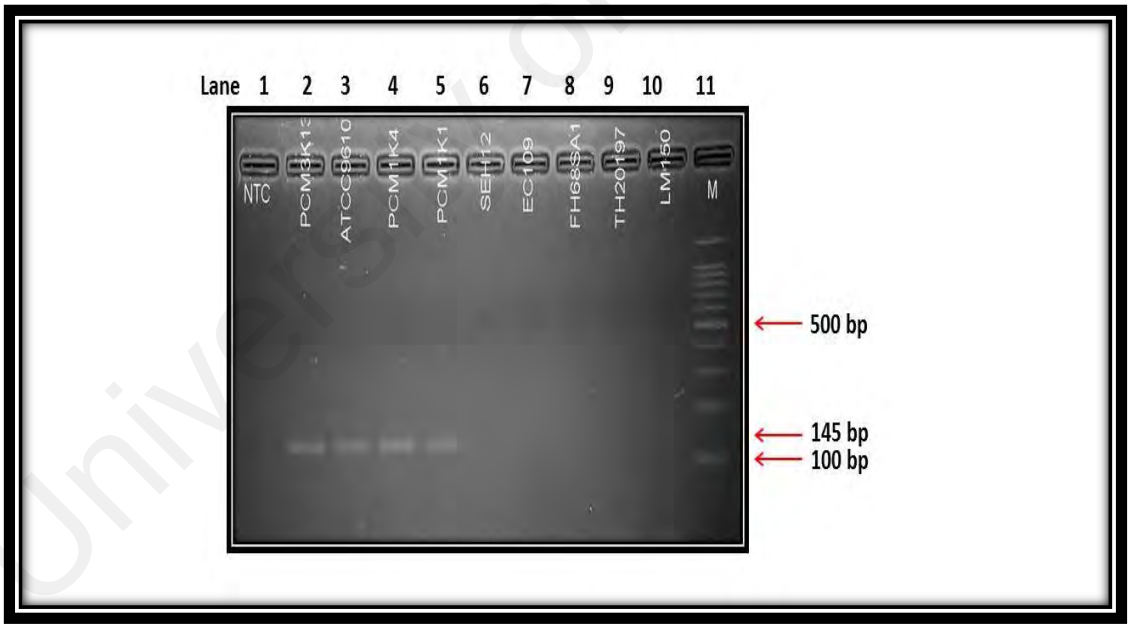


Figure 4.6: Specificity test of myst primers specific to *Yersinia enterocolitica* (145 bp) (Lane 1: negative control, Lanes 2-5: different *Yersinia enterocolitica* strains, Lanes 6-10: non-*Yersinia enterocolitica* strains such as *Salmonella enterica*, *E. coli*, *Staphylococcus aureus*, *Shigella* spp. and *Listeria monocytogenes* respectively & Lane 11: 100 bp DNA ladder)

The specificity test results were congruent to the results of *in-silico* PCR. The specificity test (Figure 4.1 to Figure 4.6) showed that every primer set is specific to the respective targeted foodborne pathogen except the modified phoA (mphoA) primer sets. The mphoA primer sets which amplified *E. coli* also amplified *Shigella* sp. strains. All *Shigella* strains produces band at 903 bp when tested with mphoA primers. However, *Shigella* spp. and *E.coli* are clearly distinguishable once subjected to multiplex PCR at which *Shigella* spp. produced two bands (903 bp and 272 bp) while *E.coli* produced only one band at 903 bp.

4.5 Validity of PCR Products

Representative PCR products of each target gene were purified and sequenced to confirm the identity of the sequences and targets. This was to make sure that the PCR product formed at particular band size was the expected pathogen. The sequencing results (Appendix D) showed that all selected PCR product were valid and belonged to the target pathogen's DNA. Thus, the designed primers and PCR conditions for amplification were verified. A summary of the BLAST results of the sequences are shown in Table 4.5. Screenshots of the BLAST results are shown in Appendix E.

Table 4.4: Summary of BLAST results.

No	Expected Pathogen	ID Of Representative Strain	Primer Pair Used	Percentage of Identity (%)
1	***E. coli***	EC110	mphoA Forward and Reverse	86
2	***Salmonella enterica***	ATCC13076	mhilA Forward and Reverse	99
3	***Listeria monocytogenes***	LM50	LM1 and LM2	99
4	***Shigella spp.***	TH10107	shipaH1 Forward and Reverse	100
5	***Staphylococcus aureus***	FH62SA1	sarpoB1 and sarpoB2	98
6	***Yersinia enteroclitica***	ATCC9610	myst Forward and Reverse	100

4.6 Multiplex Polymerase Chain Reaction (mPCR) Optimization

For optimization of mPCR, two parameters were taken into consideration namely the annealing temperature and primer concentration. For optimization of annealing temperature, a gradient PCR was set up for each targeted pathogen at different annealing temperature to observe difference in intensity of bands formed. The summary of gradient PCR to optimize annealing temperature is shown in Table 4.5. The results of gradient PCR are shown in figure 4.7 to figure 4.12.

Table 4.5: Summary of gradient PCR results in determining the optimal annealing temperature for multiplexing.

Representative species	**primers**	**51.5 °C**	**53.4 °C**	**55.8 °C**	**58.3 °C**	**61 °C**	**63.7 °C**	**66.1 °C**
Escherichia coli	mphoA		√	√	√			
Listeria monocytogene	LM1 LM2		√	√	√			
Salmonella enterica	mhilA	√	√	√	√	√	√	√
Shigella spp.	hipA H1	√	√	√	√	√		
Staphylococcus aureus	SArpoB	√	√	√	√			
Yersinia enterocolitica	yst	√	√	√	√			

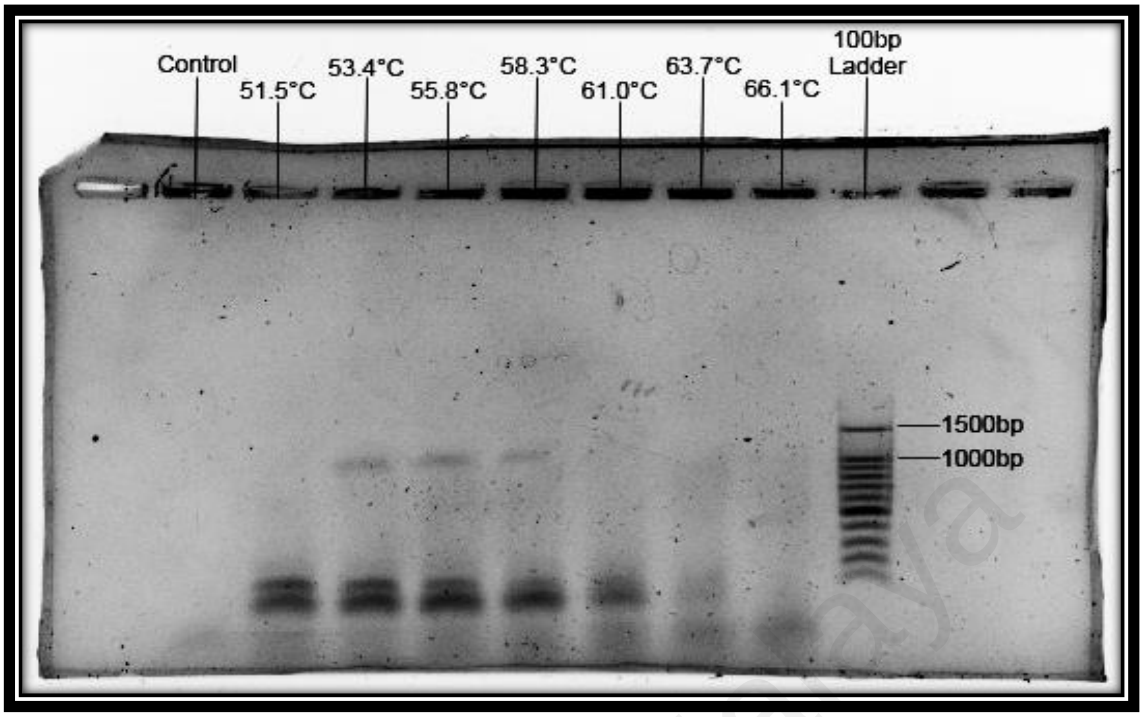


Figure 4.7: Results of gradient PCR using mphoA primers show that *E. coli* bands (903 bp) were produced at annealing temperature of 53.4 ºC, 55.8 ºC and 58.3 ºC

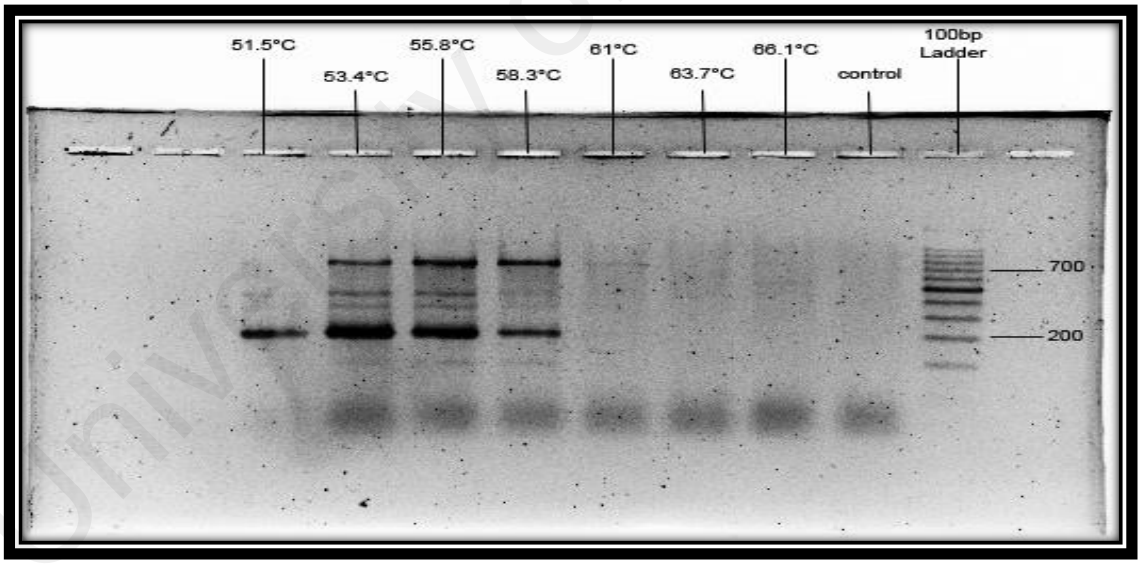


Figure 4.8: Results of gradient PCR using LM primers show that *Listeria monocytogenes* bands (702 bp) were produced at 53.4 ºC, 55.8 ºC and 58.3 ºC. There are also unspecific bands produced at 200 bp

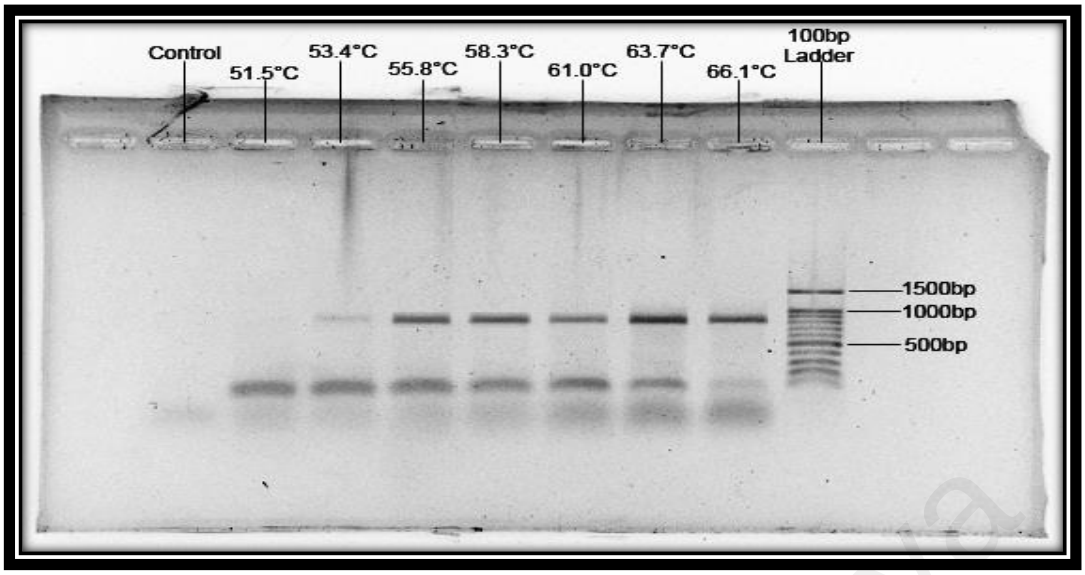


Figure 4.9: Results of gradient PCR using mhilA primers show that *Salmonella enterica* bands (784 bp) were produced at all annealing temperature except 51.5 ºC

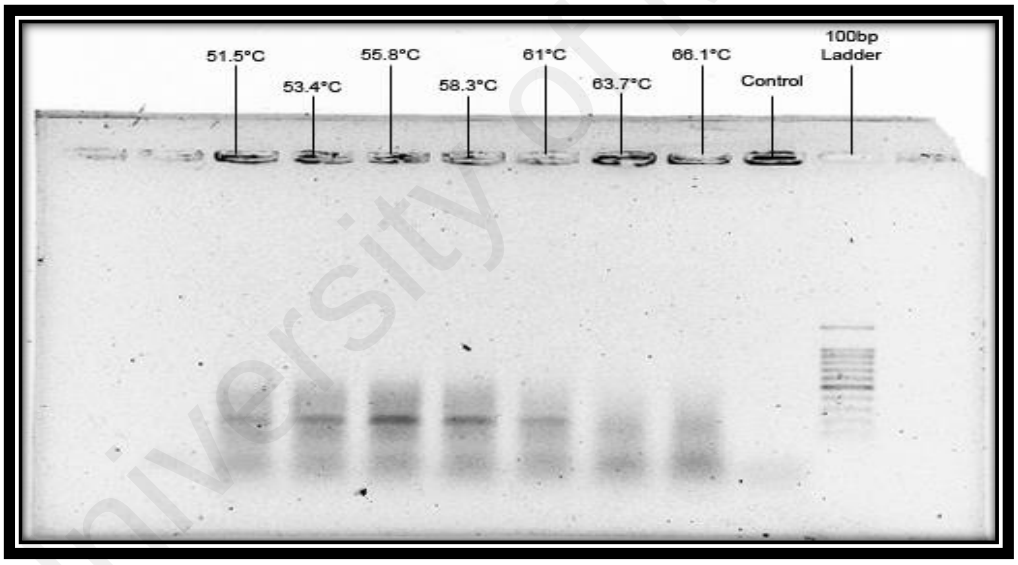


Figure 4.10: Results of gradient PCR using ipaH1 primers show that *Shigella* spp. bands (272 bp) were produced at annealing temperature of 51.5 ºC, 53.4 ºC, 55.8 ºC and 58.3 ºC

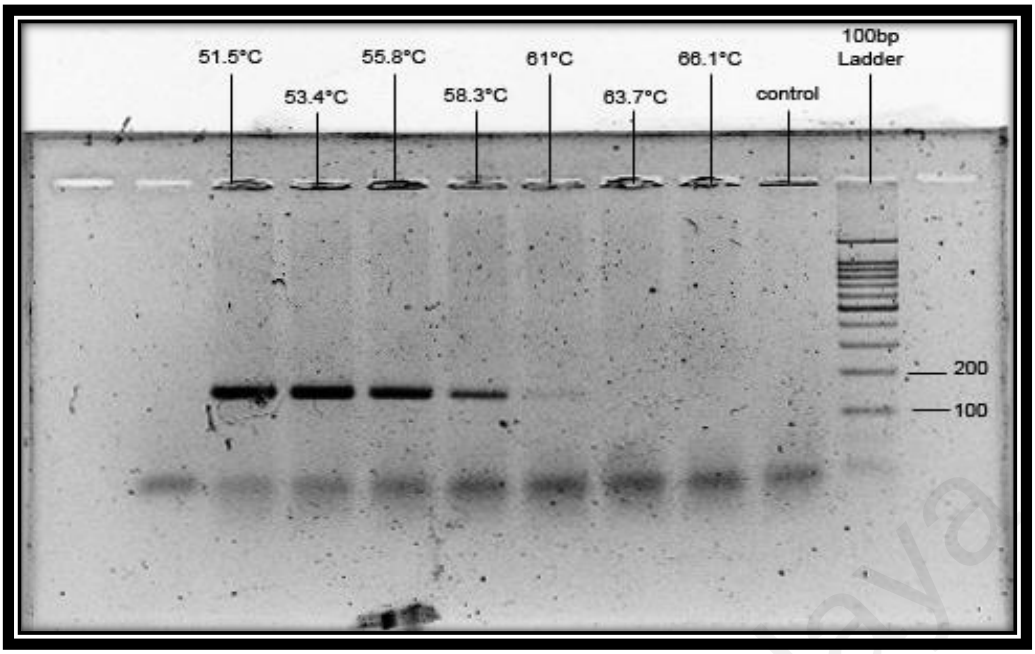


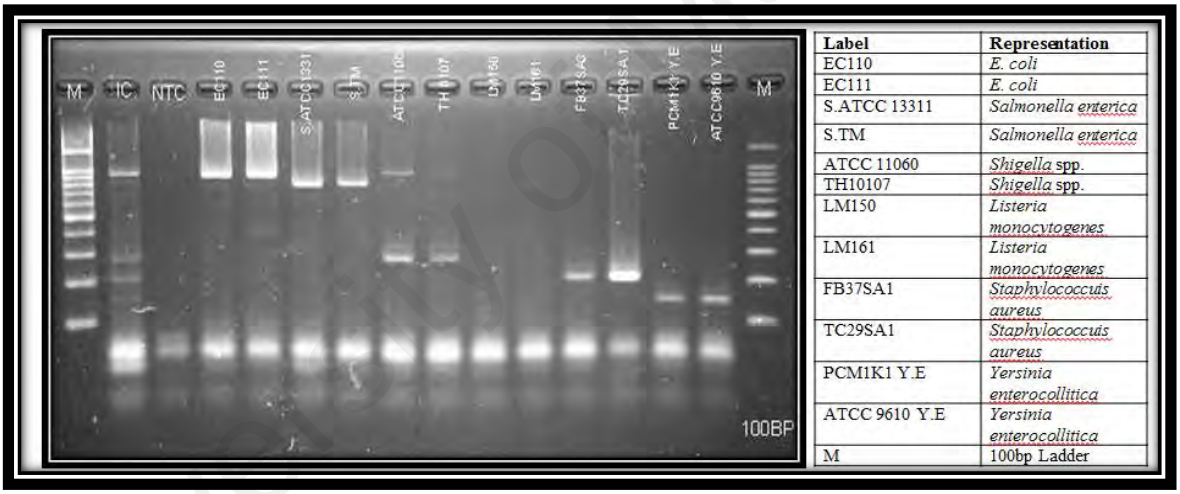
Figure 4.12: Results of gradient PCR using that myst primers show that *Yersinia enterocolitica* bands (145 bp) were formed at annealing temperature of 51.5 ºC, 53.4 ºC, 55.8 ºC and 58.3 ºC

From the Figures 4.7 to 4.12, it is clearly shown that all the six bacteria strains produced bands at annealing temperature of 53.4 ºC, 55.8 ºC and 58.3 ºC. However, highly intense bands were formed at 55.8 ºC. Thus, 55.8 ºC was rounded off to 56 ºC to be used as the annealing temperature for the subsequent experiments. However, *Listeria monocytogenes* showed formation of unspecific bands at 200 bp instead of 702 bp. This can be reduced by reducing its primer concentration.

Secondly, multiplex PCR was done by mixing all the six pairs of primers together in one reaction mixture to optimize concentration of each primer pair. Several combinations of primer concentrations were tried until well resolved bands were obtained for every pathogen. The summary of different combinations is shown in Table 4.6. The results of each primer concentration combination are shown in Figure 4.13 to Figure 4.15.

Table 4.6: Summary of optimization of primer concentration for multiplex PCR.

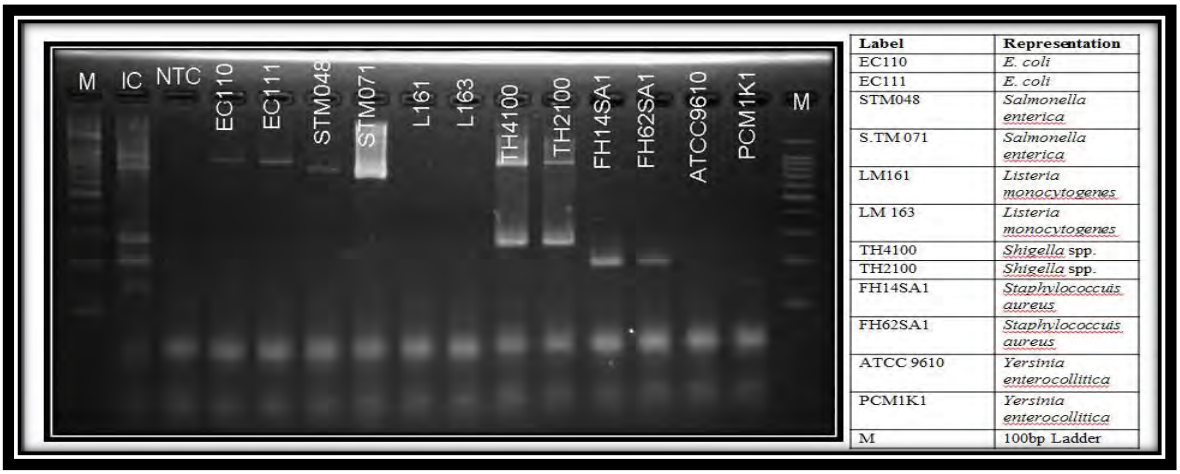
Primer Pair	Concentration in Combination 1 (µM)	Concentration in Combination 2 (µM)	Concentration in Combination 3 (µM)
mphoA F&R	0.20	0.10	0.10
mhilA F&R	0.20	0.10	0.10
shipaH1 F&R	0.20	0.10	0.10
Myst 1&2	0.20	0.10	0.10
LM 1&2	0.32	0.16	0.30
SArpoB 1&2	0.40	0.20	0.10
Comments:	All bands formed for every respective pathogen except for *Listeria*. The primer dimer was too intense.	Primer dimer reduced as the primer concentration halved. Bands for *Listeria* still did not amplify.	The primer pair corresponding to *Listeria* increased to three times the other primers. Primer concentration for Multiplex PCR was well-optimized.



Label	Representation
EC110	*E. coli*
EC111	*E. coli*
S.ATCC 13311	*Salmonella enterica*
S.TM	*Salmonella enterica*
ATCC 11060	*Shigella* spp.
TH10107	*Shigella* spp.
LM150	*Listeria monocytogenes*
LM161	*Listeria monocytogenes*
FB37SA1	*Staphylococcuis aureus*
TC29SA1	*Staphylococcuis aureus*
PCM1K1 Y.E	*Yersinia enterocollitica*
ATCC 9610 Y.E	*Yersinia enterocollitica*
M	100bp Ladder

Figure 4.13: Optimization of all the primer concentration in a multiplex PCR. The concentrations of all the primers are indicated in Table 4.6 (Combination 1). No amplifications were obtained for *Listeria monocytogenes*. Highly intense primer dimers were observed.

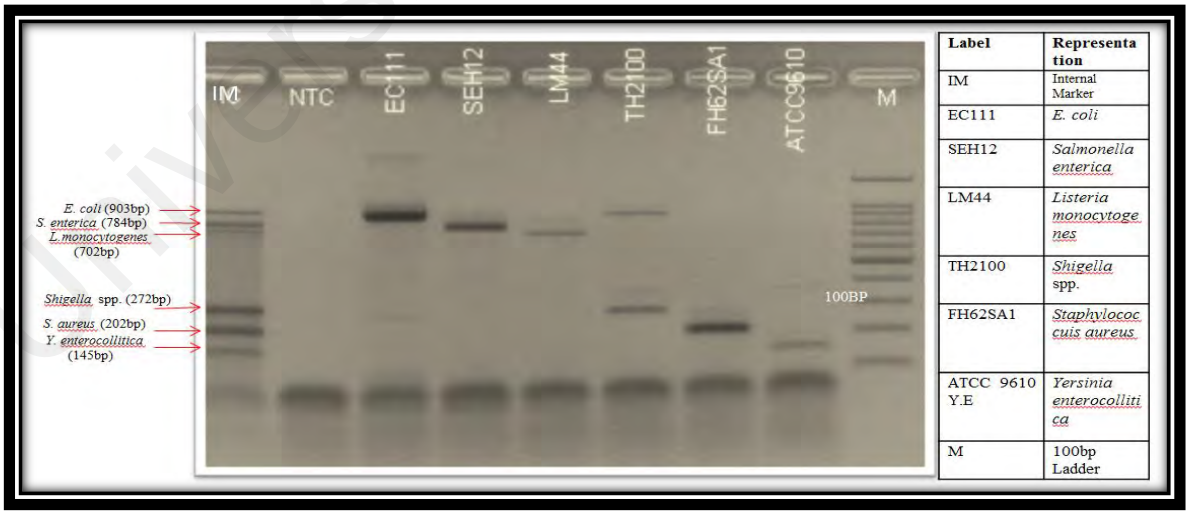
Since the primer dimers were too intense as shown in Figure 4.13, the concentration of each primer was reduced to half in the second combination. The results of second combination of primer concentration for optimization of multiplex PCR are shown in Figure 4.14 as follows.



Label	Representation
EC110	*E. coli*
EC111	*E. coli*
STM048	*Salmonella enterica*
S.TM 071	*Salmonella enterica*
LM161	*Listeria monocytogenes*
LM 163	*Listeria monocytogenes*
TH4100	*Shigella* spp.
TH2100	*Shigella* spp.
FH14SA1	*Staphylococcuis aureus*
FH62SA1	*Staphylococcuis aureus*
ATCC 9610	*Yersinia enterocollitica*
PCM1K1	*Yersinia enterocollitica*
M	100bp Ladder

Figure 4.14: Optimization of all the primer concentration in a multiplex PCR. The concentrations of all the primers are indicated in Table 4.6 (Combination 2). No amplifications were obtained for *Listeria monocytogenes* DNA. A reduction of primer dimers' intensity was observed.

As shown in Figure 4.14, the intensity of the primer dimers was successfully reduced in second combination of primer concentration for multiplex PCR optimization. However, the DNA of *Listeria monocytogenes* strains were not amplified. Eventually, the primer concentration of LM primers was increased to allow amplification of *Listeria monocytogenes* DNA as shown in Figure 4.15.



Label	Representation
IM	Internal Marker
EC111	*E. coli*
SEH12	*Salmonella enterica*
LM44	*Listeria monocytogenes*
TH2100	*Shigella* spp.
FH62SA1	*Staphylococcuis aureus*
ATCC 9610 Y.E	*Yersinia enterocolliti ca*
M	100bp Ladder

Figure 4.15: Optimization of all the primer concentration in a multiplex PCR. The concentrations of all the primers are indicated in Table 4.6 (Combination 3). Amplification of DNA of all the six bacteria was observed especially *Listeria monocytogenes*.

Thus, the third primer concentration combination is the optimum primer combination of multiplex PCR to allow amplification of all the targeted pathogens' DNA. Overall, from the optimization, the Mg2+ concentration at 2.25 μM was most effective in producing high yields of all six target genes. The primer combination found to be optimal for multiplex PCR at concentration of 0.1 μM for each yst, ipaH, mhilA, mphoA and rpoB and 0.30 μM for LM primers. Maximal band intensities of each amplicon were found at 56 °C of annealing temperature. The cycling condition for the mPCR was similar to that of monoplex PCR as previously described.

4.7 Sensitivity Evaluation of mPCR Assay Using Bacterial Species Strains

To evaluate the limit of detection or sensitivity level of the mPCR developed, a cell suspension equivalent to 10^8 CFU/ml was serially dilutedand then DNA was extracted from each diluted bacterial suspension. Crude genomic DNA was tested with developed mPCR assay. From the evaluation, the mPCR assay recorded a detection limit of 10^1 CFU/ml for detection of *E. coli, Listeria and Shigella*; 10^2 CFU/ml for *Salmonella* and *Yersinia* and 10^4 CFU/ml for *Staphylococcus aureus*. Hence, the multiplex assay developed in this study was effective for the simultaneous detection of targeted pathogens up to 10^4 CFU/ml. The representative image of each targeted pathogen is shown as follows from Figure 4.16 to Figure 4.21.

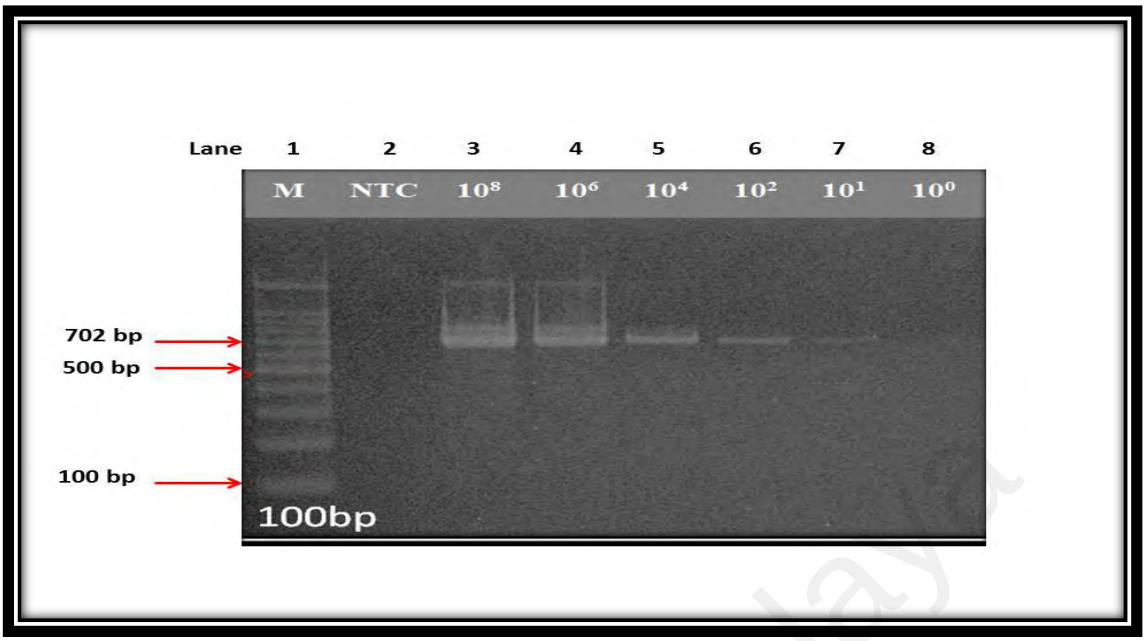


Figure 4.18: Detection limit of mPCR for *Listeria monocytogenes* (702 bp). (Lane 1: 100 bp DNA ladder, Lane 2: negative control, Lanes 3-8: limit of detection; 10^8 CFU/ml, 10^6 CFU/ml, 10^4 CFU/ml, 10^2 CFU/ml, 10^1 CFU/ml and 10^0 CFU/ml respectively)

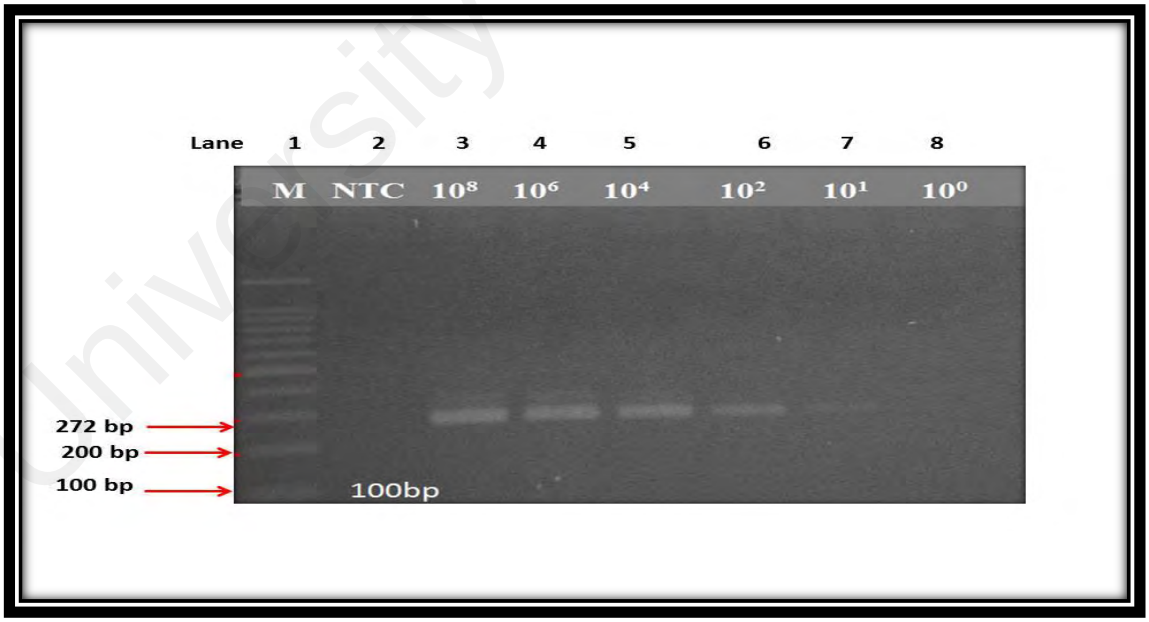


Figure 4.19: Detection limit of mPCR for *Shigella* spp. (272 bp). (Lane 1: 100 bp DNA ladder, Lane 2: negative control, Lanes 3-8: limit of detection; 10^8 CFU/ml, 10^6 CFU/ml, 10^4 CFU/ml, 10^2 CFU/ml, 10^1 CFU/ml and 10^0 CFU/ml respectively)

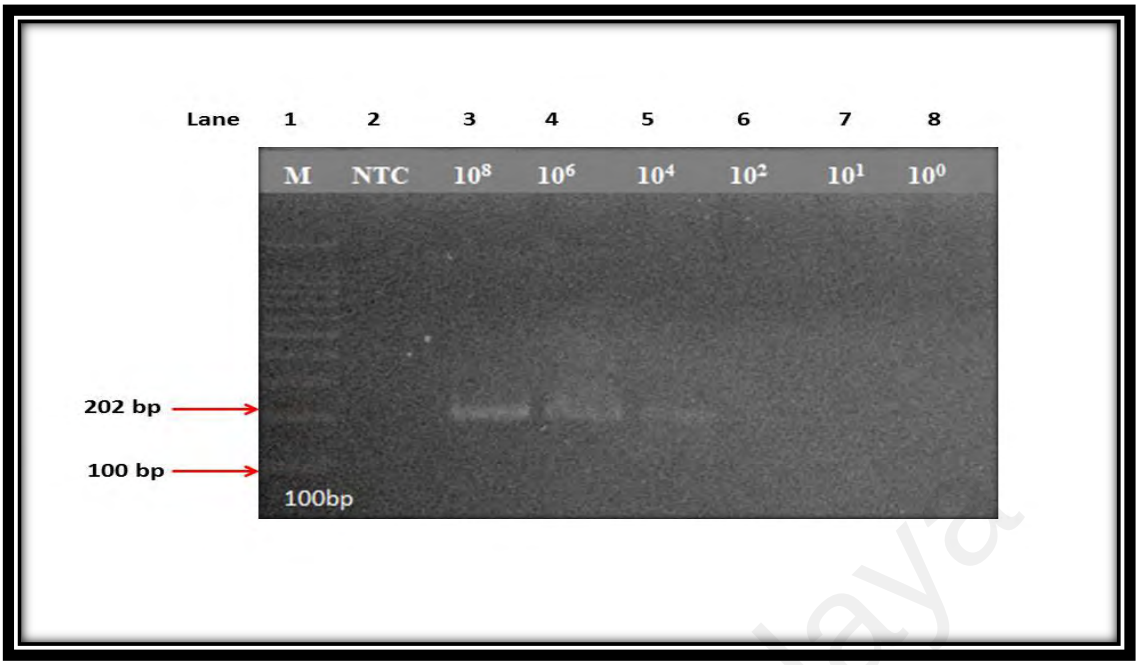


Figure 4.20: Detection limit of mPCR for *Staphylococcus aureus*. (202 bp). (Lane 1: 100 bp DNA ladder, Lane 2: negative control, Lanes 3-8: limit of detection; 10^8 CFU/ml, 10^6 CFU/ml, 10^4 CFU/ml, 10^2 CFU/ml, 10^1 CFU/ml and 10^0 CFU/ml respectively)

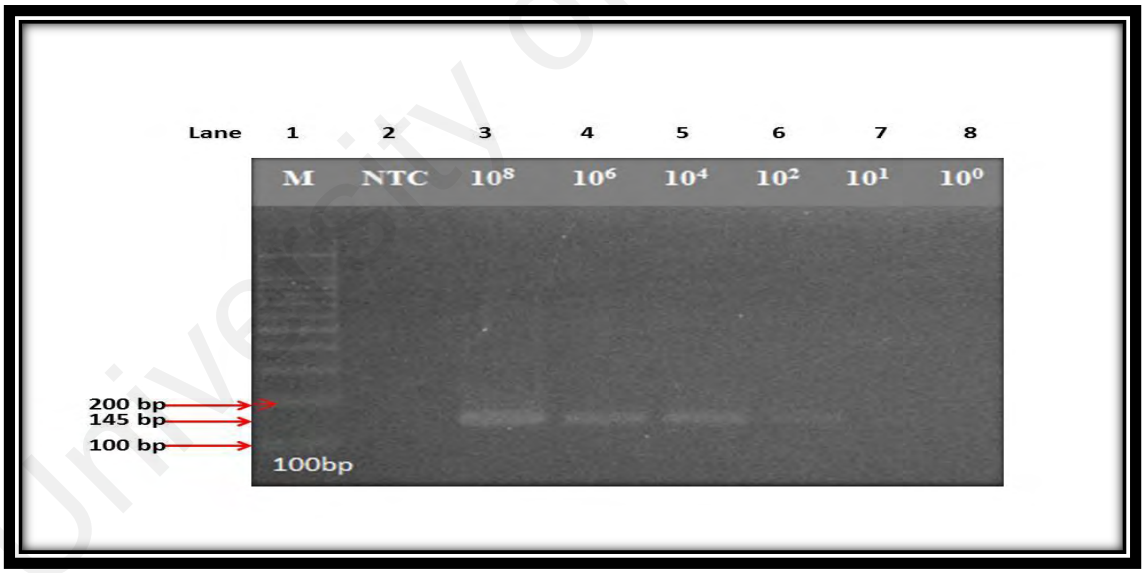


Figure 4.21: Detection limit of mPCR for *Yersinia enterocolitica*. (145bp). (From right: Lane 1 representing 100bp ladder; Lane 2 representing negative control, Lane 3-8 representing limit of detection; 10^8 CFU/ml, 10^6 CFU/ml, 10^4 CFU/ml, 10^2 CFU/ml, 10^1 CFU/ml and 10^0 CFU/ml respectively)

Table 4.7: Summary of sensitivity evaluation using different bacterial species strains.

Foodborne pathogen	Detection Limit in Known Bacterial Strains (CFU/ml)
E. coli	10^1
Salmonella enterica	10^2
Listeria monocytogenes	10^1
Shigella spp.	10^1
Staphylococcus aureus	10^4
Yersinia enterocolitica	10^2
Simultaneous Detection Level: **10^4 CFU/ml**	

4.8 Application of mPCR Assay Using Artificially Contaminated Food Samples

The developed mPCR was evaluated for practical use by testing with artificially contaminated food samples. The artificially contaminated food samples were pre-enriched with BPW and incubated for 2 hours before subjected to serial dilution and DNA extraction. The mPCR successfully detected all the targeted pathogens from artificially contaminated food samples. The individual detection limit of *E. coli* was 10^1 CFU/ml; *Salmonella*, *Listeria* and *Shigella* was 10^2 CFU/ml. The simultaneous detection limit of all the six pathogens was 10^6 CFU/ml. Summary of the result for application of mPCR using artificially contaminated food samples is shown in Table 4.8. The representative image of the results obtained is shown in Figure 4.22.

Table 4.8: Summary of sensitivity limit in artificially contaminated food sample.

Foodborne pathogen	Detection Limit in Known Bacterial Strains (CFU/ml)
E. coli	10^1
***Salmonella* sp.**	10^2
Listeria monocytogenes	10^2
***Shigella* spp.**	10^2
Staphylococcus aureus	10^6
Yersinia enterocolitica	10^6
Simultaneous Detection Level: **10^6 CFU/ml**	

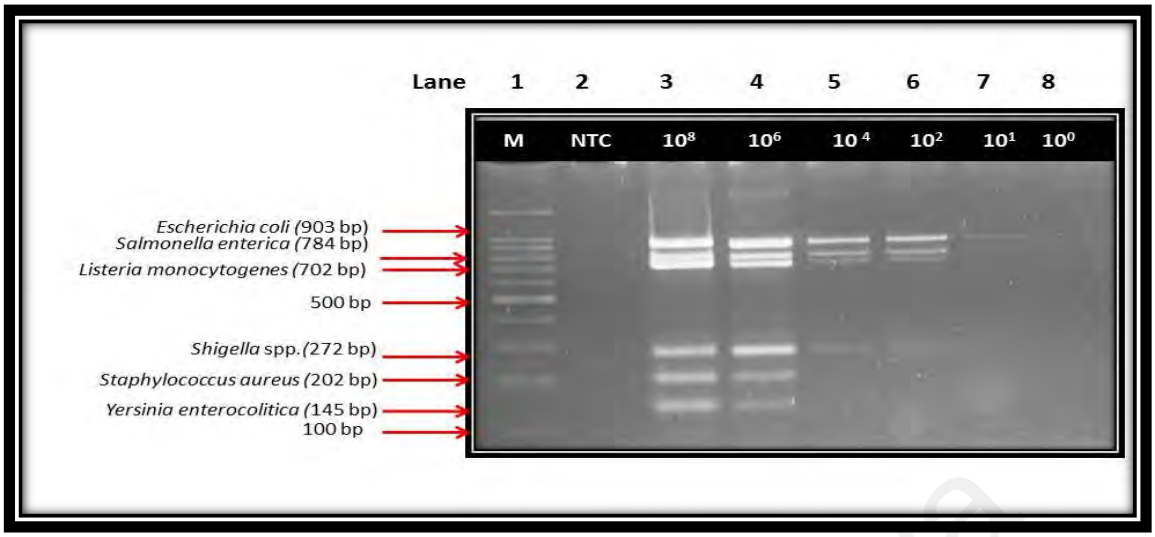


Figure 4.22: The detection Limit of mPCR when tested with artificially contaminated chicken sample. It represents *E. coli* (903 bp), *Salmonella enterica* (784 bp), *Listeria monocytogenes* (702 bp), *Shigella* spp. (272 bp), *Staphylococcus aureus* (202 bp) and *Yersinia enterocolitica* (145 bp) respectively. (Lane 1: 100 bp DNA ladder, Lane 2: negative control, Lanes 3-8: limit of detection; 10^8 CFU/ml, 10^6 CFU/ml, 10^4 CFU/ml, 10^2 CFU/ml, 10^1 CFU/ml and 10^0 CFU/ml respectively)

4.9 Application of mPCR Using Naturally Contaminated Food Samples

The developed mPCR was evaluated for practical use by testing with naturally contaminated food samples. In total 54 food samples were tested namely juice (n=1), ground beef (n=2), ground pork (n=2), tuna (n=3), cheese (n=6), egg (n=9), chicken (n=10), milk (n=10) and vegetables (n=11). The summary of number of naturally contaminated foods tested is shown in Figure 4.23.

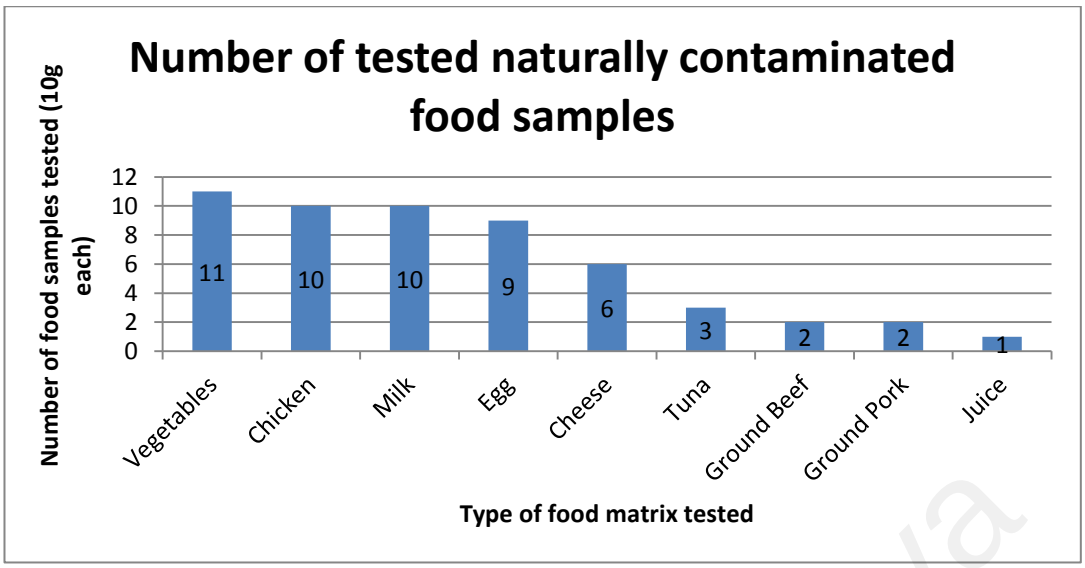


Figure 4.23: Number of tested naturally contaminated food samples representing number of each type of food matrix tested.

The food samples were pre-enriched with BPW for 2 hours followed by extraction. The mPCR testing showed 33 out of 54 tested food samples (61 %) were positive for either one or more pathogen present. The detection results are shown in Figure 4.24.

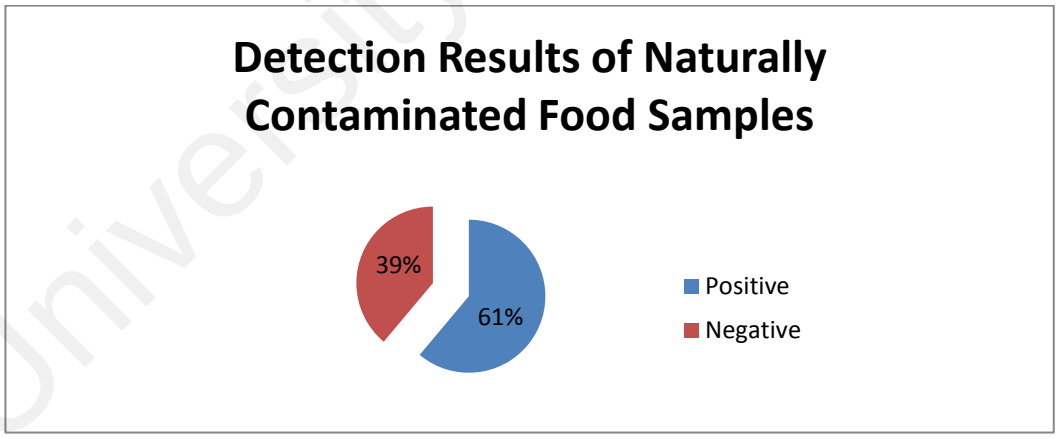


Figure 4.24: Detection Results of Naturally Contaminated Food Samples represents that 61% (33 of 54) of tested samples were produced positive results.

Specifically, *E. coli* DNA was detected in 15 samples, *Staphylococcus aureus* DNA was detected in 13 samples, *Salmonella enterica* DNA was detected in 12 samples and *Listeria monocytogenes* DNA was detected in 2 samples. Notably, none of the tested food samples showed positive results for *Yersinia enterocolitica* and *Shigella* sp. Notably, 6 food harboured more than one pathogen. The positive food homogenates were then propagated on selective media on the same day of test. All the results of selective enrichment for positive food samples were congruent to mPCR results.

The detection result of each food sample is shown in Table 4.9 while the representative gel images of tested natural food samples are shown in Figure 4.25 to Figure 4.28.

Table 4.9: Detection results of naturally contaminated food samples

Food Sample ID	Food matrix tested	Pathogen detected
F1	Cheese 1	*E. coli*
F2	Vegetable 1	Negative
F3	Vegetable 2	*Staphylococcus aureus*
F4	Ground beef 1	*E. coli*
F5	Chicken 1	*Salmonella enterica*
F6	Milk 1	*E. coli, Staphylococcus aureus*
F7	Milk 2	Negative
F8	Milk 3	Negative
F9	Egg 1	*Salmonella enterica*
F10	Egg 2	*Salmonellla enterica*
F11	Chicken 2	Negative
F12	Chicken 3	Negative
F13	Chicken 4	Negative
F14	Cheese 2	*E. coli*
F15	Cheese 3	*E. coli*
F16	Milk 4	*E. coli*
F17	Milk 5	Negative
F18	Vegetable 3	*Staphylococcus aurues*
F19	Vegetable 4	Negative
F20	Juice 1	*E. coli*
F21	Ground pork 1	Negative
F22	Ground pork 2	Negative
F23	Tuna 1	*Staphylococcus aureus*
F24	Tuna 2	Negative
F25	Milk 6	*Staphylococcus aureus*
F26	Vegetable 5	*Staphylococcus aureus*
F27	Vegetable 6	Negative

Table 4.9, continued.

Food Sample ID	Food matrix tested	Pathogen detected
F28	Chicken 5	*Salmonella eneterica*
F29	Egg 3	*Staphylococcus aureus*
F30	Egg 4	Negative
F31	Ground beef 2	*E. coli*
F32	Egg 5	*Salmonella enterica*
F33	Egg 6	Negative
F34	Egg 7	*Salmonella enterica*
F35	Vegetable 7	Negative
F36	Milk 7	Negative
F37	Milk 8	Negative
F38	Vegetable 8	Negative
F39	Vegetable 9	Negative
F40	Vegetable 10	*Staphylococcus aureus*
F41	Chicken 6	Negative
F42	Chicken 7	Negative
F43	Chicken 8	*E. coli, Salmonella enterica*
F44	Egg 8	*Salmonella enterica*
F45	Tuna 3	*Staphylococcus aureus*
F46	Vegetable 11	*Staphylococcus aureus*
F47	Milk 9	*Listeria monocytogenes*
F48	Cheese 4	*E. coli*
F49	Chicken 9	*E. coli*
F50	Cheese 5	*Listeria monocytogenes*
F51	Cheese 6	*E. coli, Salmonella enterica*
F52	Milk 10	*E.coli, Salmonella enetrica, staphylococcus aureus*
F53	Egg 9	*E. coli, Salmonella enterica, Staphylococcus aureus*
F54	Chicken 10	*E. coli, salmonella enterica, staphylococcus aureus*

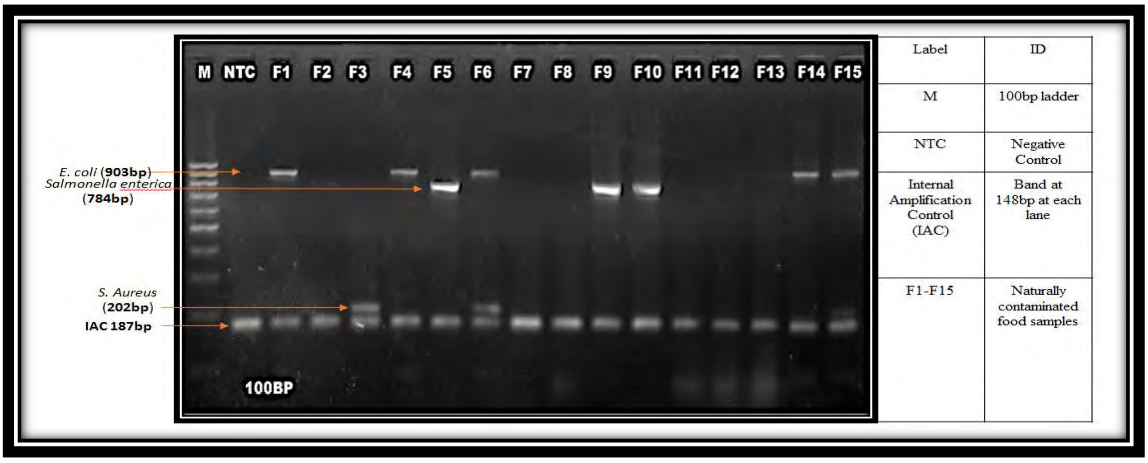


Figure 4.25: Results of mPCR tested with naturally contaminated food samples. F1-F15 represents each food sample respectively

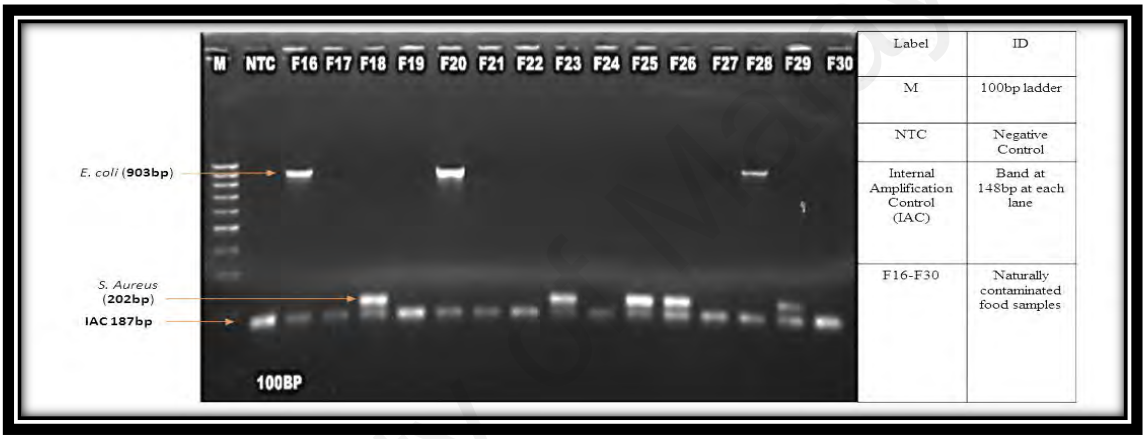


Figure 4.26: Results of mPCR tested with naturally contaminated food samples. F16-F30 represents each food sample respectively

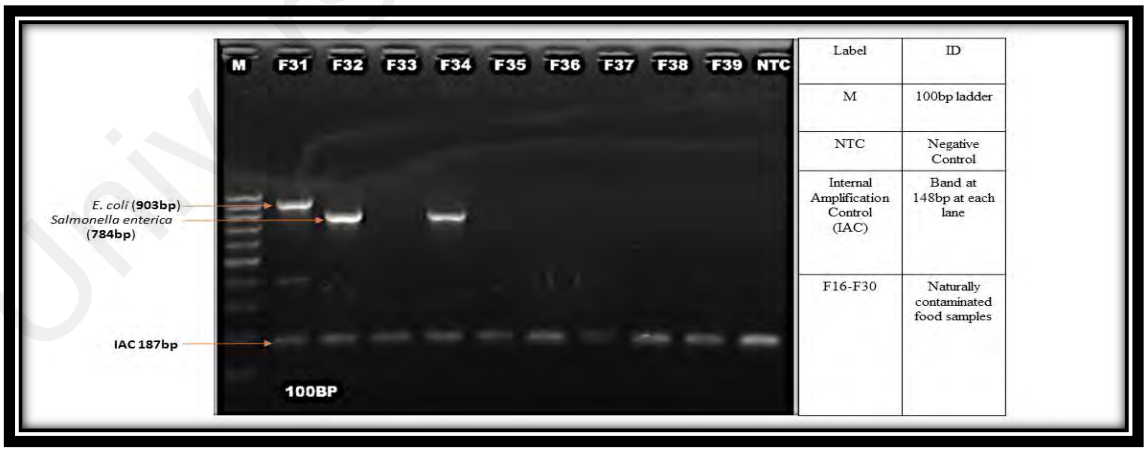


Figure 4.27: Results of mPCR tested with naturally contaminated food samples. F31-F39 represents each food sample respectively

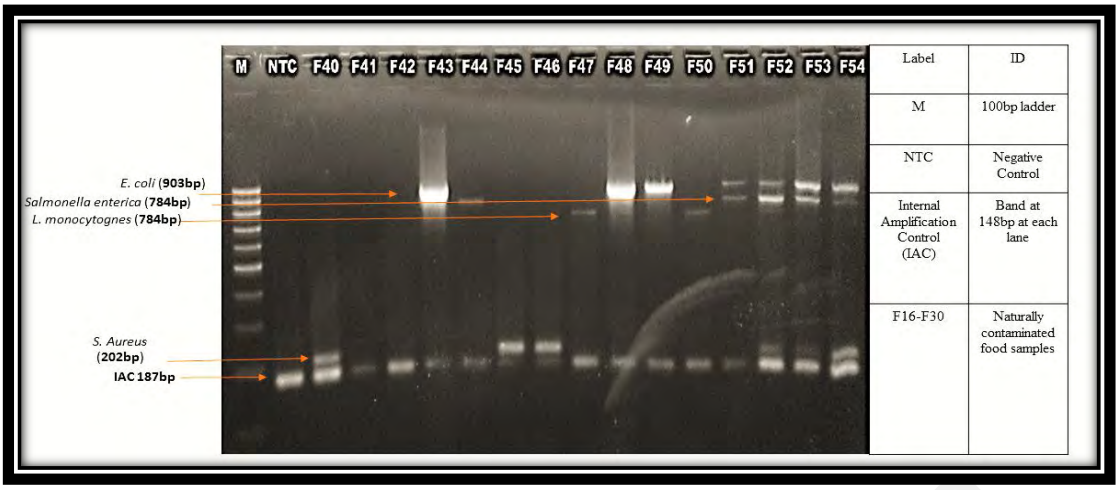


Figure 4.28: Results of mPCR tested with naturally contaminated food samples. F40-F54 represents each food sample respectively.

CHAPTER 5: DISCUSSION

Foodborne outbreaks are becoming more common and deadly in this fast pace world. Moreover, food products such as fruits, vegetables, dairy, seafood, meat, poultry, and ready-to-eat (RTE) foods are mostly responsible for multistate foodborne outbreaks according to the Foodborne Outbreak Online Database (CDC, 2015). Thus, it is very crucial to detect the foodborne pathogens rapidly and to take immediate treatment or remediation. Previously available microbiological culture method is sensitive, generally inexpensive, and simple. However, it is time-consuming and laborious. In addition, some bacterial species may enter a viable but non-culturable state where they are still viable but not culturable on routine agar, which impairs their detection by culture-based techniques (Li et al., 2014). While conventional culture methods and cultural enrichment are sometimes still necessary (Taskila et al., 2012), emerging methods are targeted on developing more rapid and less laborious methods. Rapid methods are very important for the food industry as the presence of pathogens in raw and processed products can be detected immediately and the proper control of the contaminated products can be conducted accordingly. Eventually, molecular methods such as Polymerase Chain Reaction (PCR) started to be developed as they are very precise and relatively rapid.

Despite the advantages, conventional PCR is only able to detect one particular gene or pathogen at a time. Thus, in this study mPCR method was used to detect six foodborne pathogens. This method allowed detection of multiple foodborne pathogens in a single run. Besides that, this method is also ideal for conserving costly polymerase and templates in short supply. There are also other more advanced methods of detection available such as real-time PCR and biosensor. However, real-time PCR is more expensive (Li et al., 2016) than mPCR while development of biosensor is time consuming (Zhao et al., 2014). Thus, mPCR was chosen as a detection tool for my study

provided it is rapid, cost-effective, highly specific, sensitive and can be developed within the limited time constraint.

In this study the objective to develop a relatively rapid and cost-effective multiplex PCR (mPCR) for simultaneous detection of six foodborne pathogens namely *E. coli, salmonella enterica, Listeria monocytogenes, Shigella* spp., *Staphylococcus aureus* and *Yersinia enterocolitica* in food samples was successfully achieved. This assay was also relatively rapid as it requires about only 5 hours including sample preparation, PCR and gel electrophoresis unlike the microbiological culture method which needs overnight incubation (Zhao et al., 2014).

The boiling method successfully extracted sufficient amount of DNA to be tested using mPCR. The extraction method saved time, eliminated intensive labor and was cost efficient for pure bacterial cultures with was generally proven to be higher than 10^5 CFU/mL concentration (Li et al., 2016). However, the recovery of DNA of *Listeria* was lower than the rest of the bacteria. This might be caused by the thicker cell wall of gram-positive bacteria than that of Gram-negative bacteria (Ma et al., 2014). Unlikely, *Staphylococcus aureus* which is also Gram-positive bacteria were recovered efficiently. Thus, the poor recovery of *Listeria monocytogenes* DNA could be due to the need of longer incubation period than other food-borne bacteria (Goulet et al., 2013).

5.1 Selection of Genes and Primer Design for PCR Amplification

Primer pair selection is very critical in the multiplex PCR assay for the simultaneous detection of six foodborne pathogens in order to ensure specificity and sensitivity as well as to avoid cross-interactions. Development of primer pairs specific to each targeted pathogen was performed with the aid of Primer-BLAST. Factors that were taken into consideration include similar annealing temperatures (Tm) and distinguishable PCR product size (Zhao et al., 2014).

The *phoA* (alkaline phosphatase) region was selected for *E. coli* as it is proven for reliable detection of *E. coli* that enables its differentiation from biochemically and phylogenetically related bacteria. This gene is located, precisely at 8.6 min on the E. coli W3110 genome (Bachmann & Low, 1980; NAIST, 2006) and encodes bacterial alkaline phosphatase. The alkaline phosphatase of *E. coli* is synthesized under low phosphate conditions and is secreted across the inner membrane to the periplasmic space where it plays a central role in the breakdown of organic phosphate esters. This gene has been proven to be a universal marker (Kong et al., 1999) and many previous studies demonstrated the stability and usefulness of *phoA* for identification of *E. coli* (Rathi et al., 2009, Yu & Thong, 2009 and Thong et al., 2011). In this study, the *phoA* gene was successfully amplified at 903 bp as reported in the literature.

Besides that, the *hilA* region was chosen to detect *Salmonella* because it has recorded 100% specificity to *Salmonella* in previous studies (Pathmanathan et al., 2003; Thong et al., 2014). *hilA* is a member of the OmpR/ToxR family of transcription regulators based on homology of its N-terminal domain to the conserved OmpR/ToxR DNA binding and transcription activation domain. Members of this family typically bind degenerate direct repeats as a dimer. Although *hilA* also appears to activate gene expression by binding to direct repeats, other features of *hilA* deviate from those of

well-characterized family members (Rodriguez et al., 2002) .The presence of a 854 bp band considered a positive indicator for *hilA* DNA from *S.* enteritidis strain (Carvajal et al., 2017) . However, in the current study the previously designed primers modified to allow product size differentiation by just using agarose gel electrophoresis.

The *hlyA* gene that codes for the action of listeriolysin protein (*hlyA*), the main virulence gene of *L. monocytogenes* (Soni et al., 2014), was considered as the target gene for *Listeria monocytogenes* detection in the study. This is because listeriolysin protein allows *L. monocytogenes* to escape from phagosomes into the cytosol without damaging the plasma membrane of the infected cell. This allows the bacteria to live intracellularly, where they are protected from extracellular immune system factors such as the complement system and antibodies being important factor causing food-poisoning. There were various studies which showed specificity of *hylA* gene in detection of *Listeria monocytogenes* in clinical samples (Mehmetii et al., 2017) and food samples (al-Ali et al., 2018).

On the other hand, the invasion plasmid antigen H (*ipaH*) gene was chosen for detection of *Shigella* sp. as it is carried by four *Shigella* species (Shao et al., 2011; Chen et al., 2012). *Shigella* possesses 12 *ipaH* genes, which reside on both the large plasmid and the chromosome. The encoded *ipaH* proteins are injected into host cells via the T3SS (Ashida et al., 2007, Ashida et al., 2013). *IpaH* family proteins contain N-terminal leucine-rich repeats (LRRs) and have E3 ubiquitin ligase activity in their conserved C-terminal regions (Rohde et al., 2007; Ashida et al., 2013). Initially, the differentiation of *Shigella* and *E. coli* was very complicated especially the enteroinvasive *E. coli* (EIEC) that possesses the ability to cause dysentery using the same method of invasion as Shigella does. Thus, ipaH was proven to successfully differentiate *E. coli* from Shigella spp (Beld & Reubsaet, 2011). Since *E. coli* is also involved in the current mPCR development, this would be a very relevant character of

ipaH gene to be considered as a target region. Many previous publications showed successful identification of *Shigella* spp. using *ipaH* gene (Wang et al., 2015; Hu et al., 2014 and Chen et al., 2012). In current study, this gene successfully differentiated *E.coli* from *Shigella* strains.

For detection of *Staphylococcus aureus,* the *rpoB*, gene that encodes the highly conserved β-subunit of the bacterial RNA polymerase was chosen. This gene was also previously known for accurate identification of *Staphylococcus* isolates (Drancourt & Raoult, 2002).

Finally, the yst (*Yersinia* Stable Toxin) gene was chosen for detection of *Yersinia enterocolitica.* The toxin YST is encoded by the genes ystA and ystB, is a membrane-acting virulence factor. It is also heat-stable enterotoxin that is important in causing diarrhea in the host and only known to present in virulent strains of *Y. enterocolitica* . Besides that, it stimulates the cGMP synthesis in the intestinal lining (Duan et al., 2017; Duan et al., 2014) which leads to an overall effect of fluid loss due to a lack of fluid absorption making it a relevant target gene. Moreover, it has been utilized to differentiate *Yersinia enterocolitica* from other *Yersinia* species (Singh & Virdi, 2004).

Even though, the target regions selected were utilized in many previous publications, the sequences were modified to adapt for the current multiplex assay. This was to ensure that all the amplicon sizes are below 1000 bp and to have well separable band for each pathogen by using gel electrophoresis technique which would be cost-effective.

5.2 Primer Specificity Test and Validation of PCR Products

The specificity test shows that every primer set is specific to the respective targeted foodborne pathogen except the modified *phoA* (m*phoA*) primer sets. The m*phoA* primer sets which corresponding to *E. coli* and also when tested with *Shigella* sp. strains. Thus, all *Shigella* strains produced band at 903 bp when tested with mphoA primers. However, *Shigella* spp. and *E. coli* were clearly distinguishable once subjected to multiplex PCR at which *Shigella* spp. produces two bands (903 bp and 272 bp) while *E. coli* produced only one band at 903 bp. This can be explained by close evolutionary relationship between *E. coli* and *Shigella* at which *E. coli* shares 95 – 97 % similarity in genome with certain species of *Shigella* (Mitra et al., 2015). This is because the *phoA* gene is a house-keeping gene which exhibits high similarity of the conserved sequences with other members of Enterobacteriaceae besides E.coli. However, the gene was still required to produce bands for *E.coli* such that it can be viewed together with other targeted pathogens by using 100 bp ladder. Besides that, the invasion plasmid antigen H (*ipaH*) gene used in this study was the main target for detection of *Shigella* in many other researches (Shao et al., 2011). This allows clear differentiation of *Shigella* from *E. coli* at which a band will be produced at 272 bp with the presence of *Shigella* sp. only. On the other hand, development of mPCR using 4 targets by Maheux et al. (2009) reported the appearance of 4 bands corresponding to the primers to be *E. coli* and 3 or less bands to be non E. coli resolved the issue. Similarly, in this study it was recorded that the appearance of one band to be *E. coli* and two bands to be *Shigella* spp. upon mPCR.

Moreover, to confirm the specificity of primer pairs, sequencing of each representative bacterium was done. All the sequenced PCR products were the expected DNA signature of the tested pathogens. Thus, the specificity of primers designed was high enough to distinguish the six targeted foodborne pathogens.

5.3 mPCR Optimization

For a multiplex PCR assay to be successful, the relative concentrations of primers, PCR buffer concentration, the balance between magnesium and DNA, cycling temperatures, amounts of template DNA and Taq DNA polymerase are very important (Markoulatos et al., 2002). In the current study, the six targeted foodborne pathogens were successfully differentiated from each other by optimizing the annealing temperature and primer concentration only. Unlike, previously developed mPCR assays, the initial concentration of $MgCl_{2:}$ which was 2.25 µM able to produce bands for all the targeted pathogens. Thus optimization was not required for Mg2+ concentration. The optimum annealing temperature was 56ºC which found to be within range described in Guidelines of optimization. The concentration of each primer also obey the guideline (Clontech, 2015) stated that it should from 0.2–0.3 µM (each 0.05-0.10µl in reaction mixture). It was ensured that same sterile stock primer, pcr machine as well techniques used throughout the optimization process to increase consistency and reproducibility.

5.4 Sensitivity Evaluation of mPCR Using Bacterial Strains

The multiplex assay developed in this study was effective for the simultaneous detection of targeted pathogens up to 10^4 CFU/ml. However, the individual detection limit was even higher at which 10^1 CFU/ml for detection of *E. coli, Listeria and Shigella*; 10^2 CFU/ml for *Salmonella* and *Yersinia*; 10^4 CFU/ml for *Staphylococcus aureus.* It showed that the DNA extraction method described in this study could successfully isolated DNA from PCR inhibitory components (such as fats, glycogen, organic and phenolic compounds, etc.).

The detection level of this study was higher than that recognized by Kim et al. (2007) and Germini et al. (2009) at which the former assessed a multiplex PCR assay,

which was able to detect at a level of 10^5 CFU/mL for E. coli O157:H7, *Salmonella*, *S. aureus*, *L. monocytogenes*, and *V. parahaemolyticus* while the latter reported a sensitivity level of 10^6 CFU/mL for detection of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*.

Besides that, the multiplex developed by Lee et al. (2014) to detect *Escherichia coli* O157:H7, *Bacillus cereus*, *Vibrio parahaemolyticus*, *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus* demonstrated equivalent detection limit as the current study when tested with bacterial strains.

Some previous studies have recorded even higher detection limit than this study but mostly when paired with other advanced techniques. A study to detect *E. coli, S. aurues* and *Salmonella* in milk (Wei et al., 2018) recorded 10^3 CFU/ml. Another study have recorded a limit of 10^0 CFU/ml when combined mPCR with large volume Immuno-magnetic Separation (IMS) (Mao et al., 2016). Other than that, a mPCR that combined with IMS and PMA treatment for detection of Salmonella in raw meat demonstrated detection limit of 10 CFU/ml (Li et al., 2016).

5.5 Application of mPCR Assay Using Artificially Contaminated Food Samples

From the evaluation using artificially contaminated food samples, it showed that even though, detection limit of *E. coli* was up to 10^1 CFU/ml; *Salmonella*, *Listeria* and *Shigella* was up to 10^2 CFU/ml, the simultaneous detection limit of all the six pathogens was 10^6 CFU/ml. This value was lower compared to the detection limit recorded when bacterial strains are tested. This might be due to presence of inhibitors in the food samples (Schrader et al., 2012). This condition necessitates that the food samples to be enriched for a few hours (2 hours) in peptone water for providing conditions for growth and multiplication of bacterial pathogens to a detectable level, dilution of inhibitory substances present in food and dilution of dead target cells, which provides some

assurance that the detected DNA belongs from viable target cells (Radhika et al., 2014). The enrichment is also useful for resuscitating injured cells (due to heat, cold, acid, or osmotic shock during food processing), increasing the level of targeted pathogens and diluting inhibitory compounds in processed food products (Gracias & McKillip 2004; Dwivedi & Jaykus 2011).

Other than that, interactions between the six pathogens in the same suspension would be complex which may have inhibited the growth of particular bacteria, reducing the detection rate of the assay (Zhao et al., 2016).

The detection limit for detection of *E. coli*, *Salmonella* and *Listeria monocytogenes* of the current study were higher than that of a study by Li et al. (2017) which exhibited detection limit of 10^2, 10^3 and 10^4 CFU/ml for *Salmonella*, *E. coli* and *Listeria monocytogenes*. Another study by Yang et al. (2013) which combined nano-beads separation with PMT and mPCR showed only 10^3 CFU/ml detection limit for *Salmonella*, *E. coli* and *Listeria monocytogenes* which is also lower than detection limit obtained in current study for the three pathogens. This proves that the current study is cost-efffective and relatively sensitive.

Besides that, there are also several studies recorded higher detection limit than current study due to longer incubation period. In previous mPCR development studies the pathogens can be detected up to 10^0 CFU/ml when tested with lettuce samples by (Lee et al., 2014). However, that was upon 8 hours incubation while the incubation time of current study is only 2 h. Similarly, a study by Chen et al. (2012) reported a congruent sensitivity level with current study before subjecting them to overnight incubation. It is proved that, the current study produced relatively sensitive results in shorter incubation time.

5.6 Application of mPCR Assay Using Naturally Contaminated Food Samples

From the mPCR testing on 54 naturally contaminated food samples, 61% were positive. The percentage of positive results is higher than the study by Lee et al. (2014). This represents that the developed mPCR is appropriate for practical use in food testing. Food homogenates that showed positive results were streaked on respective selective agar to re-confirm with culture method. Results observed with multiplex PCR and traditional cultures were similar. This confirms that the developed mPCR is relatively reliable than previously available mPCR platforms at which mPCR developed by Chen et al., (2012) had 2 food samples which weren't consistent with culture method.

The false negative results can be caused by the inhibitory substances, incorrect PCR mixture, malfunction of the PCR apparatus (Wardyn et al., 2014). The application of IAC (Thong et al., 2014) that of 187bp was effectively eliminated false negative results. Even though the naturally occurring food samples tested in the present study are low in number, the results obtained are valuable and highly promising.

5.7 Limitations of Study

The developed mPCR is only applicable for detection of six food borne pathogens namely *E. coli*, *Salmonella*, *Listeria monocytogenes*, *Staphylococcus aureus*, Shigella spp. and *Yersinia enterocolitica*. Specificity of primers designed for *E. coli* also amplified *Shigella* due to close evolutionary relationship. Only 54 natural food samples were tested with the developed mPCR due to the time constraint.

5.8 Recommendations for Future Research

More specific primer pair for *E. coli* can be designed by utilizing advanced visualization techniques. The designed mPCR can be integrated into real-time PCR to allow quantitation. Reliable quantitation foodborne pathogens in artificially inoculated or naturally contaminated foods is critical to obtain highly reliable research data to address various issues related to predictive microbiology, epidemiology, risk assessment and regulatory testing (Auvolat & Besse, 2016). Besides that, the developed mPCR also could be combined with other advanced detection methods for improved efficiency and even more rapid detection.

CHAPTER 6: CONCLUSION

The multiplex PCR assay described here can simultaneously detect six foodborne pathogens namely *E. coli*, *Salmonella enterica*, *Listeria monocytogenes*, *Shigella* spp., *Staphylococcus aureus* and *Yersinia enterocolitica.*Thus, the core objective of the study was successfully achieved. It was found to be sufficient in specifically and simultaneously detecting as few as 10^4CFU/mL and 10^6 CFU/ml of the six pathogens when tested with bacterial strains and artificially contaminated food samples upon enrichment for 2 hr. The developed mPCR also efficiently detected pathogens present in naturally and artificially contaminated food samples. Therefore, the multiplex PCR assay developed in this study is an effective qualitative method to detect the six foodborne pathogens in foods and will also be useful for the food industry and various regulatory agencies.

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